SYNTHESIS, MOSQUITO REPELLENCE AND APOPTOTIC ACTIVITY OF FUNCTIONALIZED MONOTERPENES

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SYNTHESIS, MOSQUITO REPELLENCE AND APOPTOTIC ACTIVITY OF FUNCTIONALIZED MONOTERPENES

By

Geoffrey Maroa Mahanga

A Thesis Submitted in Fulfillment of the Requirement for the Degree of Doctor of Philosophy (Chemistry) of the University of Dar es Salaam

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CERTIFICATION

The undersigned certify that we have read and hereby recommend for examination a thesis entitled: ***Synthesis, mosquito repellence and apoptotic activity of functionalized monoterpenes***, in fulfillment of the requirement for the Degree of Doctor of Philosophy (Chemistry) of the University of Dar es Salaam.

Signed

…………………………………

Prof. Mayunga H. H. Nkunya

(Supervisor)

Date……………………..

…………………………………

Dr. Cosam C. Joseph

(Supervisor)

Date…………………………

DECLARATION

AND

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I, **Geoffrey Maroa Mahanga**, declare that this thesis is my original work, and it has not been presented and it will not be presented to any other university for a similar and/or any other degree award.

Signature………………………….

ACKNOWLEDGEMENTS

And to my siblings, Jane Mandiga, Lydia Rhobi, Susan Bhoke and Leah Matinde I wish to thank them for the love, care, and support they accorded me during my study period. Their prayers and encouragement through phone calls made me have a glimpse of home. Lastly, I wish to express deep appreciation to my wife and our lovely daughters (Sandra Robi and Sally Ghati). Their endurance, love, support and understanding while I was away is unmatched.

For the good health and gift of life throughout the period of my studies, I thank the Almighty God for his Sustenance.

DEDICATION

This thesis is dedicated to my late beloved mother

Mary Bhoke Mahanga (RIP)

and

Daughters

Sandra Robi and Sally Ghati

Your presence will forever be felt

ABSTRACT

The investigations whose results are reported in this Thesis involved the synthesis of variously functionalized *p*-methane based epoxides, hydroxylated derivatives, *β*-amino alcohols, *β*-hydroxy sulphides and *C*-gycosidic compounds. These compounds bearing a similar skeleton motif were evaluated to determine their efficacy as mosquito repellents and their apoptosis inducing potential. Twelve epoxides obtained *via* reactions with bicarbonate activated peroxide (BAP) *meta*-chloroperoxybenzoic acid (*m*-CPBA), and *via* bromohydrin formation exhibited good to high mosquito repellence, some of which were more active than *p*-menthane-3,8-diol which was used as the standard insecticide in this study. The epoxides also exhibited good to excellent apoptosis inducing potential against two cell lines (Jurkat T and CHO) and six indicated better activity than Camptothecin, a drug that was used as the standard. Seven variously substituted hydroxylated compounds were obtained *via* oxymercuration-demercuration and *cis*-hydroxylation reactions using OsO_4, which revealed varying levels of mosquito (*Anopheles gambiae*) repellence, ranging from poor to very high repellent activity. The hydroxylated derivatives also indicated some level of apoptosis induction. Eight variously substituted *β*-amino alcohols and nine *β*-hydroxysulphides were obtained *via* oxirane ring opening with benzylamine, aniline, piperidine and benzylmercaptan also indicated some level of apoptosis induction. *C*-glycosylation catalyzed by $TiCl_4$ afforded six *C*-glycals, which also exhibited varying levels of apoptotic induction potential. Structures of the synthesized compounds were established based on analysis of spectroscopic data.

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LIST OF ABBREVIATIONS

BAP = Bicarbonate-Activated Peroxidation

CDC = Center for Disease Control

$CHCl_3$ = Chloroform

CHO = Chinese Hamster Ovary

m-CPBA = *meta*-Chloroperoxybenzoic acid

CRPF = Chloroquin Resistant *Plasmodium falciparum*

DADS = Diallyl disulfide

DAS = Diallyl sulfide

DDT = Dichlorodiphenyltrichloroethane

DEET = *N,N*-Diethyl-3-methylbenzamide

DEPA = *N,N*-diethylphenylacetamide

DFB = Diflubenzuron

DMBA = Dimethylbenzyl(a)anthracene

DMP = Dimethylphosphate

DMSO = Dimethylsulfoxide

EIMS = Electron Spray Mass spectroscopy

EtOAc = Ethyl acetate

EtOH = Ethanol

IGR = Insect Growth Regulators

IRS = Indoor Residual Spraying

IR = Infra Red Spectroscopy

ITCs = Insecticides Treated Clothes

ITNs = Insecticides Treated Nets

MeCN = Acetonitrile

MS = Mass Spectroscopy

^{1}H NMR = Proton Nuclear Magnetic Resonance

^{13}C NMR = Carbon 13 Nuclear Magnetic Resonance

NOESY = Nuclear Overhauser Enhancement Spectroscopy

PMD = *p*-Menthane-3,8-diol

R_f = Retention factor

SAC = S-allylcysteine

SAMC = S-allylmercaptocysteine

SAR = Structure Activity Relationship

TEA = Triethylamine

THF = Tetrahydrofuran

TLC = Thin layer chromatography

USDA = United States Department of Agriculture

US EPA = United States Envirnmental Protection Agency

UV = Ultra Violet

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE SURVEY

1.1   Introduction

The mosquito is perhaps the world's most dangerous organism as its bite has for centuries inflicted humans with untold suffering, in the form of malaria and other diseases. Mosquitoes rely on smell to guide them towards mates, food, and sources of blood meals. The process is highly specific, in particular for female mosquitoes which are attracted to blood sources, which they require for nourishment of their eggs. Olfaction is also species-specific. *Anopheles gambiae* (Figure 1.1) for instance, prefers to bite humans. Because this species has evolved to target humans, it will stay indoors where it can get to them more easily.[1, 2]

Fig. 1.1 *Anopheles gambiae* mosquito

Mosquitoes transmit parasites that in humans cause malaria, dengue and hemorrhagic fever[4], epidemic polyarthritis, and several forms of encephalitis[5]. Mosquitoes also transmit arboviruses that are responsible for yellow fever [3] while bancroftian filariasis

that is caused by a nematode is also transmitted by mosquitoes.[6]

1.2   Malaria Occurrence and Socio-Economic Effects

Malaria occurs predominantly, though not exclusively, in tropical and sub-tropical regions in Africa, the Middle East, Asia, China and Latin America where it has been directly or indirectly responsible for untold sufferings and economic deprivation from the beginning of recorded history. Each year, up to three million deaths occur due to malaria and close to five billion episodes of clinical illness possibly meriting antimalarial therapy are recorded throughout the world, with Africa having more than 90% of this burden.[7] In non-endemic areas, travelers and immigrants are possibly responsible in transmitting the infections. However, projected changes in climatic and other pertinent factors indicate expansion of the worldwide malaria potential occurrence zones including places as disparate as Sweden.[8]

Because of its virulence in the very countries that are less developed, malaria has continued to be responsible for much of human suffering, misery and imparing the process of socio-economic development, thereby fuelling the vicious cycle of poverty, ignorance and disease. In particular, the disease remains a major public health threat to the African continent, especially Sub-Saharan Africa and its control is critical to achieving the Millennium Development Goals (MDGs) in this sub-region. The recently published Global Strategic Plan for Roll Back Malaria 2005–2015 has stated that "six out of the eight MDGs can only be achieved with effective malaria control in place".[9]

Macroeconomic projections show that the costs inflicted by malaria generally are far greater than those of individual cases, with a substantial deleterious impact being felt on schooling of patients, external investments into endemic countries, and tourism. Poor populations are at greatest risk as 58% of the cases occur in the poorest 20% of the world's population and these patients receive the worst care and have catastrophic economic consequences from their illness.[7] Economists believe that malaria is responsible for a 'growth penalty' of up to 1.3% per year in some African countries. When compounded over the years, this penalty leads to substantial differences in GDP between countries with and without malaria endemicity, and severely restrains the economic growth of such countries.[10]

Table 1.1   Global malaria fatality scenario[7]

Region	Population	Malaria Deaths	
	(Thousand)	(Thousands)	(Percent)
World	6,122,210	1,124	100
Africa	655,476	963	85.7
Americas	837,967	1	<0.1
Eastern Mediterranean	493,091	55	4.9
Europe	874,178	<1	<0.1
Southeast Asia	1,559,810	95	8.5
Western Pacific	1,701,689	10	0.9

Table 1.1 summarizes the global malarial fatality scenario, with Africa being in the leading front, while Figure 1.2 depicts the global distribution of the disease.
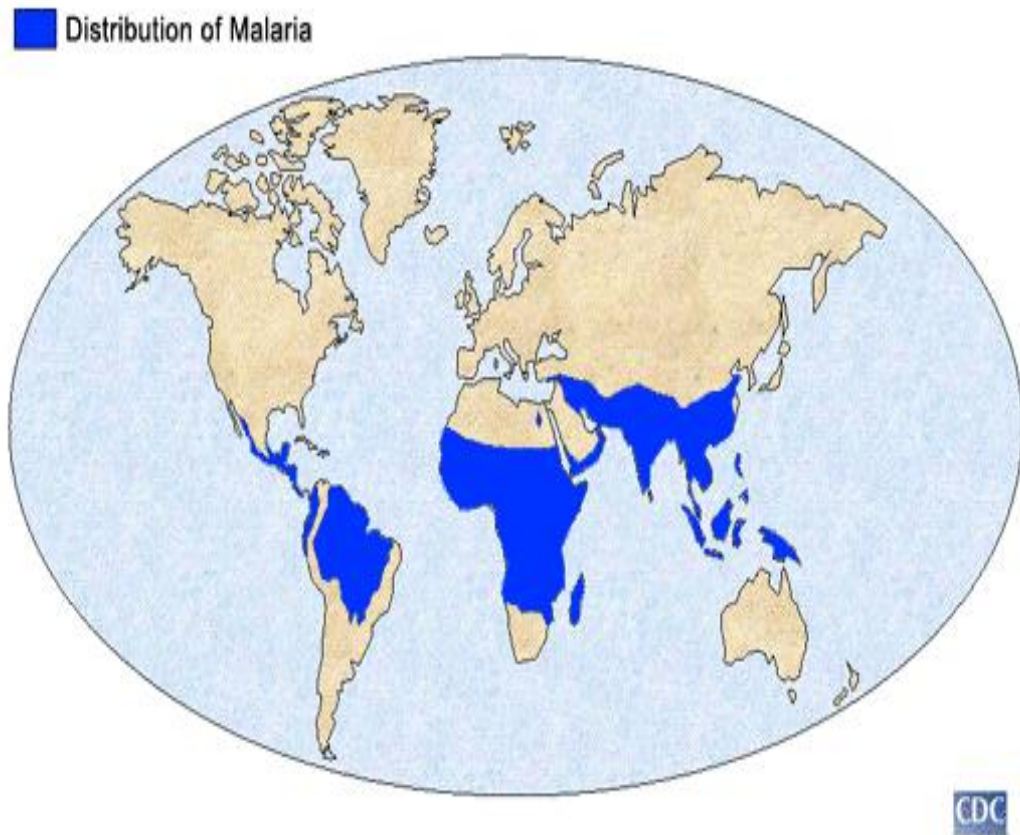
Fig. 1.2 **Geographical distribution of malaria** (Centre for Disease Control)[11]

1.3 Malaria Control

Malaria is a preventable and treatable disease. Thus, mortality caused by malaria can be avoided through judicious application of control strategies that are currently available. Prevention of malaria encompasses a variety of measures that broadly protect against infection, termed as vector control or the development of the disease in infected individuals, including chemotherapy, chemoprophylaxis and vaccination.[12]

1.3.1 Chemotherapy and Chemoprophylaxis

The control of malaria in developing countries remains a major public health concern. In this regard one of the plausible principal strategies has been to reduce mortality by rapid treatment with antimalarial drugs. However, drug resistance in many malaria-endemic countries has made this approach unsustainable. Therefore, in addition to vector control malaria control programs are adopting multi-pronged approaches encompassing personal protective measures, combinatorial drug administration with newer drugs like artemisinin derived compounds.

Chloroquine (**1.1**) and other quinine analogs have been the most widely used antimalarial drugs in most endemic regions. Since its development in the early 1940s, chloroquine became the drug of choice for malaria management. It is relatively cheap and therefore affordable in the poor countries, which are the worst affected. Other quinoline based synthetic antimalarials include Amodiaquine (**1.2**), Mefloquine (**1.3**) and Primaquine (**1.4**, **Scheme 1.1**). Quinine (**1.5**), the natural template from which the other aminoquinoline-based antimalarial drugs were developed, has been used as the last resort drug where *Plasmodium falciparum* resistance to the synthetic antimalarials is rampant, despite its toxicity and other negative side effects.[13]

The spread of *P. falciparum* Chloroquine resistance has been reported in most areas in Africa, Latin America and South East Asia. Although at first Chloroquine resistant *P. falciparum* (CRPF) strains retained some sensitivity to Amodiaquine, resistance to this

drug soon followed Chloroquine.[14] In recent years, Mefloquine, which was also widely used for the treatment of acute malaria where multi-drug resistance *P. falciparum* occurred [15], has been found to show resistance in immune compromised individuals in Thailand.[16] Although Quinine remained the main therapeutic drug for CRPF malaria for a long time, low sensitivity was reported in Brazil as early as 1910. Success rates have since fallen to below 50% in some areas, but the drug is still deployed to manage cases where other first line treatment is seen to be ineffective.[13]
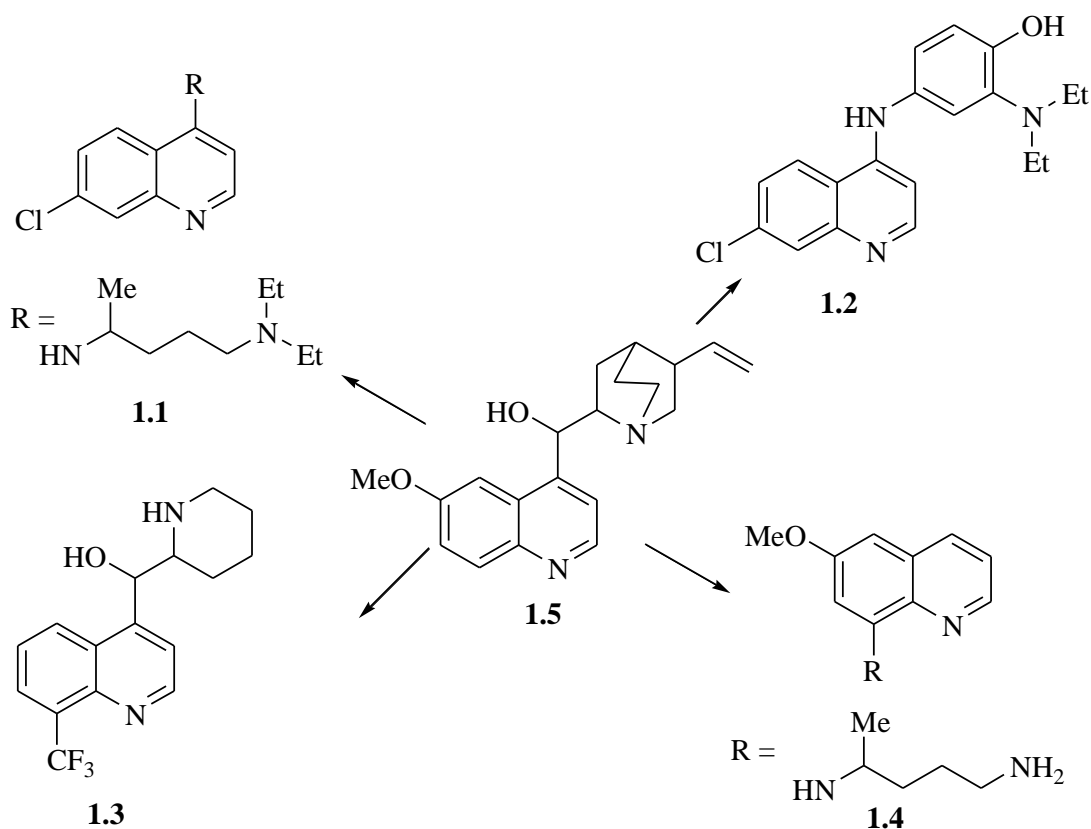
Scheme 1.1   Derivatives of Quinine (1.5)

The readily available antifoliates including Pyrimethamine (**1.6**) and Proguanil (**1.7**)

have been used as chemotherapeutics against malaria. However, resistance against these agents has also been recorded.[17]
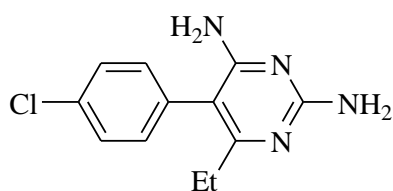
1.6
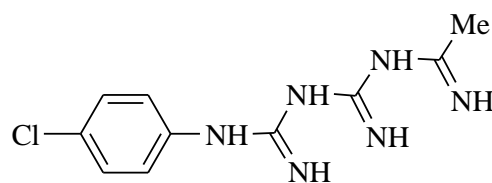
1.7

Resistance to Pyrimethamine was first described in 1953 and is now widespread,[18] while the asexual resistance of *P. falciparum* to Proguanil was also earlier detected [19] and its lack of prophylactic efficacy has been confirmed in Thailand.[20]

Sulfur based antimalarials like Dapson (**1.8**), Sulfadiazine (**1.9**) and Sulfadoxine (**1.10**) have also been used in malaria control interventions but due to the development of resistance and low tolerance amongst some patients the drugs are currently being phased out.[21]
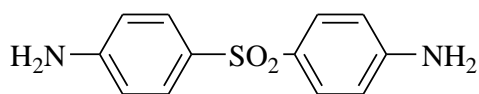
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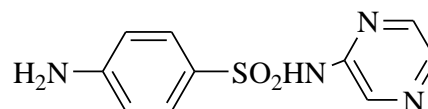
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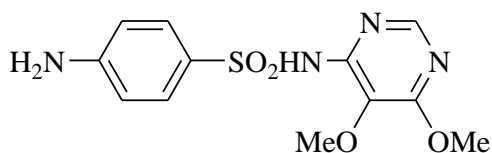
1.10

Other drugs like Halofantrine (**1.11**), a phenanthrene-methanol compound and

Atovaquone (**1.12**) have been used in malaria control and management.[22] However, resistance to both drugs has been recorded too.[22, 23]
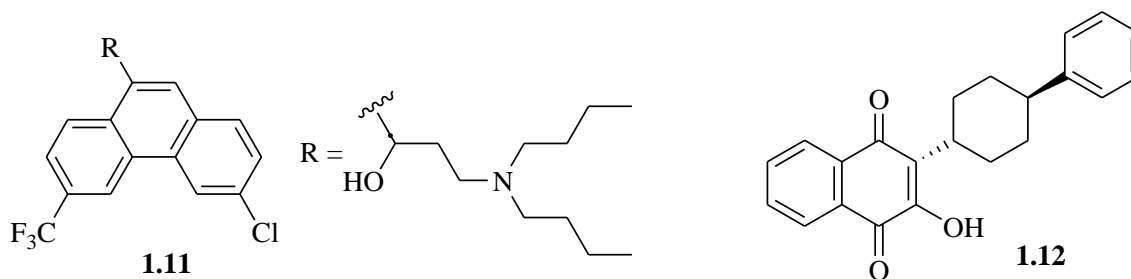
Another newer class of anti-malarials consists of the sesquiterpene lactone Artemisinin (**1.13**) and its derivatives such as Artemether, Artesunate and Dihydroartemisinin.[25]
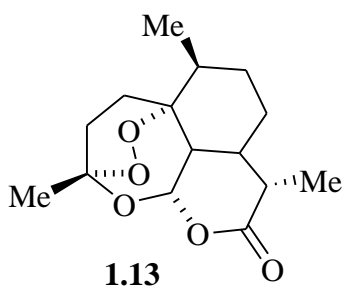
These drugs have so far shown no cross-resistance with known anti-malarials and as such they are considered to be important for treating severe malaria in areas where multi-drug resistance is prevalent.[26] However, they require long treatment courses and when used alone, recrudescence may occur.[27] Furthermore, *in vitro* resistance to artemisinin has been demonstrated.[28] Hence, the drugs need to be used sparingly so as to avoid rapid development of resistance. This entails that the search for new and effective antimalarials has to be an ongoing phenomenon, so as to outpace drug resistance that is

continuously being experienced.

1.3.2 Vaccine Development

It has long been noted that natural exposure to malaria leads to the development of partial immunity in humans, but repeated re-infection is needed to maintain this immunity.[29] As a result, this phenomenon has prompted a great deal of research directed towards the development of vaccines to protect humans against infection by *P. falciparum*. The complexity of the life cycle of *P. falciparum* is being exploited in attempts to develop malaria vaccines because it provides several potential targets for immune response.[30] Potential candidate vaccines are largely based on various antigens derived from different stages of the malaria parasite life cycle.

Worldwidely, vaccine developers are trying to develop all three types of malaria vaccines, namely pre-erythrocytic, blood stage, and transmission blocking. All these types have been tested in humans, and some have shown promising effectiveness. [31] It is therefore anticipated that the optimal malaria vaccine will most likely combine antigens from all three stages of the malaria parasites' life cycle. Many single-antigen malaria vaccine candidates have undergone clinical trials, and combination studies are being planned.[31] For instance, the pre-erythrocytic vaccines RTS,S" [32] and SPf-66 [33] that prevent the malaria parasite sporozoites from entering or developing within the liver cells have been tried in some malaria endemic areas where they elicited protective efficacy.[29, 34, 35]

The development of a suitable malaria vaccine has however been hindered by the lack of a suitable source of parasites from which it could be prepared, the main problem being low immunogenecity of malaria parasites.[32] Vaccines would be a useful addition to both chemotherapy and mosquito control in combating malaria. However, until now there is no guarantee that the current approaches to malaria vaccine development would lead to the establishment of a viable and cost-effective vaccine. Meanwhile efforts to develop an effective malaria vaccine cannot be abandoned and it may be a reasonable anticipation that ultimately success will be achieved although this may take several years to happen. [12, 36]

Considering the existing socio-economic settings, drugs and vaccines would more easily be applicable in the industrialized world. However, in less developed countries in Africa, South East Asia, Latin and Central America and the Caribbean it is only an efficient mosquito control approach that would hold the promise for the eradication of malaria in these regions.[37] In addition, the emergence of drug-resistant parasites and the elusive nature of the parasites suggest the control of the insect vector to be the most cost-effective and practical approach in reducing the burden of malaria.

1.3.3   Mosquito Vector Control

Vector control is an essential and effective means to eliminate transmission of vector-borne diseases, especially in areas where there exists resistance of parasites against the available drugs. Since no single method of mosquito vector control is effective there is

need to evolve comprehensive control strategies, including the use of insecticides, bio-control agents and good environmental management practices. The following sections give a review of past and current approaches towards the control of malaria transmitting mosquitoes.

1.3.4 Biological Control Agents

1.3.4.1 Bacterial Agents

In the last decade, bacilli-based mosquito larvicides popularly known as biocides or biolarvicides became popular in vector control and many commercial formulations are now available that could be used in large-scale mosquito control operations.[38, 39] Bacilli-based larvicides constitute the bacterium *Bacillus thuringiensis* (*Bt*) which is a safe and effective an endospore-forming soil organism. *B. thuringiensis* is one of the most important microorganisms used as a biopesticide [40] and it consists of hundreds of subspecies, most of which on sporulation produce one or more insecticidal proteins, the so-called δ-endotoxins [41] or insecticidal crystal proteins (ICPs or Cry proteins). There are currently about 150 insect pests that are susceptible to *Bt*, among them being mosquitoes. Cry proteins bind to specific receptors in the larval midgut cells, thereby causing cellular swelling and lysis.[42]

Bt based insecticides have been used since 1961 against insect caterpillars and more recently, against mosquito and black fly larvae. However, one of the major drawbacks in their use is that the δ-endotoxins are readily inactivated, owing to their rapid

biodegradability. Thus, this requires several applications to be made so as to maintain an effective level of the insecticidal activity. Despite their increased application, mosquito resistance to *Bt* larvicides has been reported for *Pletulla xylostelle* L., *Culex. pipiens* and *Cx. quinquefasciutus*. This, therefore, poses a challenge on the versatility of the bio-larvicides towards mosquito vector control.[41-46]

1.3.4.2 Invertebrate Agents

Invertebrate predators of mosquito larvae also exist. Under natural conditions invertebrate predators such as Coleptera, Dystiscidae, Hydrophylidae, Hemiptera, Belostimidae and Notonectidae can be found particularly in rice fields. Their presence could be effective for a drastic reduction in larval populations. The mermethid or mosquito attacking nematode, *Ronanomesis culsivorax*, has also been used against mosquito larvae. However, the use of vertebrate predators in areas where agrochemicals are also applied and climatic fluctuations would be unfavourable factors, as the invertebrate predators could be eliminated or severely reduced due to such factors.[47-51]

1.3.4.3 Vertebrate Agents

Vertebrate predators like larvivorous fish have been used as biological tools for mosquito control since about 100 years ago. Thus, the fish *Gambusia affinis* has been successfully used in controlling populations of *Cx. freeborni* larvae in California rice fields, and is by far the most commonly used fish for mosquito control. However, the fish is expensive to rear and transport, apart from being a manace to many native fishes

when introduced into other ecosystems. Furthermore, generally fish may not survive in temporary breeding habitats. This therefore limits the use of fish in mosquito control programs.[52-57]

Although considered a nuisance, lizards which are commonly found in living rooms in African houses could in fact be effective predators of mosquitoes and other unwanted insects in such premises. However, so far there is no documented study to quantify such effectiveness.

For a number of reasons, biological control of mosquitoes is generally more difficult to achieve than the control with insecticides or through environmental management practices. Biological agents are slow in their action and may not be used in emergency situations like during epidemics.

1.3.5   Environmental Management

Environmental measures such as reduction of vector populations through eliminating potential vector habitats or targeting their early immature (early) stages have been applied within three broad categories, namely modification of environmental conditions, manipulation of environmental conditions and modification of human habitation or behavior.

Modifications of environmental conditions include permanent physical transformations such as drainage of water bodies, and land filling and grading. Manipulation of

environmental conditions includes recurrent measures that produce temporary effects that are unfavourable to larval production or growth sustenance. This includes regulation of water levels, removal of vegetation and stream flushing, among others. The third category involves activities that are designed to reduce human-vector contacts leading to the elimination of pathogen transmission. These include location of human settlements away from vector sources, mosquito proofing of houses, use of bednets, zooprophylaxis and other personal protection measures. Some of these procedures were effective in the past but were later de-emphasized or discarded due to their high costs and labour-intensiveness. Likewise, environmental management for malaria control requires specialist skills and commitment that are lacking in many sub-Saharan African countries where they are needed most. [58, 59]

1.3.6   Chemical Insecticides and Larvicides

Chemical insecticides and larvicides are by far the most widely used agents for the control of malaria transmitting mosquito vectors. The first efficient chemical insecticides such as organochlorines, organophosphates and carbamates were introduced in the middle of the 20th century. Before that, insect pest control was mainly based on the use of inorganic agents such as sulfur, arsenicals, hydrogen cyanide or cryolite.[60] The introduction of organochlorine, organophosphorus and carbamate insecticides led to a real revolution in insect pest control and between the 1940s and 1960s as these agents became indispensable in mosquito vector control initiatives. This led to the eradication

of malaria in many countries in the northern hemisphere including the USA, USSR, southern Europe and most of the Caribbean islands.[61]

1.3.6.1 Organochlorines

The introduction of dichlorodiphenyltrichloroethane [DDT (**1.14**)] during World War II as one of the first organochlorine insecticides was remarkable. DDT has a wide spectrum of action and a long residual activity. These properties made it for a long time to be a dependable insecticide not only in insect vector disease control, but also against agropests. Other early used organochlorines in mosquito vector control included methoxychlor (**1.15**) and benzene hexachloride (**1.16**).[62, 63]

1.14

1.15

1.16
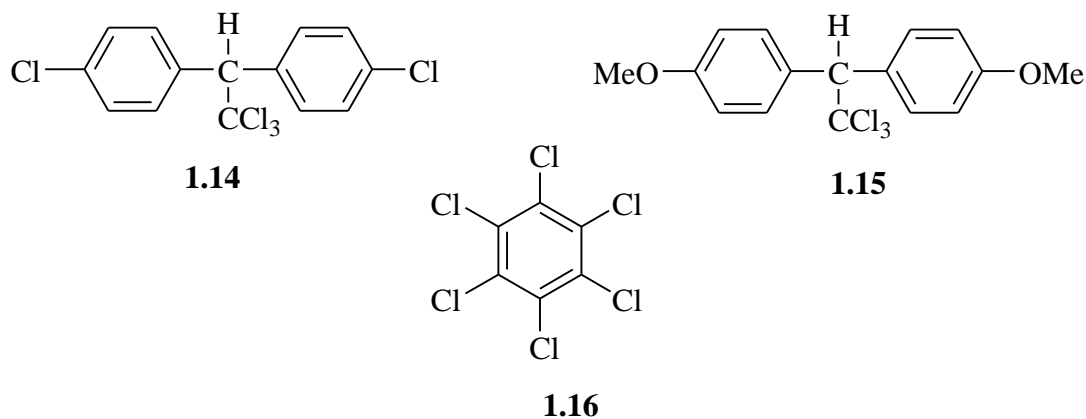
Despite their success in insect pest control upon their introduction, a few years later, organochlorine insecticides were shown to be detrimental to the environment both in terrestrial and aquatic ecosystems due to their non-biodegradability and hence being environmentally persistent. This led to their accumulation not only in the environmental ecosystems but also in marine organisms and animals through the food chain. As a

result, most of these insecticides were banned or their use largely restricted in many countries including East Africa. Studies also suggested that chronic exposure to DDT was associated with neurological impairments, accelerated ageing, and breast cancer. However, despite these shortfalls DDT is still in use in some countries where malaria is endemic.[62-68]

In the 1950s through 1970s mosquito control through DDT spraying campaigns was undertaken in many malaria endemic countries including East Africa. To a great extent, the campaigns reduced malaria incidences in such countries. However, several drawbacks were encountered, including the large costs involved and vastness of the afflicted countries. Furthermore, the prolonged exposure led to the mosquitoes developing resistance against DDT and other organochlorides. Therefore, due to these factors and consistent pressure from environmentalists, DDT use was banned in many countries in favour of other insecticides like organophosphates and the pyrethroids.

1.3.6.2 Organophosphates

Organophosphorus insecticides, frequently called organophosphates include parathion (**1.17**), fenthion (**1.18**) and temephos (**1.19**) among others and were developed by Bayer AG in the 1940s. For many years these compounds proved to be reliable and effective pest control agents.[69,70]

1.17

1.18

1.19
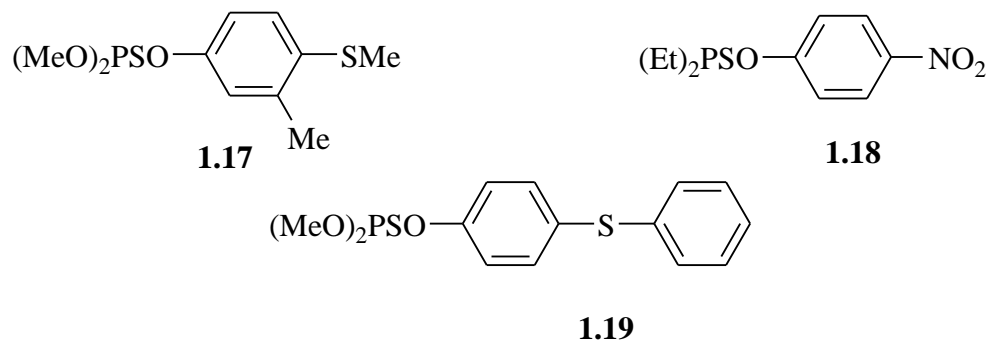
Notwithstanding their usefulness and wide applicability, organophosphorus insecticides affect the nervous system by phosphorylation of acetylcholinesterase, thereby provoking respiratory muscle weakness and neuromuscular dysfunction. They are also known to induce tumorigenic risks. The US Environmental Protection Agency (EPA) has released an organophosphorus cumulative risk assessment, which has resulted in the cancellation of a number of organophosphorus pesticides from use.[71-76]

1.3.6.3 Carbamates

Carbamates were developed as insecticides in the 1950s and have since been used for disease and agricultural insect vector control. These insecticides, which include Aldicarb® [2-methyl-2-(methylthio)] (**1.20**), propionaldehyde-*o*-methylcarbamoyloxine) (**1.21**), Carbofuran® (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methycarbamate, **1.22**), Carbaryl® (1-naphthylmethyl carbamate) and Fenobucarb® 2-(1-methylpropyl)-phenyl-*N*-methylcarbamate (**1.23**), are rapidly detoxified and excreted in warm-blooded animals and they are generally selective against targeted insect pests.[76, 77]

1.21   **1.22**   **1.23**   **1.24**
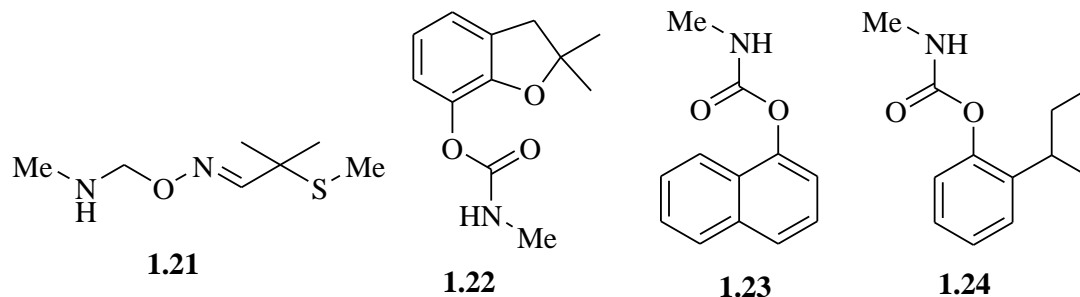
Carbamates have low persistence in soil, plants, and in the environment. Nevertheless, several studies have shown an association of long-term carbamate exposure with neuropsychological function impairment, which could be interpreted as evidence of a chronic effect of cumulative high exposure to these compounds.[76-79]

Apart from the environmental aspects caused by carbamates, there are populations of insects which have been shown to have developed resistance not only to organochlorine and organophosphorus insecticides, but also against carbamates.[80] Therefore, a lot of efforts have been made to establish new pesticides with different mechanisms of action, to replace those which have now become less effective due to resistance. Particular focus is on the development of selective and highly effective substances that would cause no harm to human health and to the environment. Therefore, currently extensive efforts to search for compounds of natural origin from plants and microorganisms with insecticidal properties are ongoing.

1.3.6.4 Insect Growth Regulators

Insect growth regulators (IGRs), are regarded as the third generation insecticides. They represent diverse groups of chemical compounds that are highly active against insects' larvae. They act by disrupting the larvae metamorphosis such that the larvae would not transform into adult insects, as the larvae would eventually die before completing the metamorphosis cycle. There are also IGRs that would allow completion of metamorphosis stages, but the emerging adults would either die before maturity, or would mature but their reproduction ability would have been curtailed by the compounds.[81]

In general, IGRs have a good margin of safety to most non-target biota including invertebrates, fish, birds and other wildlife. They are also relatively safe to humans and domestic animals. The compounds do not induce quick larvae mortality in the pre-imaginal stages treated and occur many days post treatment. On account of these advantages and the high level of activity, it is likely that IGRs could play an important role in vector control programs. They are more specific than conventional insecticides by interfering with the hormonal mechanisms of target organisms resulting in various kinds of morphological, anatomical and physiological abnormalities so that the target species does not reach the final stage of development.[81]

A large number of IGRs, both juvenoids and chitin synthesis inhibitors, have been evaluated for vector control but only very few of these have been found to be effective

and commercially feasible, like diflubenzuron (**1.25**), methoprene (**1.26**) and fenoxycarb (**1.27**).[81-87]

1.25

1.26

1.27
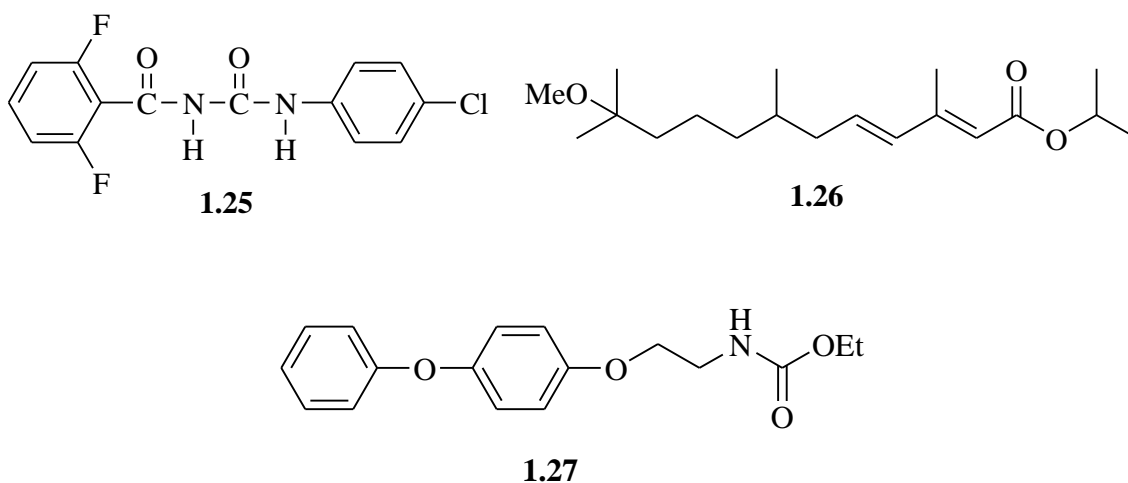
Resistance to juvenoids has however, been found in *Culex quinquefasciatus* in USA and Tanzania.[88] Resistance to diflubenzuron has also been detected in *Cx. quinquefasciutus* in Tanzania.[88]

1.3.6.5 Plant Derived Insecticides

In order to overcome insecticide resistance, it is imperative that new pesticides with different modes of action are developed. Currently, the main focus in pesticide research is in the development of new, selective and highly effective substances that cause no harm to human health and the environment. To achieve this, more extensive research has been focused on compounds of natural origin from plants and microorganisms that are shown to have insecticidal properties.

Research efforts have shown that plants are potential sources of alternative insect-control agents since they contain a range of bioactive compounds, many of which are selective and have little or no harmful effects on non-target organisms and the environment. For that reason efforts have continued to be focused on plant extracts or phytochemicals as potential sources of lead mosquito control agents.[89] This is because for self defense purposes, many plants generate chemicals that are toxic to insects. These naturally occurring botanicals comprise among others pyrethrum, nicotine, rotenone, *d*-limonene, sabadilla and ryania.[90] Such compounds are reviewed in the following sections.

1.3.6.5.1 Pyrethrins

Many oligophagous and polyphagous insect herbivores, monoterpenes have been demonstrated to act as toxins. Thus monoterpenes appear to play an important role in protecting plants from insect attack. The best known insect neurotoxins among monoterpenes are the pyrethrins. Pyrethrins are derived from the flowers of *Chrysanthemum cinerariaefolium* and were first recognized around 1800. The dried flower powders were originally used as insecticides that were later established to contain six insecticidal principles, namely pyrethrin I (**1.28**) and II (**1.29**), jasmolins I (**1.30**) and II (**1.31**), and cinerins I (**1.32**) and II (**1.33**).[91]

	R	R'
1.28	CH_3	C_5H_7
1.29	$COOCH_3$	C_5H_7
1.30	CH_3	C_4H_7
1.31	$COOCH_3$	C_4H_7
1.32	CH_3	$COOCH_3$
1.33	$COOCH_3$	C_5H_9
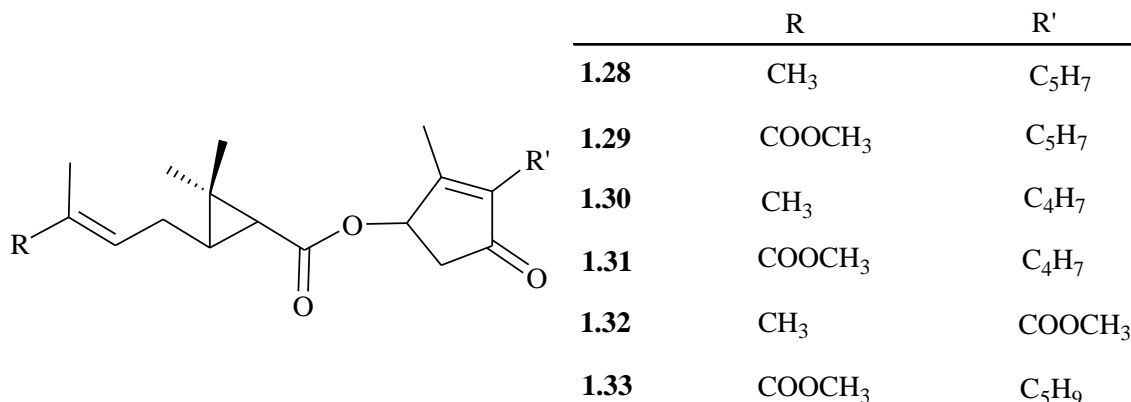
In the early days, finely ground pyrethrum daisy flowers were mixed with coconut husks or sawdust and made into mosquito coils. Presently, pyrethrum powder impregnated mosquito coils are still in use in many rural areas of Africa.

Although natural pyrethrins are effective insecticides, extreme photolability limits their broad application. This disadvantage prompted the need to prepare synthetic analogues, called pyrethroids. Currently there are a number of such analogues which are being used, thereby having eclipsed pyrethrins due to the high photostability of the synthetic ones. Nonetheless, both the natural pyrethrins and synthetic pyrethroids continue to be used and they all posses rapid insecticidal activity and repellent action.[92] Unlike natural pyrethrins, mosquito resistance to synthetic pyrethroids has emerged, despite the earlier optimism that their rapid toxicological action would not produce resistance.[93] Hence, this indicates the superiority of naturally derived substances in the management of biochemical systems in nature.

1.3.6.5.2   Other Plant Based Insecticides

Nicotine (**1.34**) is one of the oldest pesticides whose insecticidal properties were recognized as early as the mid-1700s.   Nicotine is an alkaloid that is derived from species of the Solanaceae plant family. The compound is obtained commercially from the leaves of tobacco plants (*Nicotiana tobaccum* and *N. rustica*). Anabasine (**1.35**) is another natural insecticide that is related to nicotine. It occurs in *Anabasis aphylla* (Chenopodiaceae) and has been commercially used in Russia as an insecticide.[94]   The toxicity of nicotine and the related alkaloids to non-target organisms including humans has limited the widespread use of these compounds as insecticides.
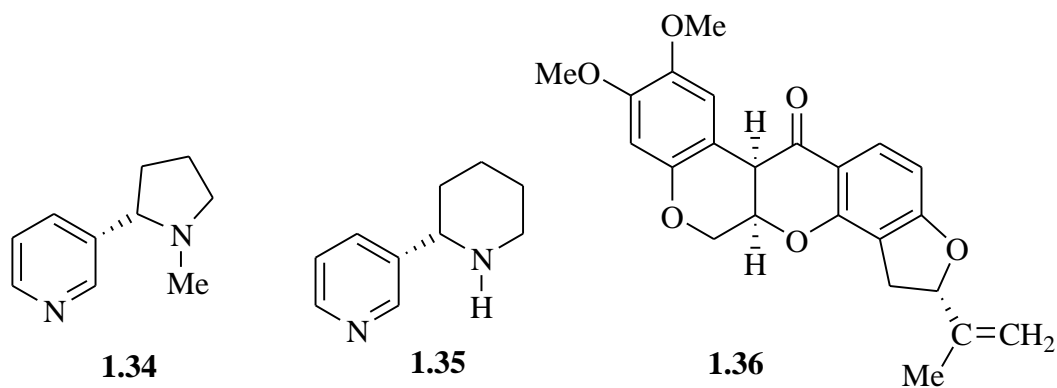
Another group of plant-derived insecticides comprises the rotenoids that occur in roots of some genera in the family Leguminosae. Six rotenoids have been isolated from roots of plant species of the genus *Derris*. These include rotenone (**1.36**) which is the most active compound among the *Derris* rotenoids. Rotenoids are also found in *Tephrosia* and *Lonchycarpus*.[95]

Rotenoids are also effective as fish toxins and have been used by native fishermen in Africa to rid undesirable species from lakes and streams.[96] The toxicity of rotenoids to non-target aquatic organisms and their skin irritation in humans has limited their large scale use as insecticides. In addition rotenone is also photolabile and hence it has limited persistence in field application.[95]

Long recognized for its insecticidal properties in India, the neem tree (*Azadirachta indica*) received a great deal of attention around the world as potentially useful and safe "bio-pesticide".[97] Azadirachtin (**1.37**) has been established as the insecticidal agent of the neem tree that grows widely in Asia and Africa.[98]

1.37
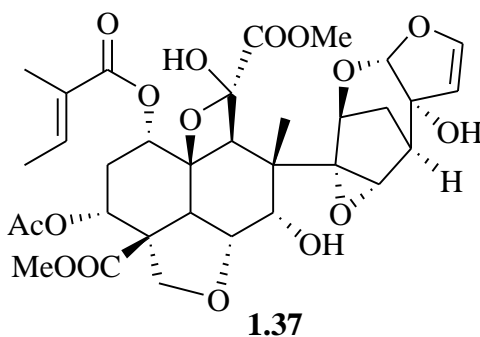
Extensive research has been carried out to evaluate the effectiveness of botanical derivatives of the neem tree and its relatives as biologically active substances.[99, 100] The research has led to the establishment of at least 35 biologically-active principles from the neem tree, of which azadirachtin is the predominant bioactive ingredient. It is found in the seeds, leaves and barks of the plant. The neem tree contents positively correlated with their toxic effects against insects. The effects may be grouped into six categories,

namely antifidancy, growth regulation, fecundity suppression, sterilization, oviposition repellency or attraction and changes in biological fitness.[101]

The repellency of neem oil to hematophagous insects has been tested, although the results have been variable. However, experiments using neem oil from the seeds have shown better protection. It was also proposed that neem may be used to repel mosquitoes by adding it to kerosene lamps used to light homes throughout the developing world.[102] Neem products are currently used for vegetable and flower pest control in USA.[103]

1.3.7   Personal Protection

Personal protective measures have become an important tool against mosquito nuisance and are often mentioned as the last line of defense targeting the adult mosquito. These are measures that protect the vertebrate host from mosquito bites and therefore preventing malaria pathogen transmission. Personal mosquito protection methods have generally involved the direct application of repellents, the use of insecticide treated clothes (ITCs), insecticide treated bednets (ITNs) and indoor residual spraying (IRS) of insecticides on walls.

The WHO "Action Plan for the Reduction of Reliance on DDT in Disease Vector Control" recommends research into "the effectiveness, sustainability, and affordability" of insecticide-treated materials as one of the handful of approaches to replacing DDT use in malaria control.[104]   This has led to intensified research towards the establishment

of viable anti-mosquito agents, including mosquito repellents, mosquitocides and larvicides.

1.3.7.1 Insecticide Impregnated Bednets

ITNs have widely been tested in the control of malaria and have shown great potential in reducing both morbidity and mortality due to malaria. They are being promoted as the fundamental strategy to Roll Back Malaria in Africa.[105-109] The impregnation of synthetic pyrethroid insecticide (deltamethrin) into ropes, bed nets and curtains in the human dwellings has so far been found to be promising against *Anopheles* and *Culex* mosquito species.[110, 111] Mosquito nets treated with a water dispersible formulation of deltamethrin (K-O TAB) was evaluated and found to be effective against malaria vector mosquitoes.[112] The WHO recommends olyset nets that are permethrin insecticide impregnated and these are currently in use in rural malaria endemic areas.[113] However, many people still do not own nets, particularly ITNs. Therefore, campaigns against malaria have of late included strategies to enable populations in malaria endemic areas to acquire ITNs, including offering subsidies to buy the nets or simply distributing them freely.

1.3.7.2 Indoor Residual Spraying (IRS)

An approach that formed the mainstay of malaria vector control in Africa between 1940 and 1970 was spraying of interior walls of homes with insecticides, termed as IRS. In this regard, the use of insecticide impregnated paint has also been established. Thus,

Vernacide is an example of insecticide-impregnated paint effective against mosquitoes.[114] This paint is safe and can be conventionally applied in public places where pest free conditions are desirable for considerably longer time.

Unfortunately, due to various factors including high costs and environmental awareness, the indoor spraying programmes are no longer practiced in most African countries. However, a number of studies on the comparative efficacy, cost effectiveness and affordability of ITN and IRS suggest the later to be cheaper, but this may vary depending on the insecticide used and the delivery system employed. Nonetheless, both methods are not useful for mosquito vectors that tend to rest outdoors. For instance *Anopheles darlingi* and *An. dirus* exhibit tendencies for outdoor resting while *An. minimus*, and *An. sinensis* prefer outdoor feeding, making them less susceptible to the IRS strategy. In such cases ITNs may be useful, particularly if supplemented by an effective insect repellent.[114-117]

1.4 Historical Review on Mosquito Repellents

It is likely that the use of repellents against biting arthropods developed since time immemorial. Several species of primates have been observed anointing their pelage by rubbing it with millipedes and plants including *Citrus* species*, Piper marginatum,* and *Clematis dioica*.[118-120] Wedge-capped capuchins (*Cebus olivaceus*) have been observed rubbing the millipede *Orthoporus dorsovittatus* onto their coat during the period of maximum mosquito activity.[121,122] The millipede is known to contain insect repellent

benzoquinones. It was hypothesized that the anointing behaviour was designed to deter biting insects. Laboratory studies have shown a significant repellent effect of benzoquinones against *Aedes* (*Stegomyia*) *aegypti* (the yellow fever transmitting mosquito) and *Amblyomma americanum* (the lone star tick).[123, 124]

In Italy which was once a swampland infested by malaria vectors *Anopheles labranchiae, An. sacharovi,* and *An. superpictus* prior to the malaria eradication programme of 1947, the Romans recorded methods of repelling insects (gnats) which may have included mosquitoes.[125] The use of smoke against biting insects was carried out by European settlers as recorded from settlers to The Black Swamp in Ohio.[126]

1.4.1   Traditional Repellent Currently in Use Today

Poor rural populations throughout the tropics that cannot afford modern shop-bought personal mosquito protection agents, extensively use natural fumigants to drive away mosquitoes. Thus, the use of traditional fumigants is particularly widespread in Africa. For instance, studies have indicated that 13% of rural Zimbabweans use plants and 15% use coils to deter mosquitoes. Similarly, 39% of Malawians burn wood, dung or leaves and a good percentage of Kenyans burn plants to repel mosquitoes, while about 55% of the people in Guinea Bissau burn plants or hang them in homes to repel mosquitoes.[127, 128] The most commonly used plant mosquito repellent in Africa include the neem (*Azadirachta indica*), *Hyptis* spp. (bushmint family), *Ocimum* spp. (basil family),

Corymbia spp. (formerly *Eucalyptus* spp.) and *Daniellia oliveri*, all of which are reported to be more than 70% effective against *Anopheles gambiae* in field trials.[129, 130]

Waste plant materials are frequently burned in Sri Lanka as mosquito repellents, even though indoor residual spraying has been carried out by the government for many years.[131] A number of surveys have also revealed that fire from coconut husks, papaya leaves and other waste materials is the most prevalent form of personal protection from mosquitoes in Solomon Islands, Mexico and Guatemala.[132]

The use of smoke, although effective, requires continuous production in order to repel biting insects when used as an outdoor mosquito repellent.[133] However, smoke has a residual repellent effect when used in houses,[134] but indoor combustion of biomass has severe health consequences.[135] Therefore, safer, more modern methods of repelling mosquitoes are desirable.

1.4.2   Pyrethrum, Mosquito Coils and Area Repellents

Pyrethrum is natural plant oil comprising six esters **1.28-1.33**. It is relatively harmless to mammals and it is currently incorporated into coils to repel mosquitoes. Presently, mosquito coils are widely used, whereby about 29 billion mosquito coils are sold each year, 95% of them in Asia. They command substantial household expenditure in many developing countries. There is ample evidence that mosquito coils effectively repel mosquitoes.[136-141]

The insecticidal efficacy of pyrethrum is derived from its effects on the central nervous systems of all types of flying and crawling insects by blocking sodium-gated nerve junctions. This leads to failing of the nervous impulses, [142] thereby knocking down the insect, which may eventually die. In lower concentrations, pyrethrum affects insect behaviour by producing the so-called 'avoidance reaction' or "exito-repellency", which results in the insect fleeing the source of the chemicals.[143] Synthetic analogues of pyrethrum were developed from the 1940s onwards and they exhibit a similar mode of action but are more potent and photostable.[144-148]

1.4.3 Progress Towards Modern Synthetic Repellents

Essential oils have received more attention as potentially useful bioactive compounds against insects, showing a broad spectrum of activity against insects, low mammalian toxicity and degrading rapidly in the environment. Many essential oils from plants have been tested and some have been used for personal protection against biting insects. Some plant species that have been reported to posses insect repellent activity include citronella, cedar, verbena, pennyroyal, geranium, lavender, pine, cajeput, cinnamon, rosemary, basil, thyme, allspice, garlic and peppermint, amongst others.[149-151] The repellency of essential oils appears to be associated with the presence of one or more volatile monoterpenoid constituents. Although effective when freshly applied, their protective effects dissipate.[152, 153] Most readily available plant derived monoterpenoids with repellent activity include, amongst others citronellal (**1.38**), citronellol (**1.39**), linalool (**1.40**), cineole (**1.41**), camphor (**1.42**), α-pinene (**1.43**) and β-pinene (**1.44**).[2]

1.38   **1.39**   **1.40**   **1.41**

1.42   **1.43**   **1.44**
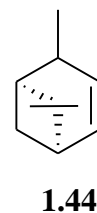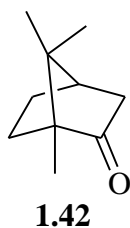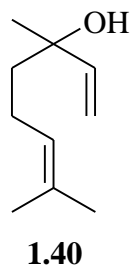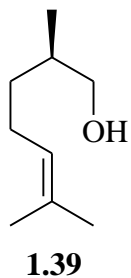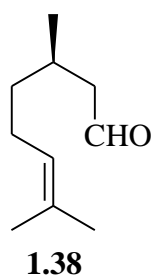
The first repellents used in the military contained essential oils derived from plants. For instance, the Indian Army was issued with an insect repellent comprising citronella, camphor, and paraffin. However, the repellents had limited duration and tended to give transient protection. This prompted intensive research during the Second World War to establish long-lasting insect repellents motivated by the enormous burden of disease suffered by troops fighting in areas endemic with malaria and other insect borne diseases.[151]

Interestingly, not many efforts have been made in deploying monoterpenoid essential oils constituents as leads in the development of highly effective insect repellents.

1.4.4 Synthetic Repellents

Between 1942 and 1945, over 7,000 potentially repellent compounds were tested by the United States Department of Agriculture (USDA). One of the first chemical repellents to be developed was dimethyl phthalate [DMP (**1.45**)], followed by Indalone® (butyl-3,3-dihydro-2,2-dimethyl-4-oxo-2*H*-pyran-6-carboxylate, **1.46**) that was patented in 1937, and ethyl hexanediol (2-ethyl-1,3-hexanediol, **1.47**) also called Rutgers 612, which became available in 1939.[152]

| **145** | **1.46** | **1.47** | **1.48** |
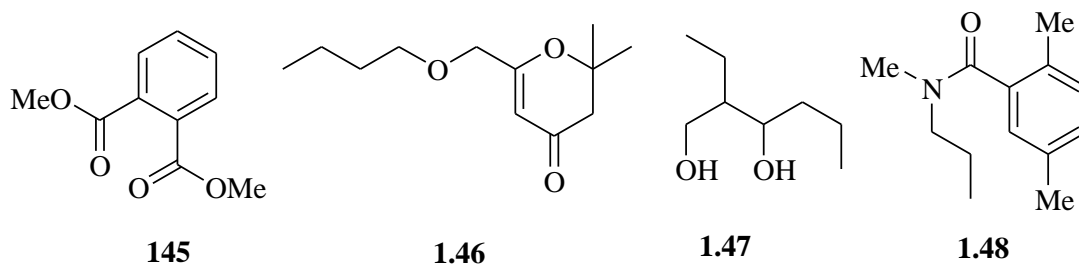
The use of these earlier insect repellents was eclipsed by the discovery of DEET (*N,N*-diethyl-*3*-methlybenzamide or *N,N*-diethyl-*m*-toluamide, **1.48**) in 1953.[153]   This was perhaps the single most important event in the evolution of insect repellents and DEET remains the principal, and still generally considered, the most effective repellent in use today, more than 50 years after its discovery.[154] DEET is a broad spectrum repellent that is highly effective against most mosquito species.[155-161] Each year an estimated 15 million people in the UK, 78 million people in the USA, and 200 million people globally use DEET. There has been much speculation on the safety of DEET following reports linking it to seizures and encephalopathy, particularly in children.[161-166]

1.4.5 Recent Discoveries of Repellents

The importance of controlling insect pests has led to the development of a variety of insecticides that prevent agriculture losses and spreading of diseases. Toxicological studies based on acute and chronic effects upon exposure have revealed that many classical insecticides are highly toxic not only to non-targeted insect species, but also to mammals and humans. Consequently, the search for safer alternatives for pest control is needed. Thus, intensive research is currently being carried out to obtain chemically modified substances with improved insecticidal activity in terms of selectivity towards insects and low toxicity to the environment, and to non-targeted species including humans. The combination of new synthetic approaches, biological and physiological studies are geared towards the preparation of insecticides with a better environmental profile, having different mechanisms of actions, and reduced risks on living systems.

Recently, DEPA (*N,N*-diethylphenylacetamide, **1.49**), a compound developed around the same time as DEET, [153] has received renewed attention. It has similar cosmetic properties to DEET and similar dermal absorption and excretion. It also displays symptoms of acute poisoning similar to DEET.[168] DEPA may prove useful, particularly among residents of developing countries for whom cost is the main motivator in personal repellent choice.[169] However, the negative effects similar to DEET could limit its acceptability.
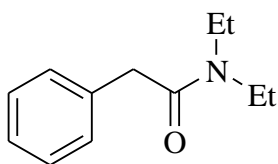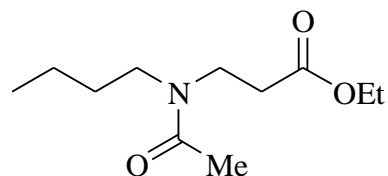
1.49   **1.50**

Insect repellent 3535 [IR3535, 3-(*N*-acetyl-*N*-butyl)aminopropionic acid ethyl ester, (**1.50**), also known as MERCK 3535, was developed in 1975 by Merck. The repellent has been on the market in Europe for the past thirty years. It has low toxicity, although it is irritating to the eyes and sometimes the skin. Nonetheless, the WHO has declared it a safe and effective repellent for human use.[170]

1.4.6   Piperidine Compounds

There has been a flurry of renewed interest in piperidine-based compounds as leads for the discovery of several new and highly effective repellents. Piperidine is a cyclic amine deriving its name due to the presence of the piperidine skeleton also found in piperine, the main active chemical agent in pepper (*Piper* sp.). The repellent picaridin [1-piperidine carboxylic acid, 2-(2-hydroxyethyl)-1-methylpropyl ester (**1.51**)] was developed by BAYER in the 1980s using molecular modelling.[171] It's most important new feature is its very low toxicity, and most importantly, it elicits practically no dermal or eye irritation nor skin sensitisation.[172] Cosmetically it is superior to DEET as it is colourless, odourless and has a pleasant feel on the skin.[173]

1.51
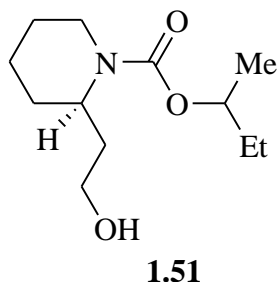
The efficacy of picaridin is excellent, and it is generally superior to DEET in terms of longevity. This is because KBR 3023 evaporates at a slower rate than that of DEET. However, it is less effective than DEET when freshly applied.[174] The combination of efficacy, safety and cosmetic appeal led to the WHO deeming KBR 3023 its "repellent of choice for malaria prevention".[175] In addition, the Centre for Disease Control (CDC) recommended both DEET and KBR 2023 for West Nile and malaria prevention.[176]

1.4.7 SS220

The latest development in synthetic skin repellents is (1*S*,2*S*)-2-methylpiperidinyl-3-cyclohexen-1-carboxamide (**1.52**), discovered by USDA and dubbed SS220. It is derived from AI3-37220 – in so much as it is the most repellent of the four racemic isomers that comprise AI3-37220, and it is 2.5 times as effective as the racemic mixture against *Ae. aegypti*.[177] Laboratory tests showed SS220 to show efficacy equal to DEET against *An. stephensi* and *Ae. aegypti*, and better than KBR 3023 against *Ae. Aegypti*.[178] Extensive toxicological tests have shown low irritation and toxicity.[179, 180] In addition, SS220 has a low rate of evaporation that leads to improved longevity.
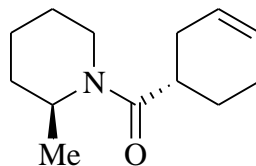
1.52

Its user acceptability is also likely to be higher because SS220 has slightly fruity odour, feels good on the skin, isn't sticky and it has little plasticizing effect.[180] The disadvantage of SS220 lies in the fact that it is a single stereoisomer, and will therefore be more costly to produce than a racemic mixture.

1.5 Structure Activity Relationship Studies: A Brief Historical Review on Selected Insecticides

The conventional strategy for pesticide and drug synthesis has largely been based on identification of active lead compounds followed by programs to synthesize analogues, based on structure activity relationship (SAR) considerations. Most of the insecticides represented by the chlorinated hydrocarbons, organophosphate esters, *N*-methyl carbamates, and benzophenylureas were derived largely from analogue synthesis and SAR optimization. This is evidenced by their closely related structural features.

Natural products of plant, animal or microbial origins are a vast source of bioactive compounds, which have been exploited only to a limited extent as models in the development of commercial insecticides. Undoubtedly, the most important and

significant application of a natural model insecticide from botanical origin can be exemplified by the insecticidal properties of pyrethrins and the synthetic pyrethroids.

1.5.1   Pyrethroids

The increased usage of chlorinated hydrocarbons and organophosphates during the 1950s and 1960s resulted in decreased demand for more expensive and less stable natural pyrethrins. However, the problems associated with these synthetic insecticides, such as insect resistance, environmental persistence and high mammalian toxicity gave renewed attention to the pyrethrins.

Studies on pyrethroid chemistry started about 1910, and have since involved two main periods. The first period was devoted to the elucidation of the basic chemical structure of the natural pyrethrins, which consists of an acid moiety, an ester linkage and an alcohol moiety. The compounds constituting the pyrethrins were during this period established to be pyrethrins-I (**1.28**) and –II (**1.29**), cinerins-I (**1.30**) and –II (**1.31**), and jasmolins –I (**1.32**) and –II (**1.33**). These were established as the active principles of the pyrethrins. In 1958, the absolute configuration of the alcohol moiety was determined and hence the complete stereochemical structure for the natural pyrethrins was unraveled. This opened up synthetic endeavours as the next period in the evolution of the chemistry of pyrethrins. During this period a number of useful pyrethroids were invented.[181-184]

Endowed with advantageous properties over the natural pyrethrins, such as enhanced insecticidal activity, improved photostability and lower cost, some of the synthetic pyrethroids were put into practical use in broad areas including household insecticides and agrochemicals. The demand for pyrethroids is still expanding as substitutes for natural pyrethrins have really not yet been established. However, the high toxicity to fish and the development of pyrethroid resistance in some pests are cited as common shortcomings for the use of pyrethrins as regular insecticides.[184] Therefore, research efforts continue to be focused on addressing the shortcomings displayed by pyrethrins, so as to establish better analogues devoid of the above stated shortfalls.

1.5.1.1 Modification of the Pyrethroids Alcohol Moiety

Various pyrethroids have been developed by retaining chrysanthemic acid as the acid moiety and modifying the alcohol function of natural pyrethrin-I (**1.28**). These modifications have led to the establishment of a number of useful compounds that have been commercially produced as active ingredients mainly for household insecticides.[185-192] Thus, through such modifications a number of insecticidal compounds have been developed, including dimethrin (**1.53**), benathrin (**1.54**), phenothrin (**1.55**), and cyphenothrin (**1.56**, **Scheme 1.2**).[193]

Other studies have been focused on furan ring compounds, leading to the development of the pyrethroids japothrin (**1.57**), furamethrin (**1.58**), proparthrin (**1.59**) and resmethrin

(**1.60**). Other studies on the alcohol moiety have led to the discovery of tetramethrin (**1.61**), allethrin (**1.62**), pralethrin (**1.63**) and empenthrin (**1.64**). [194]
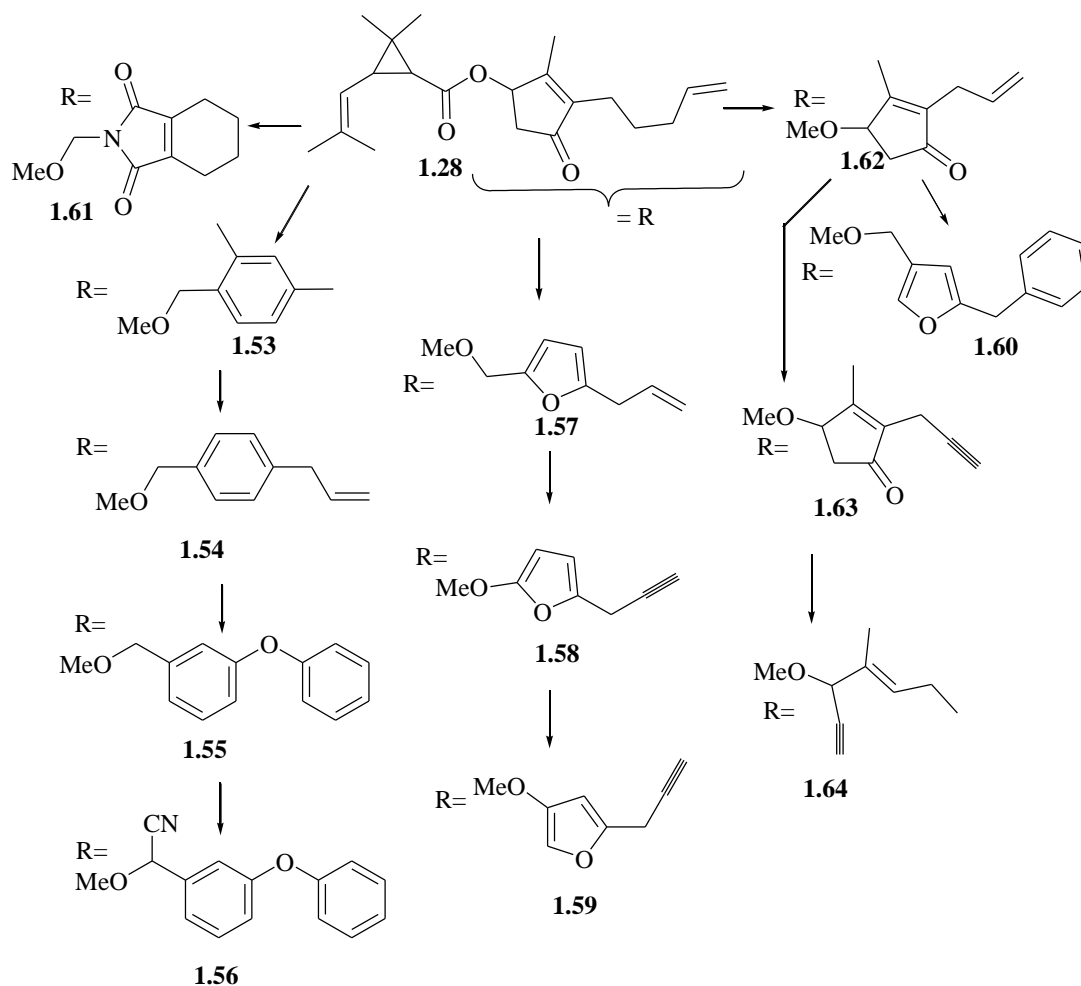
Scheme 1.2   Synthetic modification of pyrethrin-I alcohol moiety

1.5.1.2 Modification of the Acid Moiety and Ester Linkage

A number of pyrethroids having a chemically stable phenoxybenzyl group as the alcohol moiety but a modified acid moiety have also been developed. A major development in

the pyrethroid chemistry was the replacement of the isobutenyl side chain in the parent molecule **1.28** with a dichlorovinyl group, and the alcohol unit with 3-phenoxybenzyl alcohol. This led to the synthesis of permethrin (**1.65**) as the first photostable pyrethroid. Later on the substituted-cyclopropanecarboxylic acid moiety was replaced by an isovaleric acid residue, affording the commercially available insecticide fenvalerate (**1.66**, **Scheme 1.3**).[195]
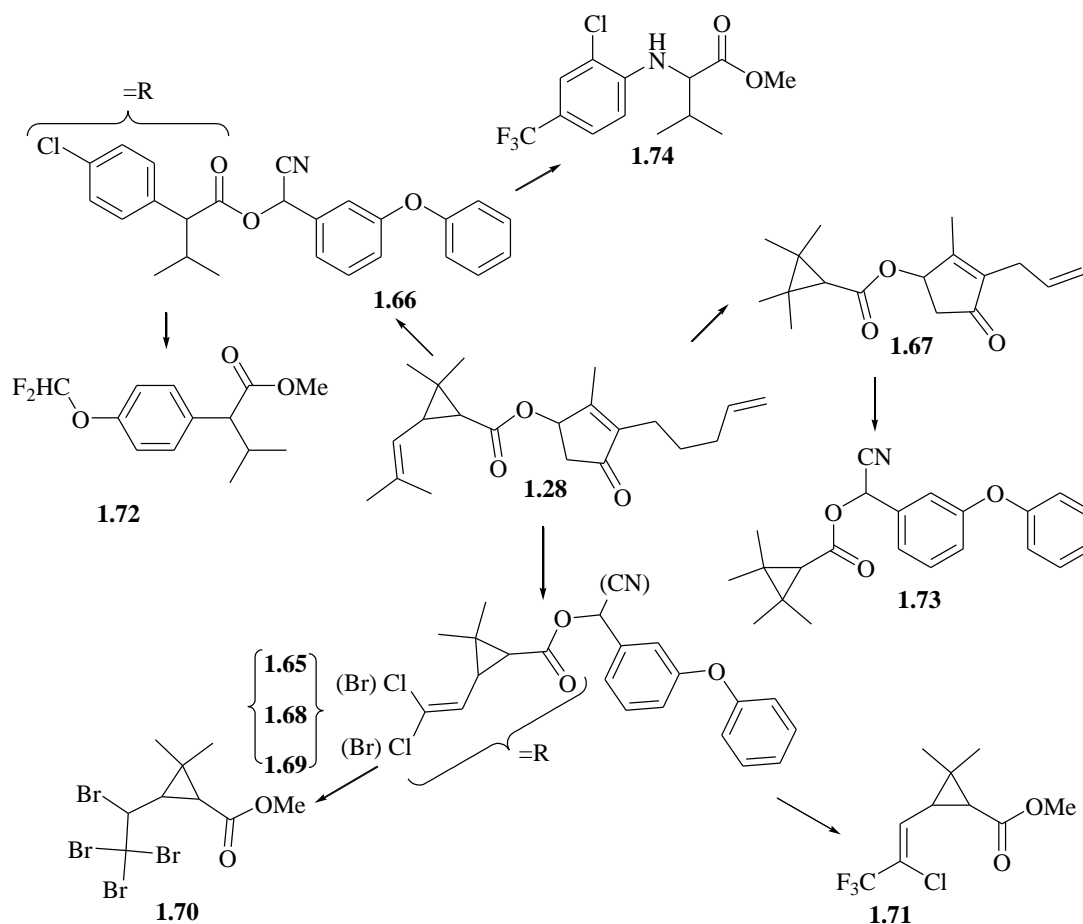
Scheme 1.3   Modification of the acid moiety in pyrethroids

Studies on this modification in the 1970s were conducted by agrochemical companies around the world and this led to the development of a number of pyrethroid analogues including terallethrin (**1.67**), cypermethrin (**1.68**), deltamethrin (**1.69**), trahalomethrin (**1.70**), cyhalothrin (**1.71**), flucythrinate (**1.72**), fenpropathrin (**1.73**) and fluvalinate (**1.74**).[195]

At present, pyrethroids account for about 20% of the total volume of insecticides used all over the world. Some of the synthetic pyrethroid analogues are highly potent in their insecticidal action. Thus, Deltamethrin is one of the most potent insecticides, having a potency rate of about 600 times that of DDT against *An. stephensi* and 34 times that of Bioresmethrin against house fly (*Musca domestica*).[196]

The evolution of pyrethroids reveals that small changes in substituents and stereochemistry are sufficient to produce compounds differing in their insecticidal potency, spectrum of activity and mammalian toxicology. Synthetic approaches have allowed the development of pyrethroids to be available not only for indoor use, but also for crop protection, veterinary and medical pest management. The availability of pyrethroids as naturally derived insecticides strongly suggests the untapped potential of lead insecticides and other biochemical modulating chemicals existing in nature, thus awaiting exploitation for further development.

1.5.2 Chitin Synthesis Inhibitors

Chitin is a polysaccharide present in certain groups of invertebrates such as insects and nematodes. Thus, chitin synthesis inhibitors (CSIs) when applied to insects, interfere with the deposition of chitin in their cuticle by inhibiting the action of chitin synthetase, the key enzyme which regulates the last step of chitin polymerization.[197] Thus, the moulting process of insects is disturbed and the larvae fail to metamorphose to the next developing stage.

The discovery of CSIs was a result of efforts to develop new herbicides. While these newly developed compounds were ineffective as herbicides, they also proved to be potent insecticides. Classified as benzophenylureas these compounds posses a number of halogen substituents. Diflubenzuron (DFB, **1.25**) is the prototypical compound in this series. More recent modifications have led to the synthesis of a number of analogous compounds, including novaluron (**1.75**), hexaflumuron (**1.76**), teflubenzuron (**1.77**) and chlorfuazuron (**1.78**). However, all these compounds have considerably higher toxicity level than DFB.[198]

1.75

1.76
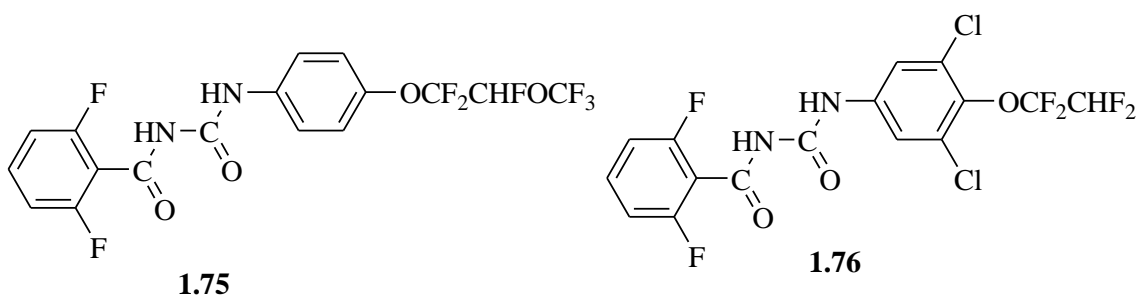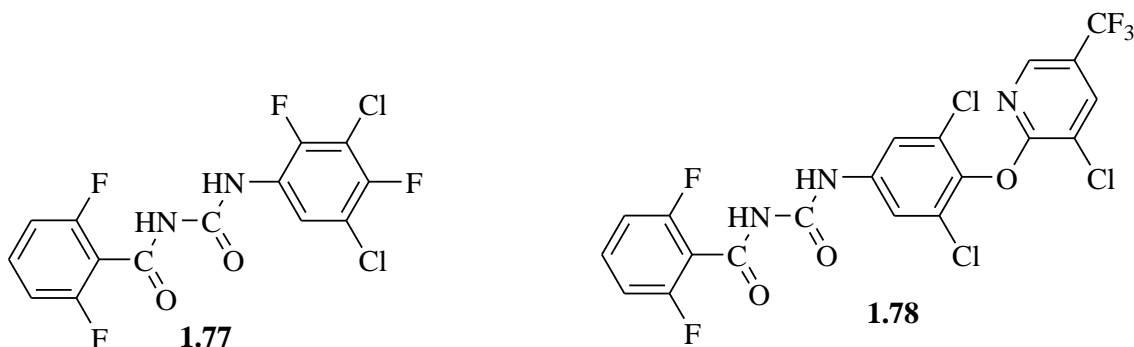
The preceding highlights represent a brief historical review on how a number of presently used insecticidal agents were discovered through the application of structural activity relationship (SAR) as a strategy to develop bioactive compounds with enhanced bio-activity and stability, and reduced mammalian toxicity, as well as environmental concerns through modification of "bioisosteres".

Bioisosteres are compounds or groups that possess near-equal molecular shapes and volumes, approximately the same distribution of electrons, and which exhibit similar physical properties.[199] The concept of bioisosterism has been coined to explain SAR phenomenon in drug discovery and development. This concept envisions bioisosteres as molecular units that would portray the same bioactivity but at different efficacy levels depending on their physical or chemical properties. Thus, in drug design exchanging one substituent for another is for the purpose of enhancing the desired biological or physical property of a compound without making significant changes in its chemical structure.[200]

The following section gives a review on SAR approaches in the establishment of mosquito repellent principles based on menthane-type monoterpenoids that share some

structural similarities with the known semi-synthetic repellent, *p*-menthane-3,8-diol (**1.79**) as the prototype. Most monoterpene components of essential oils are pleasantly smelling. They are non-toxic to humans and other mammals and hence they could be good candidates for the development of drugs for topical application, perfumes as well as insect repellents. However, only few screening programs have been implemented for assessing the insecticidal activity of essential oils and their monoterpenoid constituents.
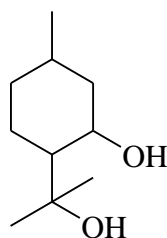
1.79

1.6   Menthanes

The menthane group of monoterpenes comprises three isomeric types, *o*-, *m*- and *p*-menthanes. The *p*-menthanes represented by **1.80** are the most widespread and arise by cyclisation of a regular acyclic monoterpenoid. The *o*- and *m*-menthanes (**1.81** and **1.82**) occur quite rarely, and presumably they are formed through alkyl migration of *p*-menthanes.[201]
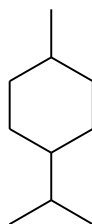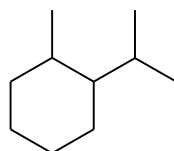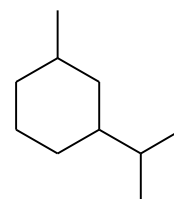
1.80   **1.81**   **1.82**

Menthanes are widely distributed in the essential oils of a number of plants. Thus, the essential oil of *Cymbopogon densiflora* consists of limonene (**1.83**), carveol (**1.84**), carvone (**1.85**) and *p*-mentha-2,8-dienol (**1.86**) as the main components.[202] On the other hand the *Calamintha nepeta* essential oil is known to constitute the unsaturated menthane limonene (**1.83**), and carvone (**1.85**).[203] The lemon eucalyptus extracts is obtainable from the plant *Corymbia citriodora* (synonyms include *Eucalyptus citriodora* and *Eucalyptus maculata* var *citriodora*), that has been known for a long time in China for its mosquito repellent properties.[204-206]

The essential oils from the leaves of *Uvariodendron gorgonis* was also found to exhibit high mosquito repellency while that from *Vepris trichocarpa* elicited moderate repellency against *An. gambiae* mosquitoes in comparison to DEET. *U. gorgonis* whose main monoterpenoid constituents are citronellal, linalool and limonene had an RC_{50} value of 1.13×10^{-4} while *V. trichocarpa* constituting over 80% of α-pinene had 0.04 in comparison to 3.95×10^{-4} for DEET under similar experimental conditions.[207] Studies have also shown that the essential oils from *Clausina anisata* and *Lantana vibrurnoides* composed mainly of monoterpenoids exhibit significant mosquito repellency against *An.*

gambiae mosquitoes. Among the major compounds in these two plants are piperitenone (**1.89**), artemisia ketone (**1.90**), verbenone (**1.91**), limonene and linalool.[208]

1.83   **1.84**   **1.85**   **1.86**

1.87   **1.88**   **1.89**   **1.90**   **1.91**
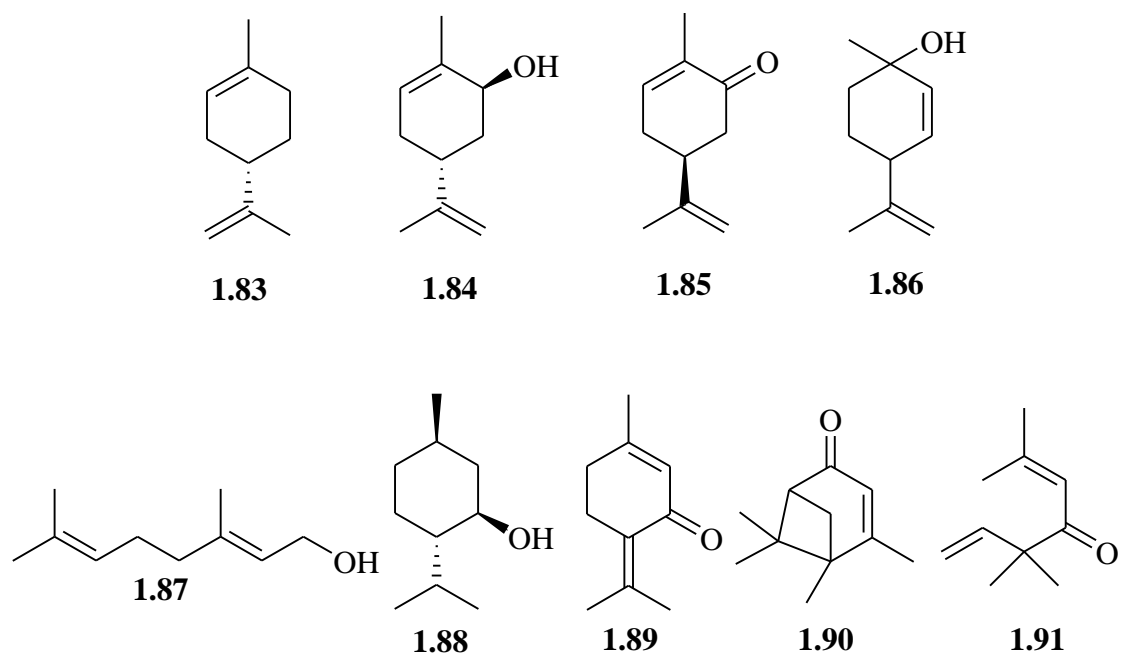
Chemical analysis of *Corymbia citriodora* showed it to contain citronellal (**1.38**), citronellol (**1.39**), β-pinene (**1.44**), geraniol (**1.87**), isopulegol (**1.88**), and a number of sesquiterpenes.[209] The essential oil extract was determined to have mosquito-repelling properties against *Aedies aegypti*, although the effects were limited to one hour.[210]

p-Menthane-3,8-diol (PMD, **1.79**) was discovered as the major constituent of the by-product of the distillate of leaves from the lemon-scented eucalyptus (*C. citriodora*). It is a relatively low volatile white waxy monoterpene that is produced as a distillate after acid modification of lemon eucalyptus essential oil.[211] Formulations of the diol compare favorably with those of DEET in repelling the most important Afrotropical malaria

vector mosquitoes, *Anopheles gambiae* Giles, *An. funestus* Giles, and *An. arabiensis* Paton.[212] PMD has undergone several trials in different parts of the world for its mosquito repellent properties. Thus, field studies in China showed that the protection time against *Aedes vexan* and *Ae. albopictus* was 2 and 5.5 h respectively when PMD was used in a 20-30% glycerol and/or alcohol formulation.[210] In studies carried out in Tanzania, 50% PMD in isopropanol was found to provide 6 h of protection against the local malaria vector mosquitoes *An. gambiae* and *An. funestus*.[212] In field experiments conducted in the Bolivian Amazon, 30% PMD in an alcohol base was established to provide 96.9% protection for up to 4 h post application from all mosquito species, compared to 84.8% protection from 15% DEET. [213]

PMD is now a well established natural product, which has proven field efficacy in repelling mosquitoes. In addition, acute toxicity studies have shown only limited toxicity.[212] For these reasons, the potential for commercial exploitation of this compound in the development of mosquito repellents is high. Currently, PMD is available commercially in several countries in Europe and the USA and it is the only plant-derived repellent that is approved for use in disease prevention by the CDC.[214] The repellent has also been found to be effective against midges, ticks and the stable fly. Moreover, its mammalian toxicity is lower than that of DEET.[204]

The high efficacy and safety of PMD as a mosquito repellent is a good insight for exploration of other closely related *p*-menthane diols for their repellent activity against mosquitoes. In existing literature, the synthesis of *p*-menthane-1,2-diol (**1.92**) using

different approaches is reported, including repellent activity against *Ae. Aegypti* in 3 hours.[215, 216]
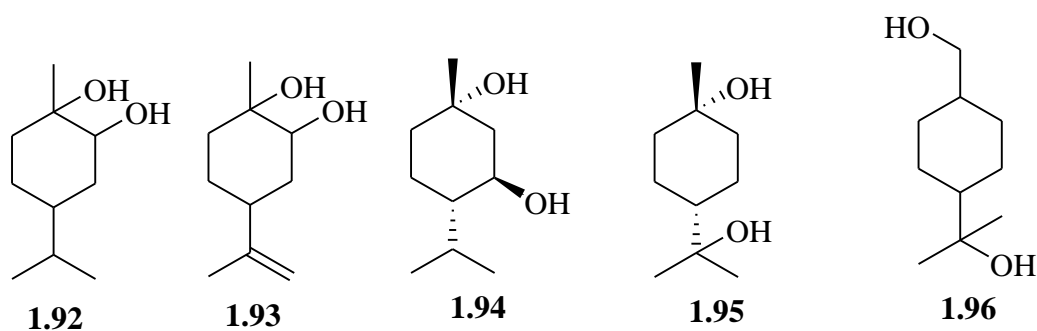
The repellent activity exhibited by the *p*-menthane diols as discussed above suggests that the presence of the diol functionality on the menthane skeleton could be responsible for the activity. Hence, this factor could be a subject for further research.

Other menthane diols that have been synthesized include *p*-menth-8-en-1,2-diol (**1.93**), [216, 217] *p*-menthane-1,3-diol (**1.94**), [218] *p*-menthane-1,8-diol (**1.95**), [219] *p*-menthane-7,8-diol (**1.96**) and *p*-menthane-2,4-diol (**1.97**).[220] A number of other closely related congeners have been isolated from plants. These include *p*-menthane-2,3-diol (**1.98**), which was obtained from the essential oil of *Mentha gentiles* L.[221] and *p*-menthane-2,5-diol (**1.99**) from peppermint oil.[222] A successful synthesis of *p*-menthane-2,3-diol has also been described.[223] However, so far all the *p*-menthane diols stated above have not been subjected to mosquito repellent assays so as to determine their efficacy. Therefore, this is a subject that required further research.

1.97   **1.98**   **1.99**   **1.100**   **1.101**
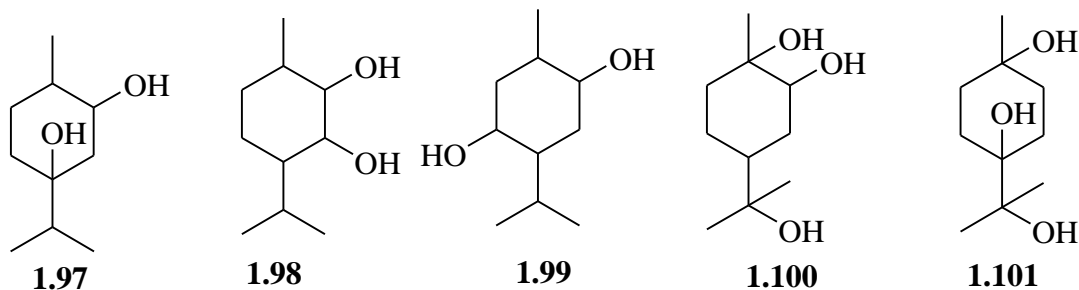
A number of menthane triols have also been synthesized including *p*-menthane-1,2,8-triol (**1.100**) and *p*-menthane-1,4,8-triol (**1.101**) amongst many others.[224, 225] However, they also need to be tested for mosquito repellence and compare any established activity with that of the menthane diols stated above.

In the recently published investigations a series of *N*-substituted *p*-menthane carboximides such as *N*-methyl-*p*-menthane-3-carboximide (**1.102**), *N*-ethyl-*p*-menthane carboximide (**1.103**) and *N*-propyl-*p*-menthane carboximide (**1.104**) have been reported to have been synthesized and tested for insect repellence. The compounds exhibited repellent activity and low mammalian toxicity.[226]

	R	R'
1.102	Me	H
1.103	Et	H
1.104	Propyl	H
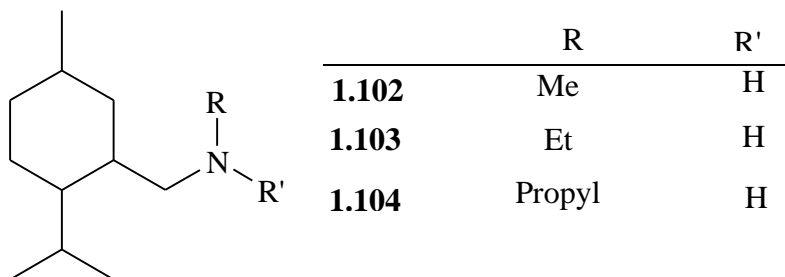
The effectiveness of these compounds as insect repellent was determined by testing them against the German cockroach and the results obtained were far better than for the benchmark repellent, DEET.

1.7   Cancer and its Causes

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells, which if not controlled can result into death. Cancer is caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. The development of most cancers is a multiple step phenomenon that occurs over many years. Certain types of cancer can be prevented by eliminating exposure to tobacco and other factors that accelerate this process. Other potential malignancies can be detected before cells become cancerous or at an early stage, when the disease is most treatable. Cancer is treatable by surgery, radiation, chemotherapy, hormone supplements, and immunotherapy.[227]

1.7.1   Cancer Cases

Cancer remains a major public health problem in the world. The disease is responsible for approximately several million deaths annually, both in developed and developing countries. It was earlier estimated that there would be more than 12 million new cancer cases in 2007 worldwide, of which 5.4 million would occur in economically developed

countries and 6.7 million in economically developing countries.[228] The corresponding estimates for total cancer deaths in 2007 were 7.6 million (about 20,000 cancer deaths a day), 2.9 million in economically developed countries and 4.7 million in economically developing countries. By 2050, the global burden is expected to grow to 27 million new cancer cases and 17.5 million cancer deaths. This has been attributed to the growth and aging of the world population.

In economically developed countries, the three most commonly diagnosed cancers are prostate, lung and bronchus, and colorectal among men, and breast, colorectal, and lung and bronchus among women. In economically developing countries, the three most commonly diagnosed cancers are lung and bronchus, stomach, and liver in men, and breast, cervix uteri, and stomach in women. In both economically developed and developing countries, the three most common cancer sites are also the three leading causes of cancer death.[228, 229]

While in the past years cancer has been regarded mainly as a group of diseases afflicting the more developed countries, the incidence of various forms of cancer is now rapidly rising worldwide. The World Health Organization database on cancer incidence and mortality indicates substantial numbers of cases of major cancers in less developed countries.[230] As such cancer prevention has become an integral part of the control of the disease with common approaches involving avoidance of exposure to known cancer-causing agents, enhancing host defense mechanisms against cancer, modifying life styles, and chemoprevention. However, even with improvement in the early detection

and treatment, overall mortality rates for most cancers of epithelial origin have not declined in the last 30 years. Carcinomas account for more than 80% of human cancers, with skin, lung, colon, breast, prostate, and uterus being the most frequent sites.[231]

1.7.2 Chemoprevention

Carcinogenesis can be viewed as a process that involves accelerated, and abnormal cellular growths in which the genes controlling proliferation, differentiation, and apoptosis are transformed under selective environmental pressure in the body. Tumor development follows three distinctive phases: initiation, promotion, and progression. Since the initiation and progression phases are irreversible events, the promotion phase of carcinogenesis may provide the best target for cancer prevention. [232]

Cancer chemoprevention has emerged as an important means of modulating the process of carcinogenesis with research indicating reduced incidence of cancer in high risk groups in the general population.[231, 233] By definition, chemoprevention refers to the use of agents so as to slow the progression of, reverse, or inhibit carcinogenesis, thereby reducing the risk of developing invasive or clinically significant disease. Consequently, an effective chemopreventive agent should intervene early in the process of carcinogenesis to eliminate premalignant cells before they become malignant. [234, 235] Thus, effective chemopreventive treatments for cancer could have an important impact on disease morbidity.

Several thousand chemical agents have been reported to possess chemopreventive activity, and more than 40 promising agents and their combinations are undergoing clinical evaluation for cancer chemoprevention. In this regard several classes of natural compounds have been evaluated for cancer chemopreventive purposes. Each of these classes of plant-derived compounds or extracts interacts with the host to confer a preventive benefit by modulating cellular signaling of proliferation and/or differentiation.[235]

Since cancer chemopreventive agents are considered to be promoters of cytostatic effects, it has been suggested that they should be administered through long-term practices, to healthy individuals who have an increased cancer risk. However, this approach has also been considered to have risks of long-term toxicity and the possibility of individuals developing resistance to chemopreventive agents, thereby limiting the feasibility and success of conventional chemoprevention. An alternative chemopreventive approach entails the use of chemical agents that quickly eliminate premalignant cells by inducing them to undergo apoptosis rather than slowing their proliferation and/or promoting some degree of differentiation thus limiting the risk of long-term toxicity and/or the development of chemo-resistance.[235]

1.7.3 Apoptosis

Apoptosis or programmed cell death is a highly organized physiological process that eliminates redundant or potentially deleterious cells whose hallmarks include cellular

membrane blebbing, cleavage of certain nucleases and polymerases, and activating

cysteine proteases known as caspases. The onset of certain cancers has been traced to a

missed apoptotic signal, and in these cases compounds that induce apoptosis [such as

doxorubicin (**1.105**) and camptothecin (**1.106**)] have proven to be powerful cancer

chemotherapeutic agents.[236, 237] Thus, apoptosis is described as the most potent defense

against cancer.

1.105   **1.106**
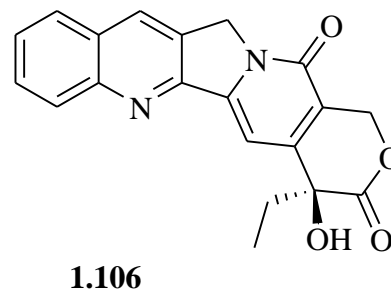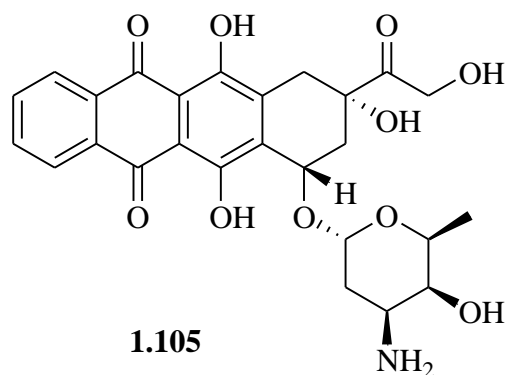
There is also growing evidence which suggests that certain chemopreventive agents are

able to trigger apoptosis in transformed cells *in vivo* and *in vitro*, which appears to be

associated with their effectiveness in modulating the process of carcinogenesis.[235]

Apoptosis has been characterized as a fundamental cellular activity needed to maintain

the physiological balance of the organism. It is also involved in immune defense

machinery and plays an important role as a protective mechanism against carcinogenesis

by eliminating damaged cells or abnormal excess cells proliferated through induction by

various chemical agents.[238] Thus, modulating apoptosis may be useful in the

management and therapy or prevention of cancer.

Apoptotic induction has thus become a new target for innovative mechanism-based anticancer drug discovery, since the life span of both normal and cancer cells is significantly affected by the rate of apoptosis.[239] Thus, the synthesis or modification of known anticancer drugs continues to be an important aspect of biomedical research. However, a vast amount of synthetic work has contributed relatively small improvements over the prototype drugs. There is therefore a continued need for new prototypes, which are new templates to be used in the design of potential cancer chemotherapeutic agents. Natural product chemistry is significantly providing such templates.[240]

1.7.4 Anticancer Monoterpenes

A number of epidemiological studies have revealed that high consumption of fruits, vegetables, and other plant products correlates with the reduction in cancer incidence. Among the cancers exhibiting reduced incidence in people consuming high levels of plant foods are epithelieal cancers of the pancreas, colon, breast and lung. Given that these cancers are especially difficult to treat with existing therapeutic modalities, the identification of dietary phytochemicals that have antitumor activity and the investigation of their mechanisms of action may lead to significant advances in the prevention of human cancer by dietary derived substances or their semisynthetic products.[241] In this regard, the fact that non-nutritive monoterpenes and other isoprenoids found in the essential oils of citrus fruits, cherry, spearmint, caraway and other plants have been known to inhibit protein isoprenylation and have *in vivo*

antitumor activities indicates the potential of the essential oil terpenoid constituents to act as templates for anticancer drug development.

Thus, the diverse therapeutic potential of essential oils has continued to inspire research efforts to test the oils for anticancer activity, as it is also envisioned that their mechanism of action is dissimilar to that of the classic cytotoxic chemotherapeutic agents.[242, 243]

Monoterpenes, which are non-nutritive dietary components, are found in the essential oils of citrus fruits, cherry, mint and herbs and physiologically function as chemoattractants or chemorepellents,[244] and are largely responsible for the distinctive fragrance of many plants. These 10 carbon isoprenoids are derived from the mevalonate biosynthetic pathway in plants but are not produced by mammals, fungi or other species. Thus, in citrus fruits, peppermint and other plants, limonene (**1.83**) is formed by the cyclization of geranylpyrophosphate in a reaction catalyzed by limonene synthase. Limonene then serves as a precursor to a host of other oxygenated monocyclic monoterpenes such as carveol (**1.84**), carvone (**1.85**), menthol (**1.107**, perillyl alcohol (**1.108**) and perillaldehyde (**1.109**).[245-248]
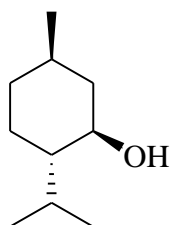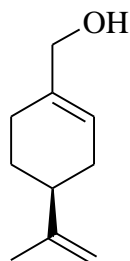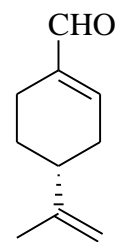
1.107   **1.108**   **1.109**

Some specific dietary monoterpenes include perillyl alcohol (**1.108**) found in cherry and spearmint, carvone (**1.85**) occurring in caraway and spearmint, and geraniol (**1.87**) which can be found in lemongrass oil, an ingredient in herbal teas. Orange oil that consists of 90–95% *d*-limonene is a commercial food flavoring agent. Furthermore, because of its pleasant citrus fragrance, *d*-limonene is commonly added to cosmetics, soaps and other cleaning products. Thus, human exposure to monoterpenes through the diet or environment is actually widespread.[249] Limonene, because of its rapid and extensive metabolization is non-toxic to mammals.[250]

Preclinical models have explored limonene as an anticancer agent. [251] Thus, in 1971, Homburger *et al*. [252] showed that co-administration of limonene and the carcinogen benzo(rst)pentaphene led to tumor development inhibition. Furthermore, Elegbede *et al*. [253] demonstrated the potential of limonene in the treatment of tumors through causing regression of chemically induced rat mammary tumors. Haag *et al*. [254] extended these findings further by demonstrating the regression of advanced rat mammary carcinomas following treatment with limonene. Further studies by Chander *et al*. [242] revealed the synergy between limonene and aromatase inhibitors in the treatment of advanced rat mammary carcinoma. The above findings led to the clinical testing of limonene in the UK.[256]

The therapeutic potential of limonene has prompted efforts to screen other monoterpenes for their chemotherapeutic activity. Thus, perillyl alcohol, which is the naturally occurring dietary hydroxylated monoterpene, on *in vivo* testing was found to induce

tumor regression, having activity potency five times that of limonene.[253] The compound has since undergone several phase I and II clinical trials, showing therapeutic potential with relatively minimal mild adverse effects. [257-259]

The acyclic monoterpene farnesol (**1.110**) that is found in lemongrass and chamomile, and geraniol (**1.87**) whose dietary sources include carrot, lemon, lime, nutmeg, orange, blueberry, and blackberry possess anticancer as well as chemopreventive efficacy against pancreatic, skin, esophageal, and mammary epithelial carcinomas.[250]
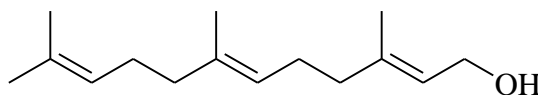
1.110

Caraway seed oil, and its principal monoterpene carvone (**1.85**) has been shown to prevent chemically induced lung and forestomach carcinoma development when administered before the carcinogen.[260] In addition, carveol (**1.84**) and menthol (**1.107**) are reported to exhibit chemopreventive activity against DMBA-induced rat mammary cancer when fed as 1% of the diet during the initiation phase.[261, 262]

Monoterpenes are thus an example of essential constituents that have potential in the development of therapeutic agents for cancer prevention and therapy, while more efficiently benefiting these areas in anticancer research.

1.7.5   Protein Isoprenylation and Cell Proliferation Activity Among Monoterpenes

Structure activity relationship studies involving monoterpenes have revealed that several limonene metabolites have greater *in vitro* cancer anti-proliferative activity than limonene itself.[250] Limonene is extensively metabolized by rats and by humans. In rats, the circulating metabolites include perillic acid (**1.111**), dihydroperillic acid (**1.112**), and their methyl esters while their urinary metabolites include uroterpenol (**1.113**) and perillic acid. In humans, perillic acid is the major circulating metabolite with carveol and urotepenol as the urinary metabolites. It was revealed that perillic acid, carveol and urotepenol are more potent chemopreventive agents than limonene itself, hence suggesting that metabolites of limonene may be the most active agents *in vivo*.[263]

Based on these observations, Crowell *et al.*[263] hypothesized that other monoterpenes might also be potent inhibitors of protein isoprenylation. This therefore prompted extensive structure-activity studies among limonene derived monoterpenes by the same research group and observed that many monohydroxylated monoterpenes were more effective inhibitors in intact cells than limonene itself. The position of hydroxylation in limonene was found to affect the inhibition of protein isoprenylation to some extent. For example, perillyl alcohol (**1.108**), 7-methylperillyl alcohol (**1.114**), and 7-ethylperillyl alcohol (**1.115**) were among the most active isoprenylation inhibitors.[263] Perillyl alcohol was slightly more active than α-terpineol (**1.116**), that has an 8-hydroxy group. The latter was found to be more active than (-)-carveol (**1.84**), that has a 6-hydroxy group.

The isomeric pair of acyclic hydroxylated monoterpenes geraniol (**1.87**) and linalool (**1.40**) were equally effective as α-terpineol. Thus, the addition of a single hydroxyl group to a monoterpene skeleton greatly increased the ability of the monoterpene to inhibit protein isoprenylation, even with slight variation of the hydroxyl group. Observed also were monoterpenes with two or three hydroxyl groups being less effective than those with a single hydroxyl group. For example, the diol sobrerol (**1.117**) was less active than (-)-carveol (**1.84**) or α-terpineol, both of which contain one hydroxyl group in the same position as one of those in sobrerol.[263]
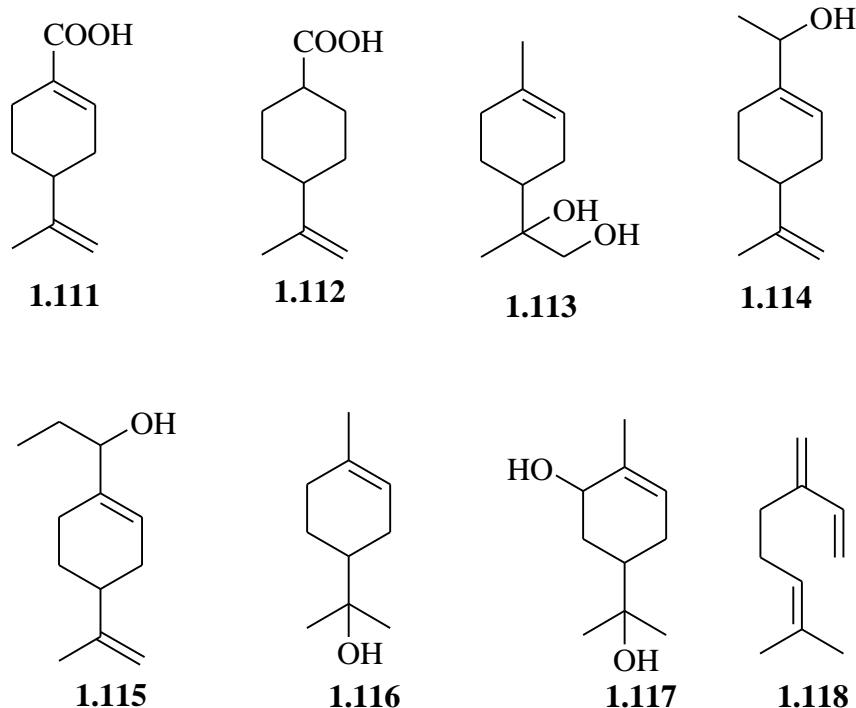
The above studies revealed that within the series of monoterpenes tested, either the less polar, such as limonene and myrecene (**1.118**), or the more polar such as perillic acid or diols/triols, were less effective inhibitors of protein isoprenylation and cell proliferation

and that those with intermediate polarity were more active. Hence, the relative polarity of the drug rather than its ring or other structural features seem to be the most important factor in determining the relative ability to inhibit isoprenylation in cells. This correlation between polarity and inhibition of protein isoprenylation could be due to the ability of these compounds to traverse through cellular membranes.[263]

1.7.6   Anticancer Principles Containing Amino Functional Group

In efforts to further explore the relative effects of the amino group towards apoptosis induction, the study whose results are reported in this Thesis included derivatization of phenylated amines and piperidine containing monoterpenes followed by examination of structure-activity relationships against Jurkat T and Chinese hamster ovary (CHO) cells. Therefore, the following sections give an overview of the anti-cancer agents that contain amino moieties that are currently in clinical use.

Among the best known and effective anticancer agents that are nitrogen containing plant derived metabolites are the vinca alkaloids vinblastine (**1.119**) and vincristine (**1.120**), isolated from the Madagascan periwinkle, *Catharanthus roseus* (*Vinca rosea* L., Apocinaceae).   Since ancient times *C. roseus* has been used traditionally for the treatment of diabetes, and that vinblastine and vincristine were first discovered during an investigation of the plant as a source of potential oral hypoglycemic agents.[264]

1.119   **1.120**
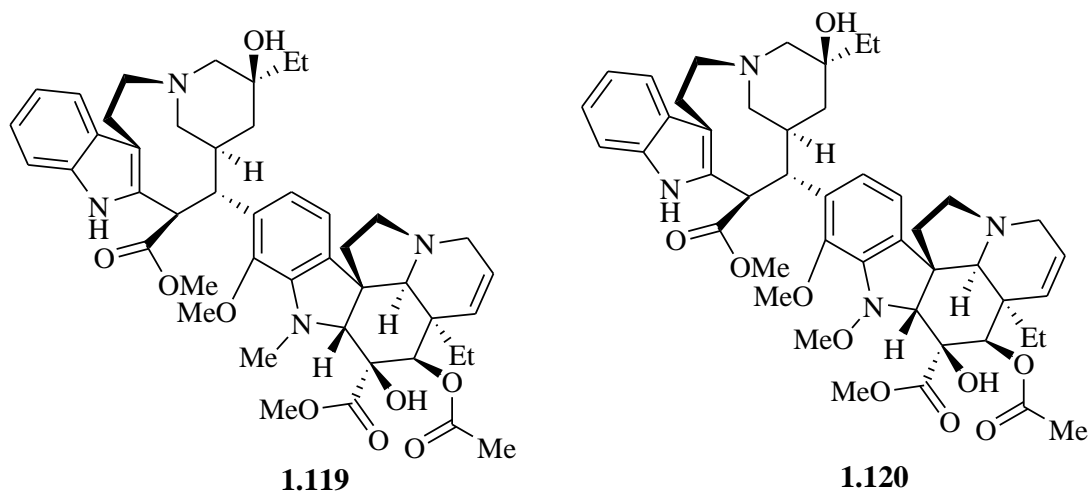
Recently, the semisynthetic compounds vindesine (**1.121**) and vinorelbine (**1.122**) have been introduced for cancer therapy. The vinca alkaloids are widely used in the treatment of hematological neoplasms.[265] They act by interfering with cell division arising from metaphase arrest of tumor cells.[266]

A very important feature of vinca alkaloids is their relatively low toxicity. Thus, vincristine while expressing only a mild myelosuppressive activity causes paraesthesias and neuromuscular abnormalities, thereby limiting its use to short courses only. Vinblastine is less neurotoxic but causes leucopenia, while vindesine is less toxic, as it exhibits moderate myelotoxicity as well as neurotoxicity.[267-269]
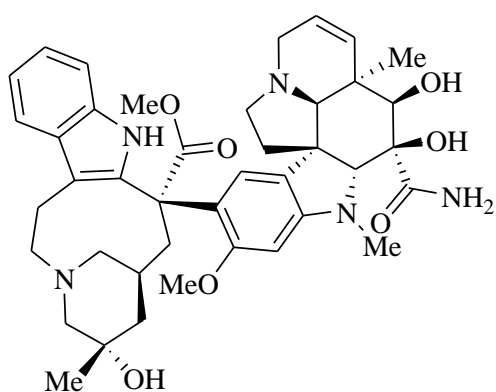
1.121
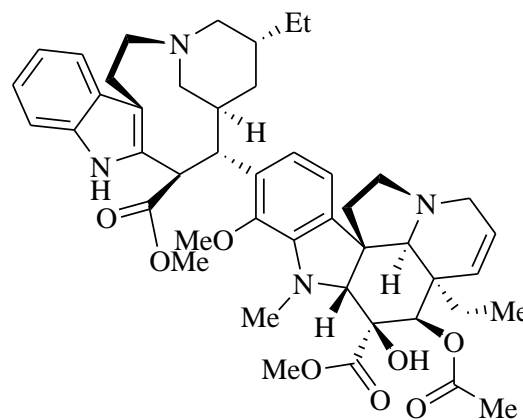
1.122

Paclitaxel (Taxol®, **1.123**) is a complex diterpene first obtained from the Pacific yew, *Taxus brevifolia* (Taxaceae) as an anticancer agent.[270, 271] Currently, it is also being isolated from several other *Taxus* species, including *T. baccata* and *T. cuspidata*.[272] Taxol®, containing the β-amino-α-hydroxy unit has significant activity against breast and lung cancer, with unwanted effects including bone marrow suppression and paraesthesias.[268, 269]

1.123
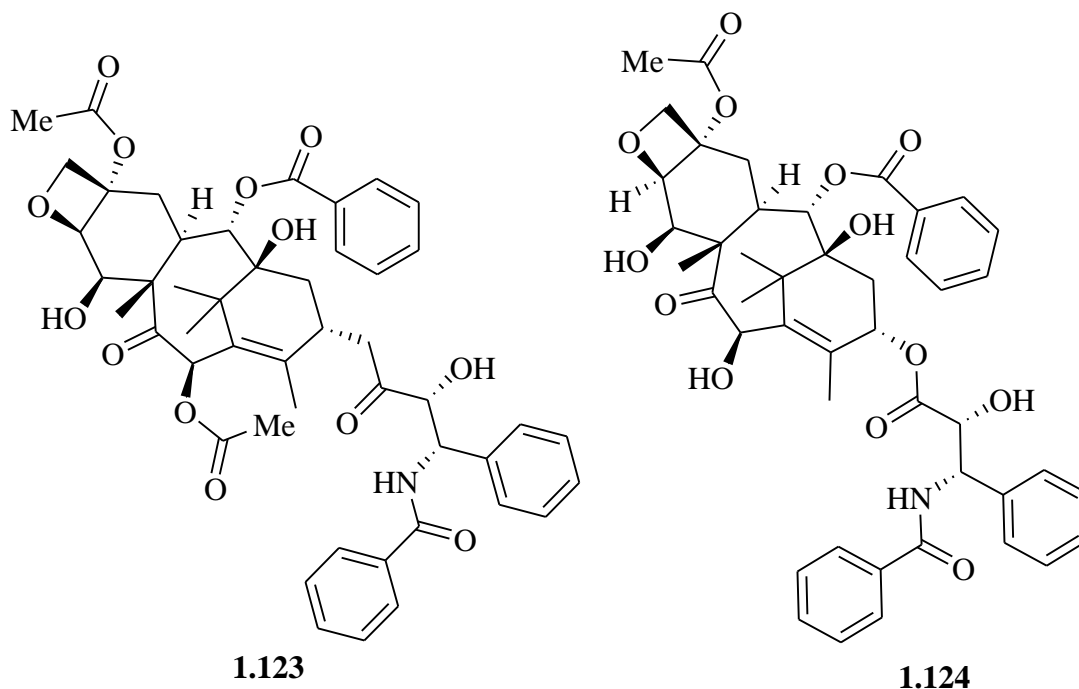
1.124

Docetaxel (**1.124**) is another anticancer taxane diterpenoid that is a semi-synthetic analogue of Taxol®. It is a clinically well established anti-mitotic chemotherapy medication used mainly for the treatment of breast, ovarian, and non-small cell lung cancer.[273]

Homoharringtonine (**1.125**) is also a plant derived anticancer agent that is in clinical use. It is obtainable from the roots and rhizomes of the Chinese tree, *Cephalotaxus harringtonia* (Cephalotaxaceae) and has shown a high chemotherapeutic efficacy on human acute agranulocyte leukemia and acute myelocitic leukemia.[265, 274]

1.125
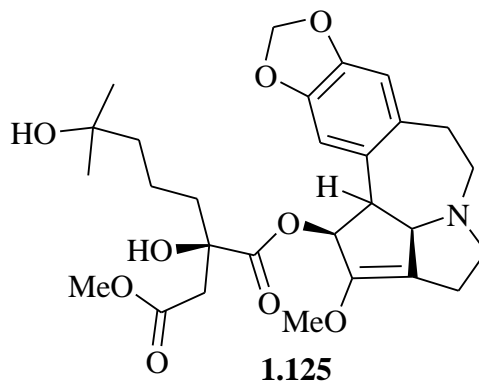
The anthracycline doxorubicin (**1.105**) is another natural product derivative which is commonly used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, and soft tissue sarcomas. The compound also has an amino group in its molecular structure. Doxorubicin was developed through minor changes in the chemical structure of the naturally occurring daunorubicin (**1.126**) produced by a mutated strain of *Streptomyces* using *N*-nitroso-*N*-methyl urethane. Doxorubicin exhibits better activity than daunorubicin against murine tumors, and especially solid tumors. It also has a relatively higher therapeutic index than daunorubicin.[275]

1.126
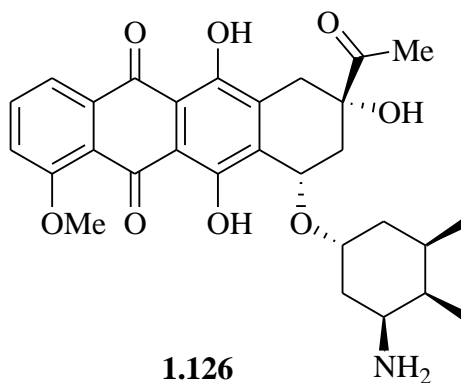
1.7.7 Sulfur Containing Anticancer Agents

Some of the investigations whose results are discussed in this Thesis focused on derivatization of monoterpenoid epoxides using sulphur-containing nucleophiles, leading to mercaptan derivatives which when evaluated for apoptotic activity *in vitro* showed activity. Sulfur-containing functionalities are known to confer anticancer properties to several natural and synthetic products. For example, epidemiological studies have shown that the intake of garlic is closely related to the reduction of various cancers, such as stomach, colorectal and prostate cancer. Garlic and its organosulfur compounds are becoming more appealing as anticarcinogenic agents due to their ability to induce apoptosis and inhibit both the initiation and promotion stages of tumorigenesis in animal studies. [276-281]

Depending on the conditions of its cultivation, garlic could contain at least 33 different organosulfur compounds, in addition to amino acids, vitamins, and micronutrients. The allyl sulfur compounds formed by enzymatic activity when garlic is minced or crushed, such as allicin (**1.127**), water-soluble (*S*)-allylmercaptocysteine (**1.128**) and (*S*)-allylcysteine (**1.129**), and oil-soluble diallyl disulfide (**1.130**) and diallylsulfide (**1.131**), probably account for the majority of these anticancer effects in the presence of (*S*)-trityl-L-cysteine (**1.132**), (*S*)-propylglutathione (**1.133**) and benzyl-(*S*)-cysteine (**1.134**).[282]
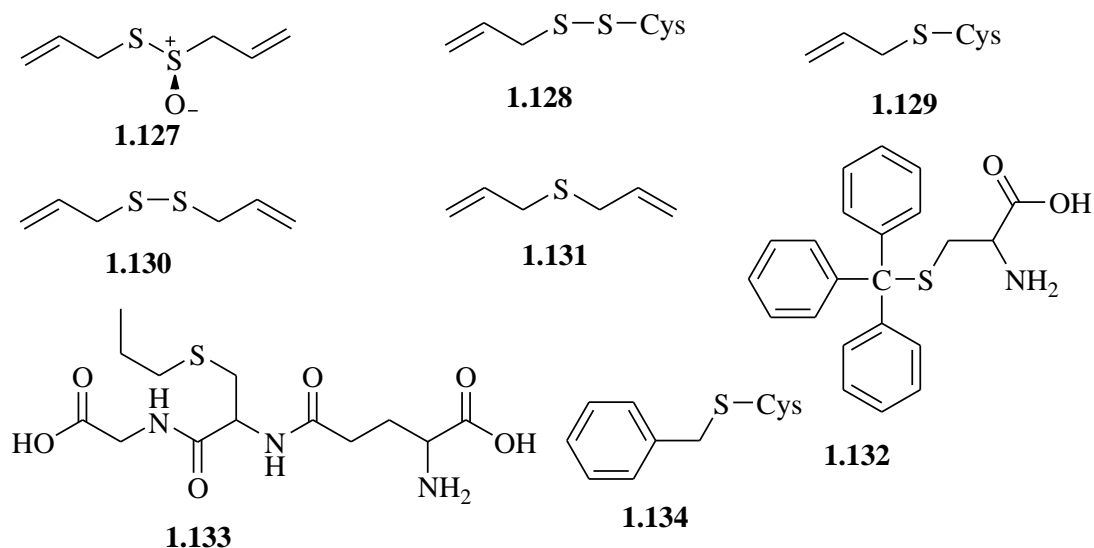
Many isothiocyanates, both natural and synthetic, display anticarcinogenic activity by reducing the activation of carcinogens and increasing their detoxification. The naturally occurring isothiocyanates are formed from glucosinolate precursors of cruciferous vegetables, such as broccoli, cauliflower, kale, turnips, collards, Brussels sprouts, cabbage, radish, turnip and watercress, and are also responsible for the typical flavour of these vegetables. Studies have shown that the isothiocyanates exhibit anti-tumor activity by affecting multiple pathways including apoptosis, and cell cycle progression.[283]

Isothiocyanates are sulfur-containing phytochemicals with the general formula R-NCS. Different molecules belong to this group, and those with the strongest anticancer effects include phenylethylisothiocyanate (**1.135**), benzylisothiocyanate (**1.136**) 3-phenylpropylisothiocyanate (**1.137**) and sulforaphane (**1.138**).[284, 285]

1.135

1.136

1.137

1.138
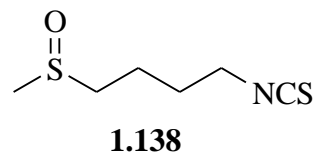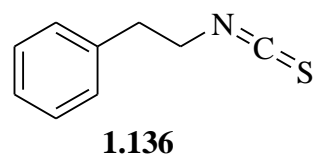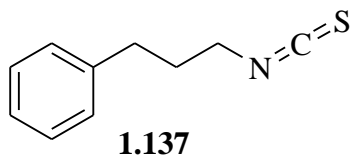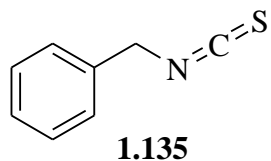
Another group of sulfur containing compounds is exemplified by sarcophine (**1.139**), a cembranoid diterpene isolated in large amounts from the Red Sea soft coral *Sarcophyton glaucum.* [286] It has been investigated for its potential as a cancer chemopreventive agent since 1998. By targeting the epoxide ring of sarcophine with ammonium thiocyanate and Lawesson's reagent (**1.140**) the sulphur containing semisynthetic derivatives of **1.139** were obtained and these recorded enhanced anticancer and anti-inflammatory activities.[286, 287]

1.139

1.140
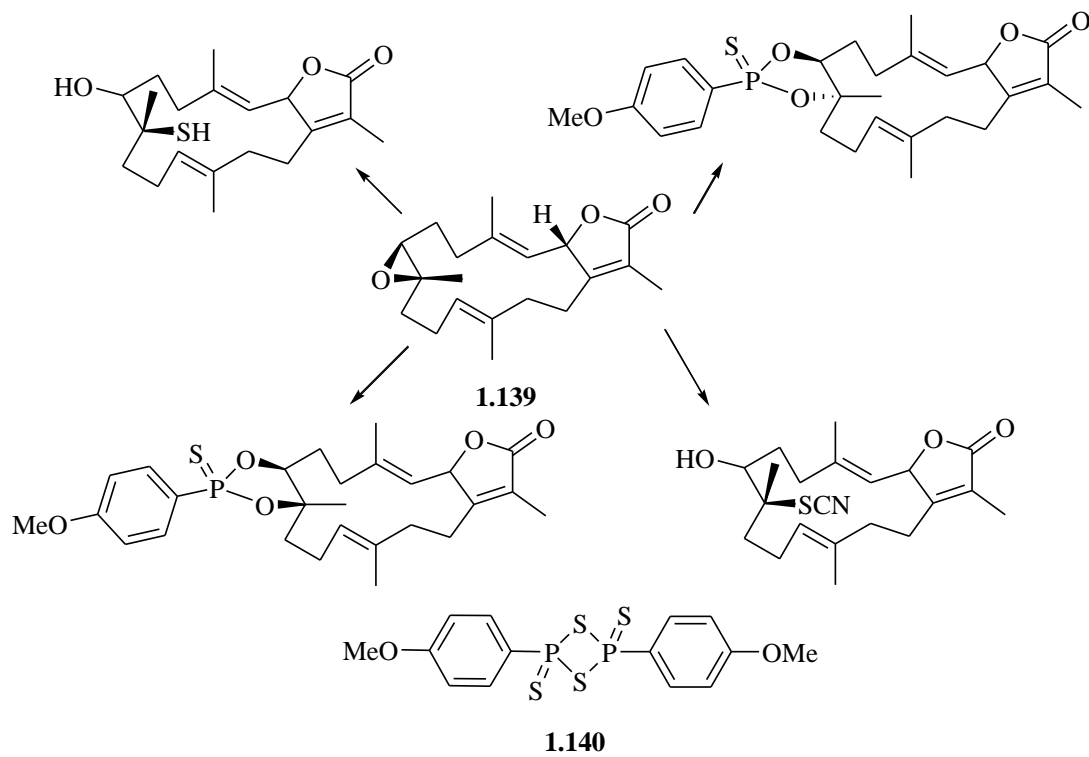
Treatment of the most common tumor diseases with these agents is still not promising, and much has to be done to achieve the ultimate goal of curative and non-toxic chemotherapy. Together with improved treatment strategies new agents are clearly needed for this series of compounds.

1.8   Objectives of this Study

The general objective of this study was to carry out synthesis of variously functionalized monoterpenoid compounds bearing the *p*-menthane skeleton and subjecting them to mosquito repellence and apoptosis induction assays so as to determine through SAR studies their potential as lead compounds for further pursuit.

1.8.1 Hydroxy- and Epoxymenthanes as Mosquito Repellents

The aforegoing sections clearly demonstrate that malaria could be prevented through a number of ways, including chemotherapy, chemoprophylaxis, and vector control. Despite the availability of drugs and/or tools to support the above strategies malaria still remains a challenge especially in the developing countries. Most drugs suffer the limitation of resistance while the currently available insecticides have been criticized for being environment unfriendly. Also the malaria vector mosquitoes are reported to have developed resistance against some of the available insecticides. Furthermore, the available agents used through direct application as repellents have negative effects to users, thereby limiting their acceptability.

From the above background this study was conceived in the hope of developing environmentally and eco-friendly repellents from naturally occurring monoterpenoids as leads. Naturally occurring cyclohexyl monoterpenes were the target precursors for chemical modifications through hydroxylation and epoxidation reactions. The precursor compounds were those which initially exhibited repellence or are constituents of repellent essential oils. The chemical modification was aimed at increasing the activities and application longevity of the derivatives so formed.

1.8.2 Amino Alcohols, Hydroxy Sulfides and Glycosidic Monoterpene Derivatives as Apoptosis Inducers

The literature review in the previous sections indicates that there are a number of cancer chemotherapeutic agents derived from monoterpenes, containing a variety of substituents as key functionalities for the inherent activity, such as hydroxyl, amino, mercapto, glycosyl or isocyanates. Therefore, another objective of this study was to derivatize variously substituted cyclohexyl monoterpenoids to form compounds having the above functionalities, and testing them for apoptosis induction.

1.9 Thesis Set Up

After the introduction and literature review under Chapter One, in Chapter Two, Synthesis (hydroxylation and epoxidation) of a number of cyclohexyl monoterpenes is discussed, together with their mosquito repellent activities. This is then followed by a presentation of results on epoxide ring opening with various nucleophiles given in Chapter Three, including the apoptotic activity of β-amino alcohols obtained *via* opening with benzylamine, aniline and piperidine while in Chapter Four, the synthesis of β-hydroxy sulphides *via* opening with benzyl mercaptan and their apoptosis inducing potential is discussed. Chapter Five contains the discussion of results from glycosylation of cyclohexyl monoterpenes, together with their apoptotic activities. Finally, this Thesis provides some concluding remarks and suggested recommendations for further pursuance in this area of research as presented in Chapter Six.

CHAPTER TWO

SYNTHESIS, MOSQUITO REPELLENT AND APOPTOTIC ACTIVITY OF OXY-MONOTERPENOIDS

Abstract

This chapter describes derivatization of some *p*-menthane-type oxygenated monoterpenes constituting 12 differently substituted epoxides and variously hydroxylated compounds from alkenyl bearing precursors, employing modified epoxidation and hydroxylation procedures and the target compounds obtained generally in low yields, but the synthesized compounds were prepared just for the establishment of mosquito repellency and apoptotic induction potential. The bioactivity results as described in this chapter indicate most of the compounds tested to have shown good level of activity both as mosquito repellents and their potential as apoptosis inducting agents.

2.1   Introduction

As discussed in Chapter One, the search for new mosquito repellents is among the various approaches for the control of malaria transmission rates. Similarly, the discovery of new apoptotic agents is an area of research that continues to command high priority. In both cases variously substituted terpenoids have been considered as interesting potential targets. This prompted the need to conduct the investigations whose results are reported in this chapter.

The cyclohexenyl monoterpenes earmarked as precursors for these investigations consisted of olefinic moieties that could be synthetically transformed, either by epoxidization or hydroxylation to yield the target molecules.

A number of cyclohexyl molecules of the *p*-menthane class of monoterpenes were selected for derivatization and subsequent bioactivity evaluations for mosquito repellency and apoptotic activity. The selected compounds included dihydrocarvone (**2.1**), limonene (**1.83**), α-terpeneol (**1.112**), dihydrocarveol (**2.2**), carveol (**1.84**), carvone (**1.85**) and α- and γ-terpinene (**2.3** and **2.4**). All the compounds are 1-methyl and 4-isopropyl substituted cyclohexanoids typically expected to exist in various conformations in which both alkyl groups would be *trans*-equatorially oriented.
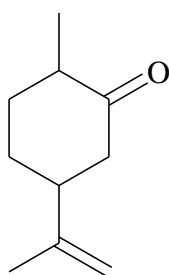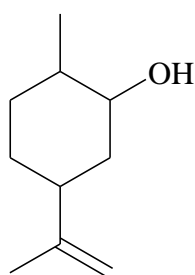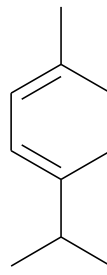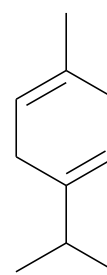
2.1   **2.2**   **2.3**   **2.4**

The olefinic group present in the precursor compounds was the important factor for this study, since the conversion of alkenes to epoxides is a useful functional-group transformation in organic syntheses, yielding compounds whose stereochemical configuration can be predicted based on steric factors. Peroxy acids and alkaline H_2O_2 are two most commonly used reagents in epoxidation reactions. Peroxy acids such as *m*-

chloroperoxybenzoic acid (*m*-CPBA) work well with electron-rich alkenes and an alkaline solution of H_2O_2 is suitable for epoxidation of α,β-unsaturated carbonyl compounds.[288]

Epoxides are important synthetic intermediates owing to the high reactivity of the strained oxirane ring.[289-291] Therefore, these compounds were prepared using *m*-CPBA or BAP as the reagents, yielding substituted epoxycyclohexanoids that were either tested for insect repellent and apoptotic activity, or were further converted into amino alcohols and hydroxylsulphides, as reported in Chapters Three and Four of this thesis.

Since peroxyacids are a source of electrophilic oxygen, the epoxidation reaction would work best with electron poor peroxyacids (e.g. *m*-CPBA) and electron rich alkenes. The reaction can also be stereoselective and would often proceed *via* attack on the less hindered side of an alkene. Generally, the reactions would proceed by polarization of the weak oxygen-oxygen bond (**Scheme 2.1**).[292]
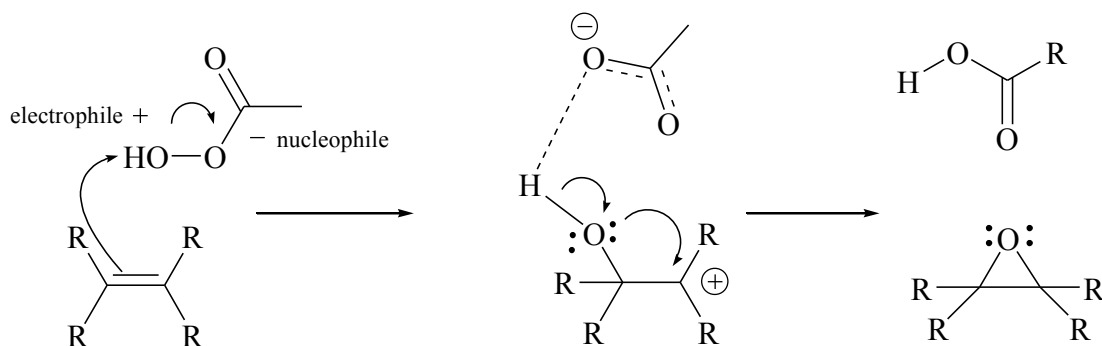
Scheme 2.1   Epoxidation reaction mechanism with *m*-CPBA

Electron-deficient α,β-unsaturated carbonyl compounds can be oxidized by an alkaline solution of hydrogen peroxide through nucleophilic addition of HO_2^- to the olefinic double bond facilitated by the C=O group, in a Michael fashion (**Scheme 2.2**).[292,293]
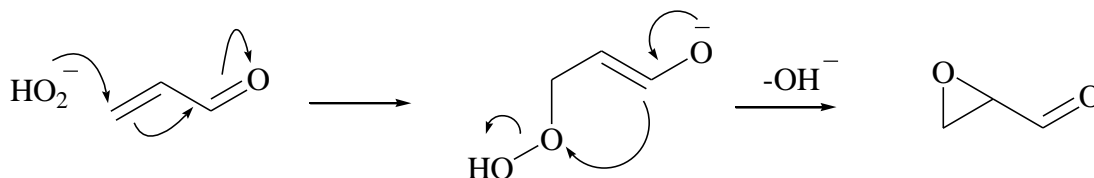
Scheme 2.2   Alkaline H_2O_2 epoxidation of α,β-unsaturated carbonyl compounds

In spite of H_2O_2 being a high oxygen content entity, it is an environmentally friendly oxidant, for which water is the sole byproduct. It is a slow acting reagent in the absence of activation due to the poor leaving tendency of the hydroxide ion.[294-296] As such, transition metal salts or complexes have been used as catalysts for alkene epoxidations with aqueous H_2O_2 or by forming reactive peroxyacids from carboxylic acids, or peroxycarboximidic acid from acetonitrile (Payne oxidation) in strongly basic solution.[297] Such systems have one or more disadvantages, such as toxicity or rapidly decomposing metal catalysts, oxidative decomposition of organic ligands, forming organic byproducts, or being strongly acidic or basic reaction conditions that decompose the desired epoxide as the product.[298]

To activate the hydrogen peroxide used, the bicarbonate-activated peroxide (BAP) system could be adopted whose active oxidant is a peroxymonocarbonate ion (HCO_4^-), presumably formed *via* perhydration of CO_2.[298]

2.2 Results and Discussion

2.2.1 Bicarbonate-Activated Peroxidation (BAP)

The need to test the applicability of the BAP method arose from the desire to obtain the targeted epoxides in an economically viable and environmentally friendly way that would be easily adopted for deriving bioactive epoxides if established to be active. By adopting the reported method,[298] the bicarbonate-activated peroxide (BAP) epoxidation reaction was carried out in water mixed with acetonitrile and H_2O_2. Without stirring, the reaction was allowed to proceed in the dark overnight. The reaction was established to be efficient for a number of the substrates used. It was therefore noted that, while the alkaline H_2O_2 preferentially reacted with electron-deficient alkenes, in some instances the BAP system afforded epoxides with electron rich alkenes.

It was therefore decided to re-investigate some aspects of the above synthetic route in connection with the overall aim of the study in order to provide a more potentially economical route for the preparation of various epoxides thereof. In this regard it was considered worthwhile to investigate the effect of stirring the reactants in the BAP system, especially considering the yield and rate (time) at which the desired product/s would be obtained.

The epoxidation of carvone (**1.85**) with the BAP system (**Scheme 2.3**) while stirring proceeded in an exothermic reaction that required cooling of the reaction mixture in an

ice bath for 2 h and not allowing the temperature to rise beyond 40 $^\circ$C to give

compounds **2.5** and **2.6** in 47.7 and 18.4 % yield, in the ratio of 5:2, respectively.

| **1.85** | | **2.5** (47.7 %) | **2.6** (18.4 %) |

$BAP = NH_4HCO_3, H_2O_2, MeCN$
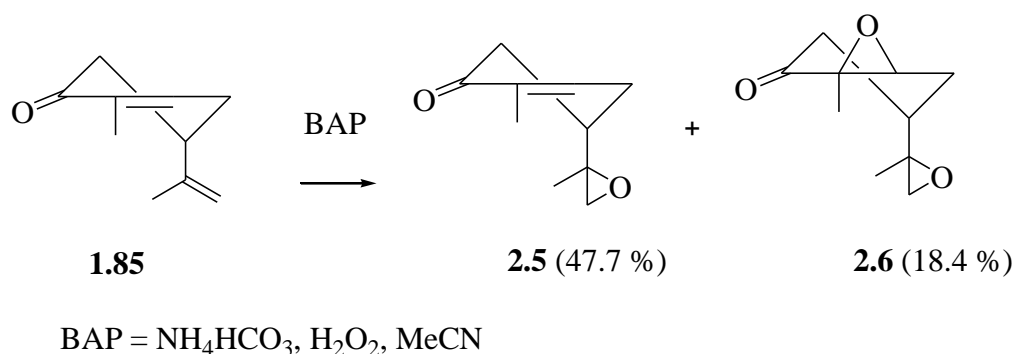
Scheme 2.3   Epoxidation products from carvone (1.85)

The mono epoxide **2.5** was formed as a result of oxidation of the more electron rich

double bond of the isopropenyl unit, as evidenced by the appearance of UV absorption

on TLC owing to the α,β-unsaturated carbonyl system retained in **2.5**.   Further

confirmation of structure **2.5** was derived from analysis of ^{1}H and ^{13}C NMR spectra that

revealed the presence of signals due to only one vinylic group.

The epoxidation reaction was not that regioselective as the diepoxide **2.6** was also

obtained. This indicated that the BAP system was also able to facilitate oxidation of the

more substituted electron deficient double bond of the α,β-unsaturated carbonyl system.

The structure for compound **2.5** was also established upon analysis of the ^{1}H and ^{13}C

NMR spectra.

p-Mentha-1,3-diene (α-terpinene, **2.3**) is a derivative of the non-planar 1,3-cyclohexadiene that has been shown by both microwave and electron diffraction spectroscopy to exists in a half chair conformation (**Scheme 2.4**).[299] Epoxidation of **2.3** under the modified BAP system afforded the diepoxide **2.7** in 42.6 % yield, whose structure was ascertained from analysis of ^{1}H and ^{13}C NMR spectral data.

2.3   **2.7** (42.6 %)
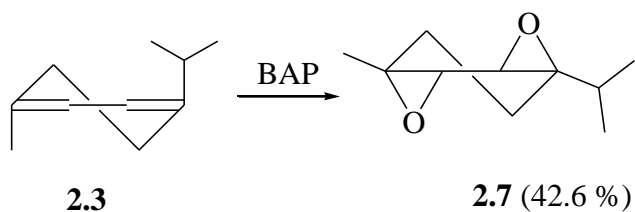
Scheme 2.4   BAP epoxidation products of 2.3

It was presumed that the exothermic nature of the reaction played a crucial role in enabling both the olefinic sites to be oxidised, since the other two expected products [**2.7 (b)** and **2.7 (c)**] were not obtained.

2.7 (b)   **2.7 (c)**
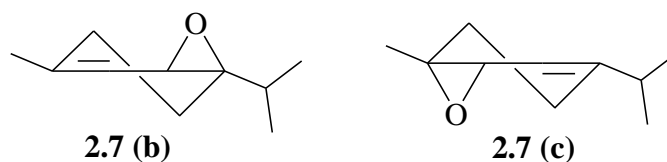
When the reaction was repeated using *m*-CPBA as the oxidant the crude product was found to consist of a multicomponent mixture (TLC) of inseparable compounds.

The BAP epoxidation of dihydrocarveol afforded the epoxide **2.8** in a moderately good yield of about 50 % and the structure was confirmed by analysis of ^{1}H and ^{13}C NMR

spectral data.   However, the ^{13}C NMR signals appeared in duplicate and this suggested the presence of two isomeric compounds as the products.
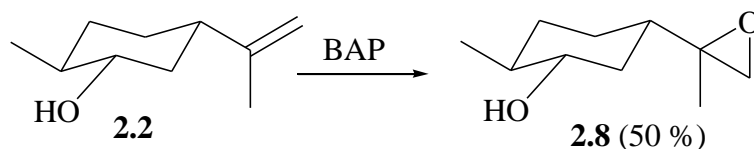
Scheme 2.5   Epoxidation of α-terpinene 2.2 to 8,9-isopropoxy-*p*-menthan-2-ol

(2.8)

2.2.2   Epoxidation Utilizing *m*-CPBA

Epoxidation reactions using *m*-CPBA for the formation of epoxides in this thesis were undertaken as reported in the literature.[13] Thus after a failed attempt to obtain an oxidation product from carveol (**1.84**) using the BAP system, *m*-CPBA epoxidation was opted, leading to reaction taking place at both the olefinic groups in the molecule to form four products (TLC, **Scheme 2.6**), which were readily isolated by column chromatography and their structures including the stereochemical configuration established on the basis of analysis of ^{1}H and ^{13}C NMR data.

Scheme 2.6 depicts the epoxidation reaction with carveol existing in the half-chair conformation. As stated earlier, the epoxidation reaction was less regioselective as both the 1,6- and 8,9- epoxides were obtained in 50 % and 8 % yield respectively.   The observed non-regioselectivity was contrary to expectation from the non-cyclic double bond being more susceptible to oxidation as compared to the endocyclic olefinic group.

This is probably due to the proximity of the electron donating hydroxyl group α to the endocyclic olefin group, thereby activating the site by electron donation.
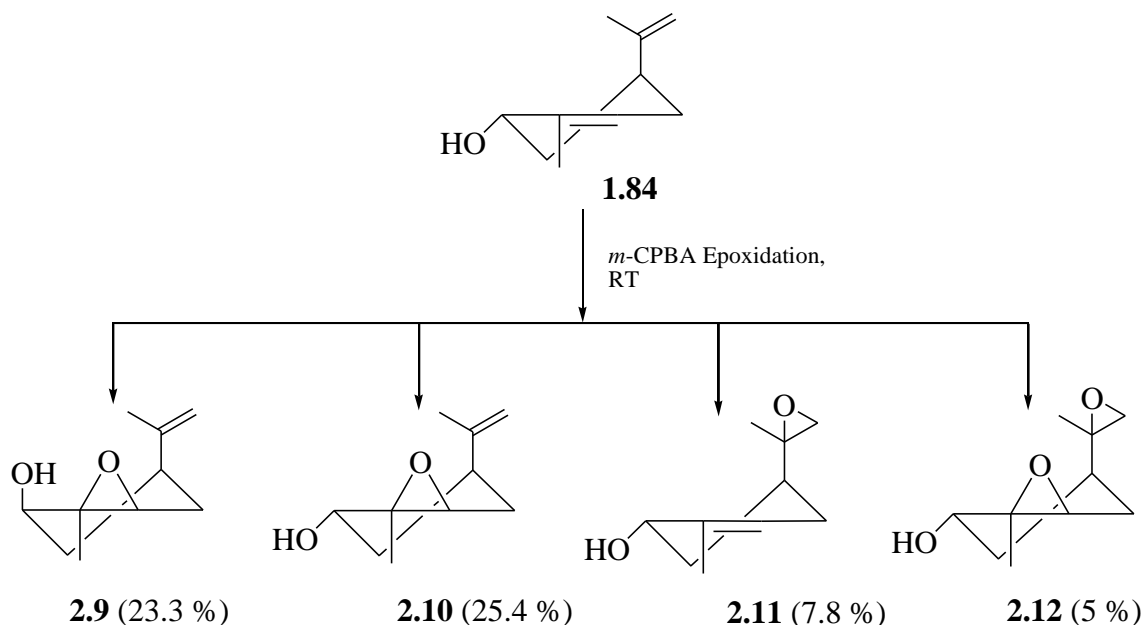
Scheme 2.6   *m*-CPBA epoxidation of carveol (1.84)

The NMR spectral data of *trans*-2-hydroxy-1,6-epoxy-*p*-menthen-8-ene **(2.10)** closely resembled those for compound **2.9**, showing only slight differences in the spatial orientation of the hydroxyl group as deduced from the NOESY spectrum that indicated strong H/H interactions between the methine proton on C-2 and the methyl group of the isopropenyl substituent (**Fig. 2.1**). This implied that the two entities were *pseudo*-axially oriented, unlike in compound **2.9** whose methine signal revealed a cross-peak with the C-7 methyl in the NOESY spectrum and none with the isopropenyl methyl proton, hence indicating a *pseudo*-equatorial orientation for the latter compound. Therefore, from this

interaction it was concluded that the hydroxyl group in **2.10** was *pseudo*-equatorially oriented and therefore in a *trans* geometry with the oxirane ring.

2.9   2.10
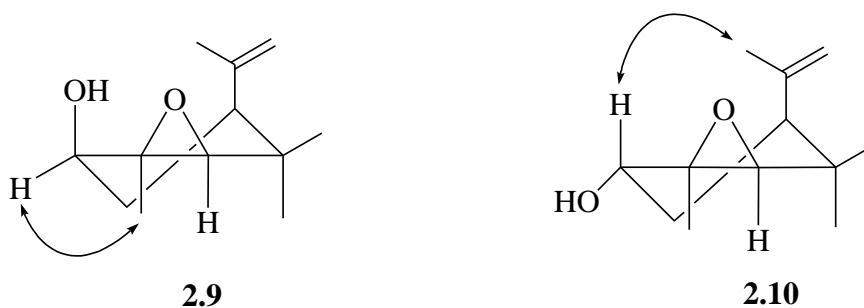
Figure 2.1   **Important NOESY interaction for *cis*-2-hydroxy-1,6-epoxy-*p*-menthen-ene (2.9) and *trans*-2-hydroxy-1,6-epoxy-*p*-menthen-ene (2.10)**

That 2-hydroxy-8,9-isopropoxy-*p*-mentha-1-ene (**2.11**) was also obtained from the epoxidation reaction and its structure was confirmed from analysis of ^{1}H and ^{13}C NMR data. Likewise, the structure of the di-epoxide 1,6:8,9-diepoxy-*p*-menthan-2-ol (**2.12**) that constituted 5% of the reaction product was confirmed from NMR spectra. The ^{1}H NMR spectral data did not indicate any olefinic signals that were otherwise present for the mono-epoxide derivatives.

m-CPBA epoxidation of dihydrocarvone (*p*-menth-8-en-2-one, **2.1**) furnished the epoxide 8,9-epoxy-*p*-menthan-2-one (**2.13**) in 47.3 % yield, whose structure was confirmed from analysis of ^{1}H and ^{13}C NMR spectral data. The spectra also revealed a

mixture of stereo isomers by showing two sets of resonances that were not well resolved in the ^{1}H NMR but visible in the ^{13}C NMR spectra.
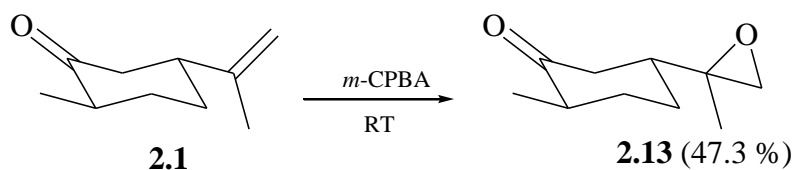
Scheme 2.7   **Epoxidation of 2.1 with *m*-CPBA**

1,2:4,5-Diepoxy-*p*-menthane (**2.14**) was obtained in 38.5 % yield upon reacting γ-terpinene (**2.4**) with *m*-CPBA and its structure established on the basis of analysis of the ^{1}H and ^{13}C NMR spectra. The yield was comparable to what was obtained by reacting the conjugated structural isomer α-terpinene (**2.3**) in the BAP system whose reaction with *m*-CPBA was not successful.
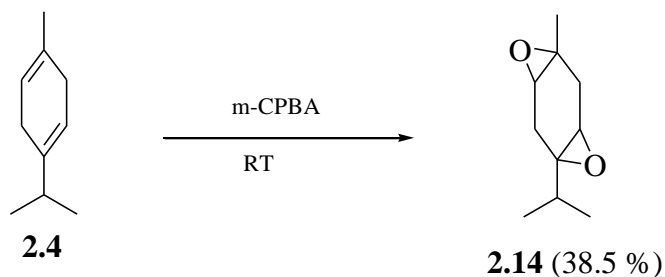
Scheme 2.8   **Epoxidation of 2.4 with *m*-CPBA**

The planar *trans*-dioxide moiety in product **2.14** with the two alkyl substituents adopting a *trans* di-axial orientation was deduced upon analysis of the NOESY spectrum. In addition the NOESY spectrum indicated strong interaction between H-5 and the methyl

protons on C-7 (**Fig. 2.2**), thereby confirming the *trans*-diaxial geometry of **2.14** whose alkyl groups were also axially oriented.
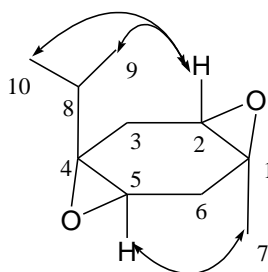
Figure 2.2   **Important NOE interactions for 1,2:4,5-diepoxy-*p*-menthane (2.14)**

2.2.3   **Epoxidation of Limonene (1.83) *via* Halohydrin Formation**

Unlike for the reactions discussed in the previous sections, the epoxidation of limonene (**1.83**) was carried out through the formation of a halohydrin followed by a nucleophilic epoxide ring closure involving an elimination reaction (**Scheme 2.9**). Both the reactions as undertaken *in situ* were expected to be stereospecific since the initial formation of the halohydrin is stereospecific and the opening of the bromonium intermediate occurs via an anti-addition mechanism.[301]
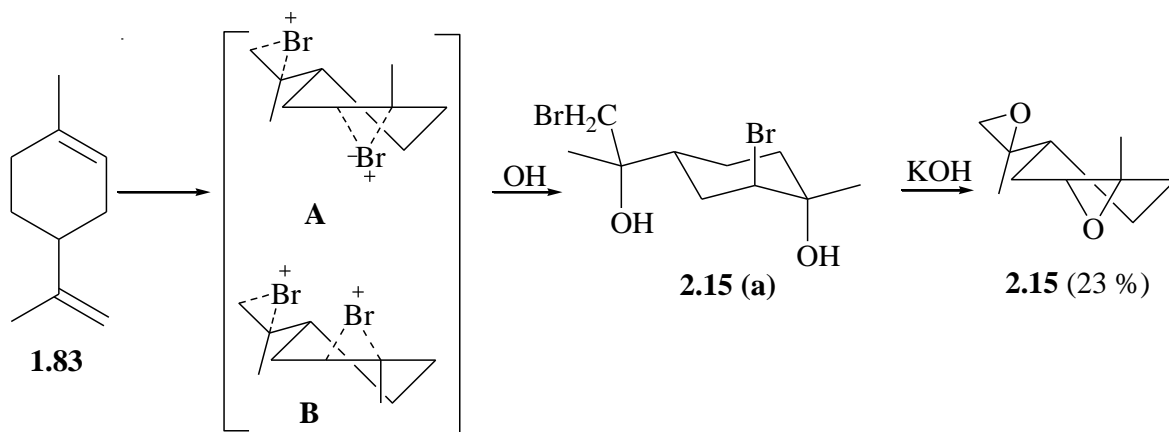
Scheme 2.9   Epoxidation of limonene (1.83) *via* a bromohydrin (2.15a)

The targeted limonene oxide (**2.15**) was obtained in rather low yield (23 %) *via* a bromohydrin (**2.15a**) that was furnished after treatment of limonene with an excess of *N*-bromosuccinimide in aqueous dioxane and subsequent dehydrobromination of the dihydroxydibromide with KOH (**Scheme 2.9**).

In the epoxidation process the initial base induced bromination of limonene was expected to proceed *via* the possible transition states **A** and **B** (**Scheme 2.9**) whereby **A** would be sterically stable and hence would be the preferred product. This would therefore determine the ultimate stereochemical configuration of the product **2.15** as shown in **Scheme 2.9**. Thus, the *trans*-diepoxy derivative **2.15** was formed as the product, whose structure was established on the basis of its spectroscopic data. The product was also assumed to have the half chair conformation due to the strained C-O-C bonds that closely resemble the C=C bond length,[300] while the isopropoxyl group would have a *pseudo*-equatorial orientation.

2.3   Oxymercuration-Demercuration (Hydration) of Alkenes

For the introduction of one hydroxyl group, an oxymercuration-demercuration reaction was applied as the mild and highly convenient procedure for the hydration of a carbon-carbon double bond. The process was expected to initially involve a reaction of the alkene double bond with mercury (II) acetate in aqueous tetrahydrofuran. The usual nucleophile in this reaction would be the solvent (water).[302]

Oxymercuration of simple alkenes is usually a stereospecific *anti* addition and the product would be consistent with the involvement of a bridged mercurinium intermediate, which is opened through nucleophilic attack. The importance of the reaction lies in the ease with which the mercury substituent can be removed by reduction with $NaBH_4$ (**Scheme 2.10**).

$$RHC{=}CHR \; + \; Hg^{2+} \longrightarrow RHC{-}CHR \xrightarrow{\text{-}OH^-} RHC{-}CHR \xrightarrow{NaBH_4} RH_2C{-}CHR$$
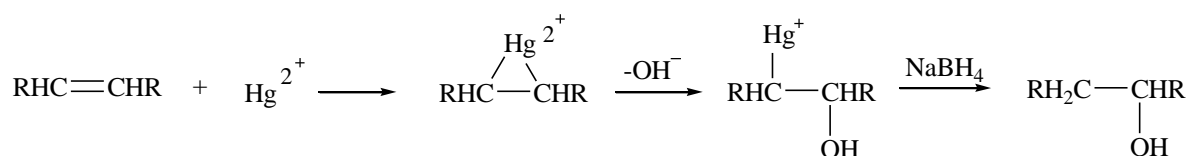
Scheme 2.10   Oxymercuration-demercuration mechanism

The reaction is highly regioselective in that the addition to the terminal and non-terminal alkenes would proceed *via* the most stable carbocation (Markownikoff). The reaction is also tolerant to the presence of hydroxyl group, which were present in some of the precursors selected for this tudy.[302-304]

The hydration of *p*-mentha-1,8-diene (**1.83**) with Hg(OAc)$_2$ as shown in **Scheme 2.11** furnished *p*-menth-1-en-8-ol (**2.16**) and *p*-menthane-1,8-diol (**2.17**) in the ratio 52 % and 15 % respectively as confirmed from NMR data.

1.83 → (1. Hg(OAc)$_2$, 2. NaBH$_4$, RT) → **2.16** (52 %) + **2.17** (14.9 %)
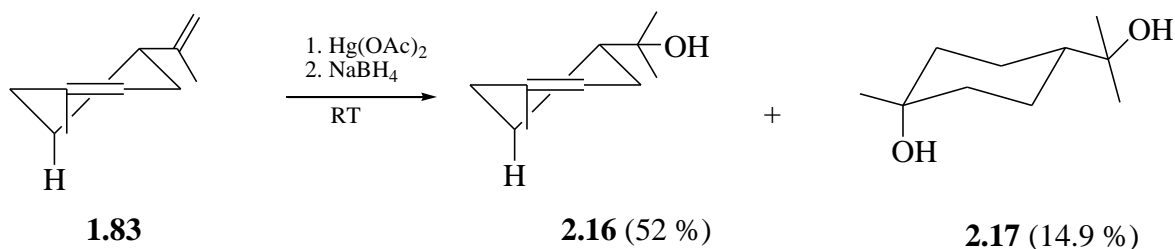
Scheme 2.11   Hydration of *p*-mentha-1,8-diene (1.83) with mercury acetate

The structure of the dihydroxyl product *p*-menthane-1,8-diol (**2.17**) was confirmed by considering the absence of any olefinic resonances and the appearance of a signal due to the introduction of the hydroxyl group on C-1 (δ 68.1) in the ^{13}C NMR spectrum.

Generally, in 1,1-disubstituted cyclohexanes the equilibrium composition depends directly on the relative conformational energy of the two substituents, as determined in mono-substituted cyclohexanes, with the "larger" substituent being preferentially equatorially oriented.[299] Thus, with higher conformational energy, the C-1 methyl group would be expected to be preferentially placed in the equatorial position instead of the hydroxyl group. Similarly, the isopropanol group would also be equatorially oriented due to its bulkiness (**Scheme 2.11**). The ^{13}C NMR resonance for the methyl substituent was observed at a relatively downfield position (δ 30.2) and this was ascribed to the deshielding effect characteristic of equatorially oriented methyl groups in a rigid six-

membered ring.[305] Likewise, the C-4 proton signal showed an upfield shift (δ 1.24), thus conforming with its axial orientation (**Scheme 2.11**).

Oxymercuration-demercuration of γ-terpinene (**2.3**) afforded *p*-menthane-1,4-diol (**2.18**) in ~11 % yield that was confirmed by the absence of olefinic proton signals in the ¹H NMR spectrum. For this case, with two "1,1" disubstituted sites both with an alkyl and a hydroxyl groups, as expected the more stable stereochemical conformation of **2.18** had the 1,4-hydroxyl groups in the *trans*-positions (**Scheme 2.12**). This was further supported by the downfield shift of the methyl proton in the ¹H NMR spectrum (δ 31.4). In addition, the axial protons were observed slightly downfield as accentuated by 1,3-diaxial interactions, thus their signals appearing at δ 1.73, while the equatorial protons signal were observed at δ1.43.

2.3   **2.18** (10.7 %)
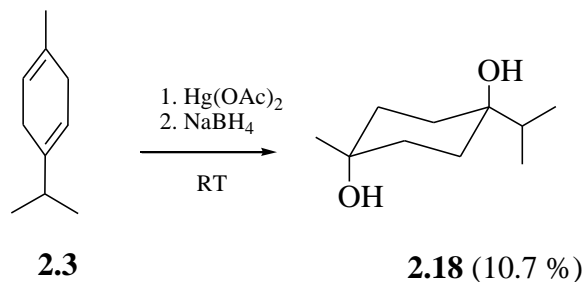
Scheme 2.12 Hydration of 2.3 with mercury acetate and product (2.18)

conformation

The low yield of the product could be attributed to the high polarity of the compound associated with two hydroxyl groups, therefore being difficult to isolate from the aqueous reaction mixture. However, another factor could have been the transition states

(**Scheme 2.13**) upon induction of the reaction and possibility to form the other two mono epoxides as byproducts (**2.18a** and **2.18b**).
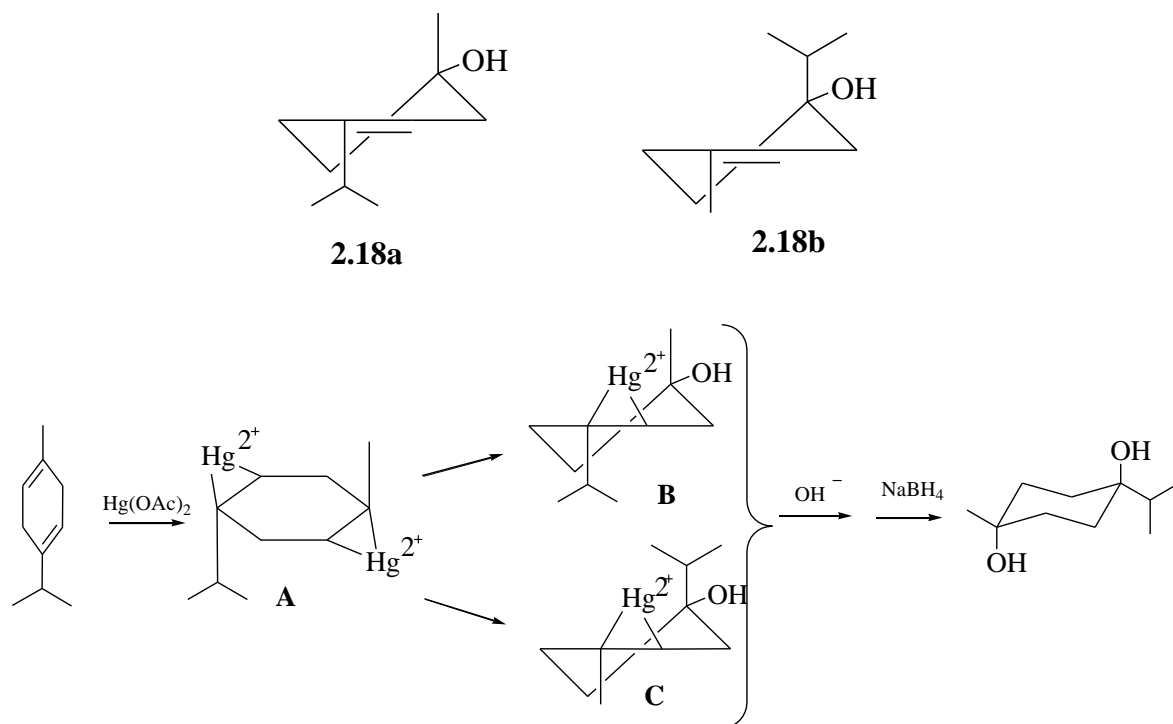
Scheme 2.13   Transition states for the formation of *p*-menthane-1,4-diol (2.18)

It was presumed that the dimercurium intermediate opened up spontaneously at both sites, leading to the formation of the diol as the sole product. However, stepwise opening of the mercurium intermediate would lead to the formation of a mixture of two intermediates (**Scheme 2.13**), both of which would be sterically controlled. Intermediate **C** would be the sterically more favoured one, and it would undergo a more facile hydration to form **2.18**.

Hydration of dihydrocarveol, (*p*-menth-8-en-2-ol, **2.2**) gave compound **2.19** in about 50 % yield. Its structure was confirmed on analysis of NMR spectra.

Conformationally, the compound may adopt either of the conformational isomers shown in **Scheme 2.14** due to two reasons. The first reason refers to consideration of conformational energy of the substituents that would dictate that the bulky alkyl (methyl and isopropyl) groups are equatorially oriented with the hydroxyl unit occupying an axial position as shown in **2.19a**.
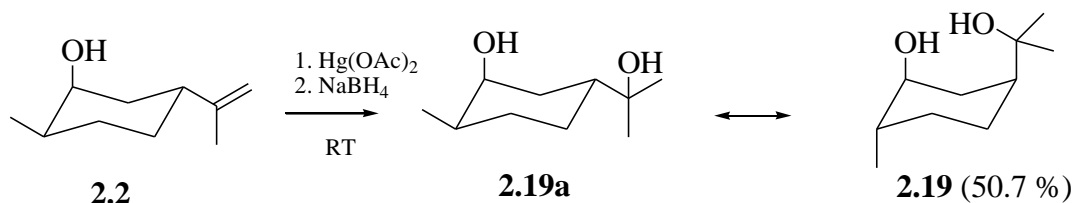
Scheme 2.14   Hydration of 2.2 and conformations of *p*-menthane-2,8-diol (2.19)

The second reason relates to the nature of interactions that would be experienced, depending on a particular orientation. As depicted in conformer **2.19**, the isopropenyl hydroxyl group positions itself in such a way as to allow intermolecular hydrogen bonding that would stabilize the axial orientation of the bulky alkyl group despite unfavourable 1,3 *syn*-axial interactions with the proton at C-6. Thus, in the ^{1}H NMR spectrum the signal that appeared at δ 2.07 was assigned to axial H-3 resonating fairly downfield due to the deshielding effect brought about by 1,3 *syn*-axial interaction with the fairly bulky C-1 methyl substituent. This analysis thus presumed the existence of *p*-menthane-2,8-diol (**2.19**).

Perillaldehyde (**1.109**) under hydration with mercuric acetate yielded *p*-menthane-7,8-diol (**2.20**) in very low yield (6%). The product was obtained as a result of the reduction of the carbonyl group into an alcohol as ascertained by the appearance of the signal at δ 3.93 in the ¹H NMR spectrum due to the hydroxy methylene protons.
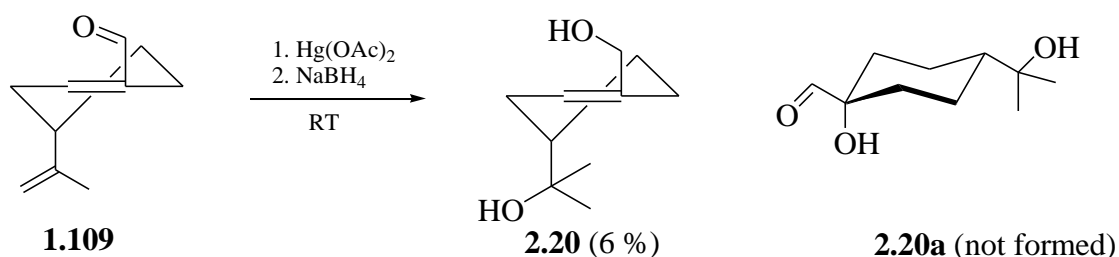
Scheme 2.15 Hydration of 1.106 to 2.20 and the expected product 2.20a

The expected product in this reaction was *p*-menthan-2,8-diol-7-al (**2.20a**) formed through the addition occurring at both the C-1 and C-8 double bonds. However, the cyclohexenyl double bond was not oxidized as indicated above. These results were a little surprising since the reaction with carvone did not yield a diol or any other compound for which the presence of the α,β-unsaturation was considered the reason. The product being a cyclohexene derivative would adopt the half-chair conformation with the isopropanol group in a *pseudo*-axial orientation (**Scheme 2.15**).

2.4   Dihydroxylation Reactions

Hydroxylation of an alkene may be carried out by using alkaline KMnO₄, forming *cis*-hydroxylated products.[304] However, attempted efforts to acquire the target compounds

using KMnO$_4$ were not successful as only complex mixtures were formed. Since reactions with KMnO$_4$ and OsO$_4$ appear to proceed by the same mechanism (**Scheme 2.16**), a repeat of *cis*-hydroxylation of the selected monoterpenes was undertaken using OsO$_4$.

OsO$_4$ hydroxylation of an alkene may be carried out in an inert solvent (e.g. ether or dioxane), forming a cyclic osmate ester intermediate (**Scheme 2.16**). This would undergo hydrolytic cleavage under reductive conditions (e.g. aqueous NaSO$_3$) to give a *cis* 1,2-diol.[306] More conveniently, a *cis*-hydroxylation process could be effected by using only catalytic amounts of OsO$_4$ in H$_2$O$_2$. H$_2$O$_2$ would cleave the intermediate osmate ester to the *cis* diol and regenerate the OsO$_4$. Because of its high cost and toxicity, OsO$_4$ was used in catalytic quantities in the presence of alkaline *t*-BuOH.[304]
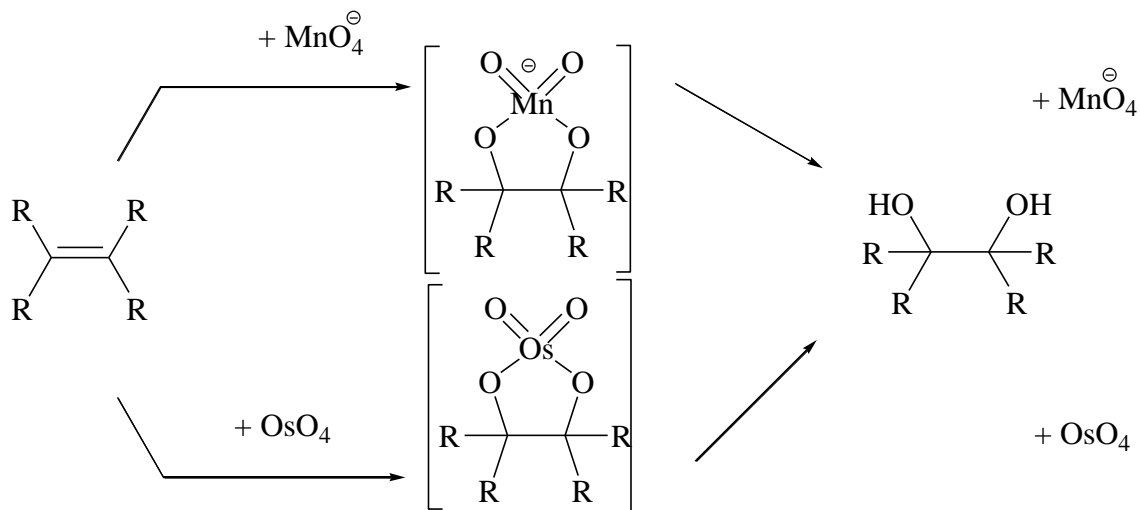
Scheme 2.16   Reaction mechanism for KMnO$_4$ and OsO$_4$ cis hydroxylation

The reaction with OsO$_4$ was conducted for *trans-p*-menth-6-ene-2,8-diol (**2.21**) and α-terpeneol (**1.112**) affording the expected products as confirmed by NMR spectra.

OsO$_4$ dihydroxylation of *trans-p*-menth-6-ene-2,8-diol (**2.21**) yielded *p*-menthane-1,2,3,8-tetrol (**2.22**) in 28 % yield, whose ^{1}H NMR spectrum indicated absence of an olefinic proton signal and presence of downfield resonances at δ 3.36 and δ 3.44 due to the carbinol methine protons H-6 and H-3 respectively. Since the ^{1}H NMR spectrum of **2.22** was run in DMSO, it was possible to observe the four hydroxyl proton signals in the spectrum, hence also confirming dihydroxylation to have taken place, as further indicated by the presence of four carbinol carbons [δ 70.3 (C-8), 70.9 (C-3), 72.2 (C-1), and 73.7 (C-2)] in the ^{13}C NMR spectrum.
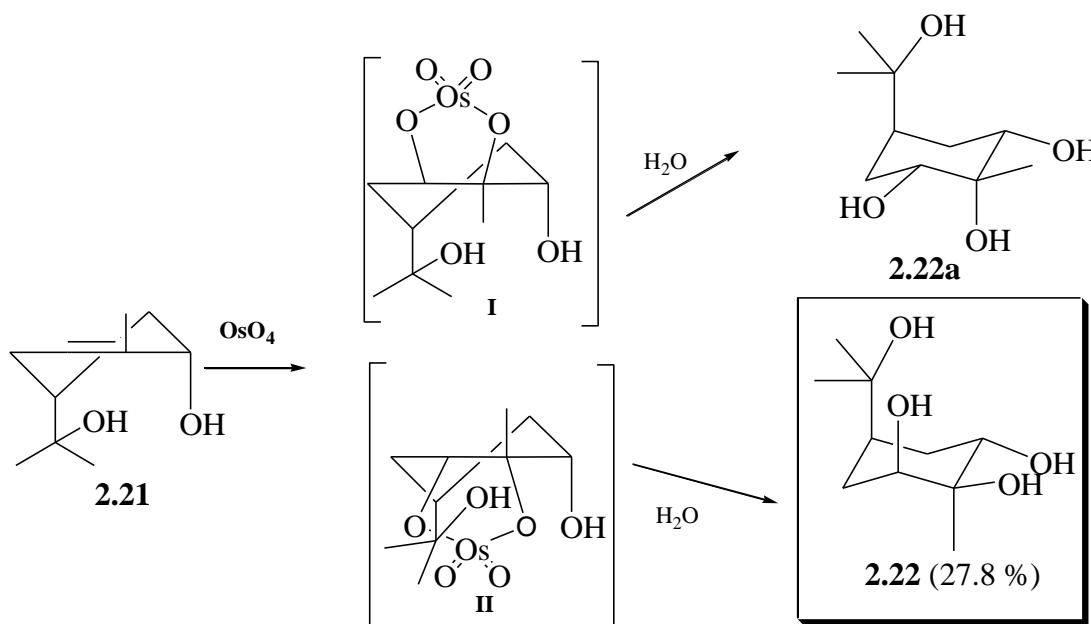
Scheme 2.17   Dihydroxylation of *trans-p*-menth-6-ene-2,8-diol (2.21)

The assignment of the stereochemical configuration could not be carried out due to limited spectral data. However, as shown in **Scheme 2.17**, intermediate **I** is more favourable with the eventual formation of compound **2.22a** in which the isopropanol moiety is axially oriented while the methyl group is equatorial. On the other hand intermediate **II** at a glance appears to be sterically less stable due to crowding as a result of the presence of an osmate moiety on the same side as the isopropanol and a hydroxyl group. It was presumed that the hydroxyl groups stabilized the osmate intermediate through hydrogen bonding, thus leading to compound **2.22** that was assumed to be the product obtained from this reaction. From this argument, it may be inferred that the hydroxyl group was responsible for directing the reaction and stereochemical configuration of the reaction process and product formed. Indeed, this is the product which was obtained as supported by the presence of the fairly upfield signal for the methyl protons and carbon (δ 1.12 and δ 23.6) appearing in the ^{1}H and ^{13}C NMR spectra respectively.

Treatment of α-terpeneol (**1.112**) with OsO_4 afforded *cis-p*-menthane-1,2,8-triol (**2.23**) as confirmed from the available spectral data. The absence of ^{1}H and ^{13}C NMR signals due to an olefinic group indicated that the reaction had taken place. That hydroxylation had occurred was confirmed by the appearance of a carbinol methine signal at δ 3.33 in the ^{1}H NMR spectrum due to H-2, whose corresponding ^{13}C NMR signal was observed at δ 75.2.

The reaction may have followed either of the routes indicated in **Scheme 2.18** on similar grounds as discussed for the reaction with compound **2.21** leading to compound **2.22** through the seemingly less stable intermediate **II**. The presumed product was corroborated by the appearance of upfield methyl signals in both the ^{1}H and ^{13}C NMR (δ_H 1.23 and δ_C 25.4 respectively) implying an axial orientation, therefore corroborating conformer **2.23** as the product.
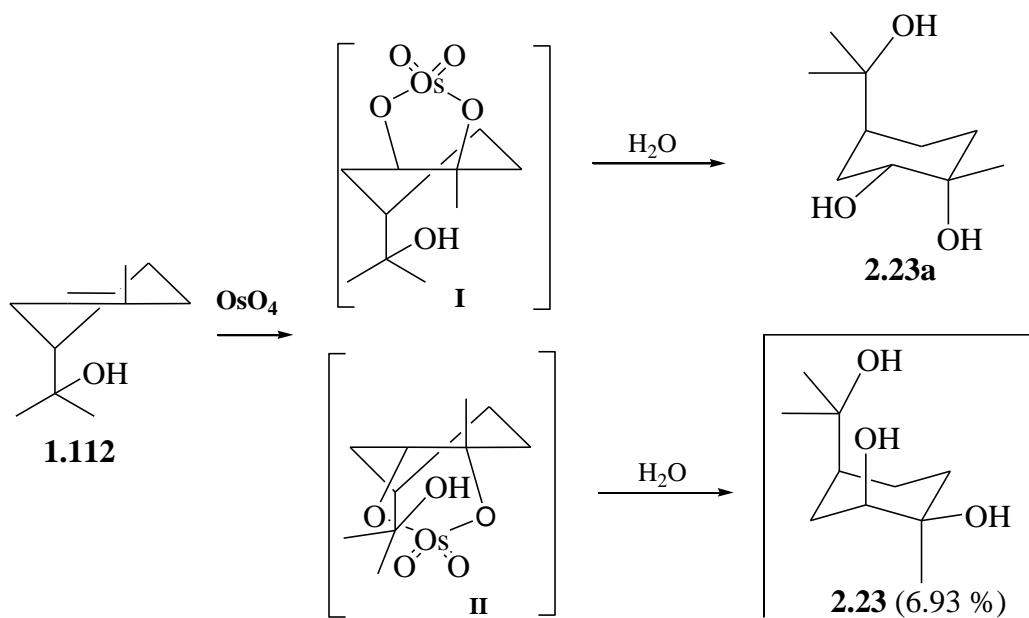
Scheme 2.18 Dihydroxylation of α-terpeneol (1.112)

2.5   Bioassay Results

2.5.1   Mosquito Repellency

Preliminary mosquito repellency assays were conducted for the precursor compounds that included (*R*)-(-)-carvone (**1.85**), (+)-dihydrocarvone (**2.1**), (-)-dihydrocarveol (**2.2**), (*R*)-(+)-limonene (**1.83**), (-)-carveol (**1.84**), α-terpinene (**2.3**), γ-terpinene (**2.4**), α-terpeneol (**1.112**), and *trans*-*p*-menth-6-ene-2,8-diol (**2.22**). The results therefore formed the basis of structure activity relationship studies in comparison with the already established mosquito repellent *p*-methane-3,8-diol (**1.79**).   The respective RC_{50} (concentration at which 50 % of the mosquitoes were repelled) values as obtained from POLO PLUS computer program are presented in **Table 2.1**.

From the dose response repellency assay carried out for the precursor monoterpenes in this study, it was indictated that all the tested compounds exhibited repellency activity (**Table 2.1**), differing only in the magnitude of the activity. Thus, carveol recorded the highest activity (RC_{50} = 3.16 x 10^{-3} mg/cm^2). The lowest activity was shown by compound **2.22** (RC_{50} = 7.69 mg/cm^2). Carvone, which is a ketonic derivative of carveol exhibited the second highest activity (RC_{50} = 3.34 x 10^{-3} mg/cm^2), followed by the monohydroxylated terpene (α-terpeneol, RC_{50} = 3.41 x 10^{-3} mg/cm^2).   The two dihydrogenated forms of carveol and carvone, that is **2.1** and **2.2** respectively showed the same activity (RC_{50} = 3.60 x 10^{-3} mg/cm^2) followed by α-terpinene (RC_{50} = 3.98 x 10^{-3} mg/cm^2) whose isomeric form γ-terpinene, showed comparatively lower activity (RC_{50} = 5.12 x 10^{-3} mg/cm^2).

These results indicate the presence of structure activity relationships. For instance the two most active compounds **1.84** and **1.85** bore the cyclohexenyl and isopropenyl moieties in addition to hydroxyl and ketonic functions, respectively. Consequently, the compounds showed differing repellent activities. The former was comparatively more active and the activity was attributed to the presence of the hydroxyl group. However, their dihydrogenated forms, namely dihydrocarvone (**2.1**) and dihydrocarveol (**2.2**) exhibited the same activity. This suggested that having the cyclohexyl skeleton both the hydroxyl and ketonic groups exhibited similar effects against mosquitoes, thereby implying that the double bonds in compound **1.84** and **1.85** did not have much influence on the repellence activity for these two compounds.

However, comparing the activity of dihydrocarveol (**2.2**) with that of α-terpineol (**1.112**), which are both mono-hydroxylated compounds and each with one olefinic group, it would be inferred that the position of the two functional groups played a crucial role towards mosquito repellency of the two compounds. The olefinic group in compound **1.112** is found on the cyclohexyl ring while that of **2.2** is attached to the isopropyl substituent. This phenomenon is also manifested by the two isomeric forms of terpinene. Thus, α-terpinene, which has a conjugated diene system was highly repellent than γ-terpinene, that has a non-conjugated diene system. The least active compound among the precursors was **2.22**, which is a diol having an isopropenyl olefinic group.

Table 2.1   **Repellency activity of precursor monoterpenes**

Compound	Percentage repellency/concentration (%-w/v in acetone)					RC_{50} (mg/cm^2)
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	
1.83	4.3±1.39	12.2±1.09	18.8±1.91	39.1±3.99	100±0	5.22×10^{-3}
1.84	7.2±1.25	13.8±2.02	32.2±2.21	55.8±9.05	100±0	3.16×10^{-3}
1.85	9.6±1.54	12.2±2.16	32.2±5.64	46.3±7.9	100±0	3.34×10^{-3}
1.112	7.4±2.33	15.2±0.75	30.5±2.41	51.6+3.99	100±0	3.41×10^{-3}
2.1	8.8±1.66	12.5±2.93	30.6±5.48	51.3±3.59	100±0	3.60×10^{-3}
2.2	6.6±2.53	15.7±1.19	32.2±2.03	47.1±3.30	100±0	3.60×10^{-3}
2.3	3.8±0.95	13.5±0.98	21.6±2.15	42.3±5.01	100±0	3.98×10^{-3}
2.4	3.4±1.74	11.2±0.99	18.2±2.50	44.0±5.99	100±0	5.12×10^{-3}
2.22	9.4±2.86	10.6±1.37	22.6±3.50	44.9±2.82	44.8±3.94	7.69
1.79 (STD)	22.5±3.41	37.2±3.53	42.5±6.21	85.8±2.39	100±0	8.4×10^{-4}

$RC_{50} =$ Concentration at which 50 % of the mosquitoes were repelled

The results in **Table 2.1** are a good indication of the potential of the tested compounds as mosquito repellents. In fact some of the monoterpenes have been registered for use as repellents for mosquitoes and other insects. For instance, carvone is registered in the USA under the Environmental Protection Agency (EPA) as a biopesticide.[307] Likewise, limonene has been registered under the same body for use as an active ingredient in products used for the control of fleas, ticks, mosquito larvicide, and as a repellent for insects on humans.[308]

Despite their use as pesticides, monoterpenoids have a shortcoming in applicability due to transient action attributed to their high volatility. Thus, several research initiatives are ongoing to establish suitable ways and formulations that would make them available for a longer duration after application. One such endeavour involves the use of polymeric cyclodextrin, which constitutes monochlorotriazinyl-β-cyclodextrin molecules, as a fixative agent for limonene impregnation onto fabrics.[309] Another important approach is through chemical modification of the monoterpenoids, which entails introduction of different functional groups that would likely lower their volatility while maintaining or enhancing their repellent potency. This synthetic route was adopted in this study as outlined by the preceding sections, and the efficacy of the derivatized compounds is presented for the epoxides (**Table 2.2**) and hydroxylated compounds (**Table 2.3**).

The mosquito repellency assay of the epoxides tested (**Table 2.2**) indicated some level of improvement as compared to their precursors. This implied that the presence of an epoxide moiety in the *p*-menthane skeleton would play a protagonistic role towards their effectiveness as repellents. Indeed, some of the epoxides tested showed greater repellence than *p*-menthane-3,8-diol (**1.79**), whose RC_{50} value was found to be 8.4 x 10^{-4} mg/cm^2 (**Table 2.2**). The highest level of improvement was recorded for limonene diepoxide (**2.16**) that appeared to be approximately 19 times more effective than its precursor limonene (**1.83**), with an RC_{50} value of 2.7 x 10^{-4} mg/cm^2 against 5.22 x 10^{-3} mg/cm^2 for limonene. The same compound exhibited the highest repellent activity amongst all epoxides tested. Likewise, carvone epoxide (**2.13**) exhibited high repellent activity (RC_{50} value = 3.9 x 10^{-4} mg/cm^2) while the diepoxide derivative (**2.14**) of

carvone showed an RC_{50} value of 1.00×10^{-3} mg/cm^2. This observation implied that the presence of α,β-unsaturation in **2.13** might have been responsible for the high repellent properties, in addition to the epoxide function and that an additional epoxide group may not necessarily mean an increase in activity. This was inferred from comparing the RC_{50} values of the five diepoxides. Whereas the reaction products **2.8** and **2.14** contain hydroxyl and carbonyl groups respectively, compounds **2.10**, **2.11** and **2.16** do not have such groups but only epoxides at different positions.

The α-terpinene derivative **2.7** showed a slightly higher activity than the γ-terpinene derivative **2.14**, implying that the nature of substitution played an important role towards the effectiveness of the compounds as mosquito repellents. Evidently, among the tested compound the diepoxides with an isopropoxyl group showed higher activity compared to the cyclohexanyl epoxides.

Table 2.2   **Bioassay results of epoxide derivatives**

Compound	Percentage repellency/concentration (%-w/v in acetone)					RC_{50} (mg/cm^2)
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	
2.5	5.2±2.58	12.8±3.59	32.1±3.84	61.6±3.55	100±0	2.55 x 10^{-3}
2.6	15.9±2.98	19.1±5.08	58.2±6.55	64±5.65	100±0	2.04 x 10^{-3}
2.7	14.0±3.80	25.9±4.29	36.9±9.02	57.7±7.93	87.7±7.31	8.9 x 10^{-4}
2.8	9.8±2.56	15.2±1.40	32.2±3.04	94.0±3.07	100±0	1.08 x 10^{-3}
2.9	10.3±2.94	24.4±5.68	41.3±5.01	85.7±9.05	100±0	1.06 x 10^{-3}
2.10	16.2±1.89	21.1±4.66	57.2±6.89	62.5±6.58	98.3±1.67	1.08 x 10^{-3}
2.11	14.1±3.43	22.6±4.47	41.9±4.26	54.6±8.7	100±0	1.48 x 10^{-3}
2.12	5.1±1.79	19.5±3.19	28.8±4.63	78.9±6.07	100±0	1.74 x 10^{-3}
2.13	6.7±5.44	11.9±2.66	31.8±4.48	97.9±1.34	100±0	3.9 x 10^{-4}
2.14	18.9±3.24	38.8±5.05	55±3.80	97.6±1.69	100±0	1.0 x 10^{-3}
2.16	13.9±4.53	35.9±2.83	79.6±6.38	83.9±6.93	100±0	2.7 x 10^{-4}
1.79 (STD)	22.5±3.41	37.2±3.53	42.5±6.21	85.8±2.39	100±0	8.4 x 10^{-4}

Another notable attribute observed from these results indicated that both the carbonyl and hydroxyl groups could have had some influence on the mosquito repellency efficacy, depending on the substitution pattern of the compounds and the presence of other functional groups. Thus, compounds **2.5** and **2.6** exhibited the lowest repellent activity, which was associated with the presence of an isopropylene (olefinic) group,

despite having the hydroxyl group directly attached to the cyclohexanyl ring, while compound **2.7** exhibited high activity, probably as a result of its olefinic group being within the ring.

Table 2.3   **Bioassay results of hydroxylated derivatives**

Compound	Percentage repellency/concentration (% w/v in acetone)					RC_{50} (mg/cm^2)
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	
2.17	14.0±1.40	22.0±2.44	27.9±2.12	35.7±3.10	79.9±1.33	1.48
2.18	11.9±2.91	16.1±4.04	32.8±4.15	48.5±6.8	64.8±3.93	1.36
2.19	12.4±3.27	21.6±4.03	39.8±6.84	79.2±5.30	99.1±0.93	1.19×10^{-3}
2.20	32.6±2.76	43.1±1.84	66.8±5.28	81.7±2.22	94.8±3.28	2.9×10^{-4}
2.21	19.3±2.55	27.4±2.83	32.2±2.15	90.7±3.86	99.1±0.93	8.1×10^{-4}
2.23	-9.86±9.8	12.3±6.61	26.2±4.21	35.7±4.21	39.9±3.61	-
2.24	-5.93±7.2	10.8±1.65	19.5±2.38	44.1±7.61	92.4±3.58	-
1.79 (STD)	22.5±3.41	37.2±3.53	42.5±6.21	85.8±2.39	100±0	8.4×10^{-4}

- implies that no figure was obtained by the computer program (POLO PLUS) used

The hydroxylated derivatives indicated a varying degree of activity as shown in Table **2.3**, with the dihydrocarvone derivative **2.20** showing the highest activity (RC_{50} = 2.9 x 10^{-4} mg/cm^2) amongst the tested compounds, being, more than twice as active as the prerillaldehyde derivative **2.21** whose RC_{50} value was 8.1 x 10^{-4} mg/cm^2, the activity being comparable to that of *p*-menthane-3,8-diol (**1.79**). The two compounds are both diols, differing only in the hydroxyl substitution pattern, the former and most active being 2,8- while the later is 7,8- substituted. The third most active compound in this series was the 1,4-substituted diol **2.19** derived from γ-terpinene, that had an RC_{50} value

of 1.19 x 10^{-3} mg/cm^2. The limonene derivative **2.18** was also active. However, its activity was comparatively lower (RC_{50} = 1.369 mg/cm^2). This compound has its hydroxyl groups at C-1 and C-8 on the *p*-menthane skeleton.

An interesting observation was made for compounds **2.23** and **2.24**, whose RC_{50} values could not be determined by the computer program used. However, the results indicated that the two compounds attracted mosquitoes at lower concentration and gradually repelling them as the concentration was raised. The attracting characteristic was more pronounced for the tetrol **2.24** attracting an average of 9.86 % of the mosquitoes tested, while at a similar concentration compound **2.23** attracted 5.93 % of the mosquitoes. However, at the highest concentration (10^{-1} mg/cm^2) compound **2.23** exhibited good repellency activity by repelling over 90 % of the mosquitoes.

These results indicate considerable improvement in the repellency activity of dihydrocarveol and γ-terpinene upon hydration, with the former rising 12 folds and the latter by about 4 times. Conversely, the other derivatives recorded lower repellent activities compared to their precursors. Thus, the diol **2.18** was 3 times less active as compared to its precursor compound (limonene), while compounds **2.23** and **2.24** showed some attracting tendencies at lower concentrations unlike their precursors **2.4** and **2.22** respectively.

In general, the SAR studies showed that the position of the hydroxyl groups was a crucial determinant for mosquito repellent activity in this series of compounds. Furthermore, while the hydroxyl group was important in imparting repellent properties

to these compounds, a higher number on the molecule, probably above two would lead to a negative effect towards their repelling potential.

2.5.2 Apoptosis Induction Results of the Precursor *p*-Menthanes and their Structure Activity Relationships

The preliminary testing of five precursors in this study indicated above 50 % apoptotic activity against both cell lines (**Table 2.4**) and on average showed higher apoptosis induction against Jurkat T cells with, four compounds recording the presence of above 90 % non-viable cells.

Table 2.4   **Apoptosis activity for the studied *p*-menthane precursors**

	% Non-viable (apoptotic) cells	
Compound	**Jurkat T**	**CHO**
1.83	96.7 ± 2.56	88.2 ± 1.42
1.84	91.9 ± 5.11	54.9 ± 9.50
1.85	98.1 ± 0.59	60.8 ± 7.64
2.1	98.3 ± 0.61	62.2 ± 12.39
2.4	57.1 ± 6.55	71.9 ±1.58
Camptothecin	81.9±1.72	55.5±7.03
-ve control	33.6±5.03	21.7±0.62
DMSO	37.7±2.65	37.3±11.46

The most active compounds against Jurkat T cells were carvone (**1.85**) and dihydrocarvone (**2.1**), both having a ketonic group, followed closely by limonene (**1.83**)

with γ-terpinene (**2.4**) as the least apoptotic compound (57.06 %). On the other hand, compound **1.83** exhibited the highest activity (88.15 %), followed by **2.4** (71.85 %) against the Chinese Humster Ovary (CHO) cell line.

The least active compound against CHO cells was carveol (**1.84**) that recorded 54.87 % cell death, which was comparable to the positive control Camptothecin. Against Jurkat T cells, all compounds tested except **2.4** exhibited higher activities than he positive control Camptothecin.

Structurally the most active compounds against Jurkat T cells have a ketonic group on the cyclohexyl ring. This may suggest that the group could be responsible for the high apoptotic potential of the corresponding compounds. However, in comparison this may not be the situation for apoptotic induction against CHO cells as indicated in **Table 2.4 and Fig. 2.3** where the activity was relatively low. Furthermore, comparison between compounds **1.83** and **2.4** suggested that two cyclohexyl olefinic groups might be playing an antagonistic role against Jurkat T cells, as observed for the later compound. However, the compound exhibited stronger protagonistic activity against CHO cells but the activity was not as high as for compound **1.83**, which consists of an isopropylene substituent.

The selective activity of compound **1.84** against the two cell lines could probably imply that as much as the hydroxyl group might have enhanced the activity against Jurkat T cells, its influence tended to diminish for activity against CHO cells.
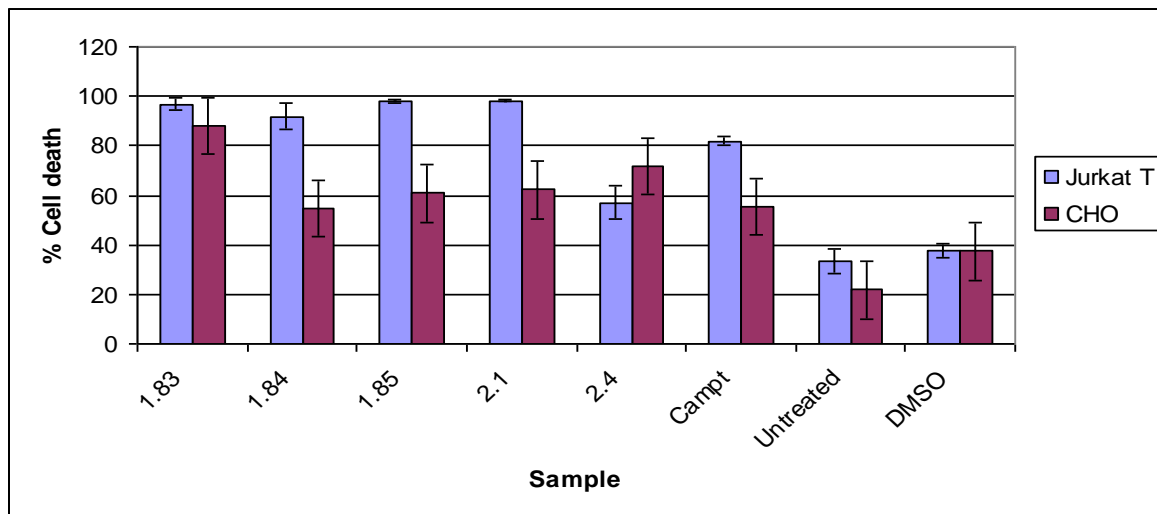
Fig. 2.3   **Apoptosis activity for the studied *p*-menthane precursors 1.83-1,85 and 2.1 and 2.4**

2.5.3   Apoptosis Induction Results of the Synthesized *p*-Menthane Epoxides and their Possible Structure Activity Relationships

The synthesized epoxides were tested against the two cell lines (Jurkat T and CHO cells) by flow cytometric analysis to evaluate their apoptotic potential. Results are summarized in **Table 2.5**. All compounds tested induced some level of apoptosis against both cell types, the activity ranging from low to very high. For instance, compounds **2.5**, **2.6**, **2.9** and **2.15** recorded above 95% non-viable Jurkat T cells followed by **2.10** and **2.11** whose activity was above 80% cell death on the same cell line. On the other hand, the activity of these compounds appeared to diminish against the CHO cell line, thus indicating some level of selective activity between the two cell lines. For example, compound **2.15** that induced 97.4% cell death against Jurkat T cells exhibited an average death of 51.2

% against CHO cells. However, the level of selectivity in activity differed amongst the tested compounds (**Fig. 2.4**). The lowest activity against Jurkat T cells was observed for compounds **2.7** and **2.12**, **2.13** and **2.14** while against CHO cells it was observed for compounds **2.7**, **2.10**, and **2.12** all of which registered below 40% non-viable cells. This could be attributed to the various and differently substituted functional groups on the compounds analyzed.

Table 2.5   **Flow cytometric results for apoptotic activity of the synthesized *p*-menthane epoxides against CHO and Jurkat T cells at (μM)**

	% Non-viable (apoptotic) cells	
Compound	**Jurkat T**	**CHO**
2.5	97.5 ± 0.79	81.8 ± 8.47
2.6	97.9 ± 0.65	76.1 ± 6.54
2.7	38.1 ± 4.71	34.7 ± 3.34
2.8	60.9 ± 13.6	51.7 ± 9.18
2.9	95.1 ± 3.35	57.6 ± 4.95
2.10	83.2 ± 4.39	36.9 ± 9.59
2.11	81.9 ± 4.92	71.8 ± 1.58
2.12	38.1 ± 4.71	34.7 ± 3.34
2.13	39.9 ± 4.91	58.4 ± 1.86
2.14	37.1 ± 4.01	62.8 ± 2.56
2.15	97.4 ± 0.44	51.2 ± 7.17
Camptothecin	81.9 ± 1.72	55.5 ± 7.03
-ve control	33.6 ± 5.03	21.7 ± 0.62
DMSO	37.7 ± 2.65	37.3 ± 11.46
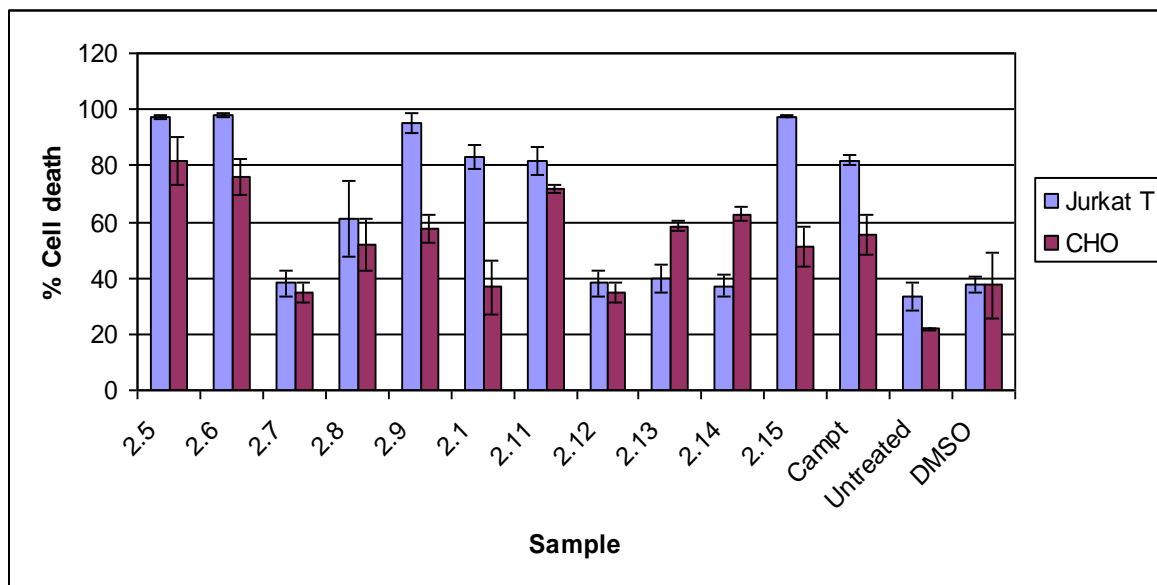
Fig. 2.4 Apoptosis activity of the derivatized *p*-menthane epoxide

These results suggested a pattern that could be related to structure activity relationships for the tested epoxides. Thus, against Jurkat T cells, the results suggested that an α,β-unsaturated carbonyl system might have played a significant role towards induction of apoptosis, as indicated by the carvonoid **2.5**. In addition the other carvonoid **2.6**, which had a β-carbonyl group relative to the epoxide unit, also exhibited similarly high activity. This, observation was further substantiated by the low activity exhibited by the dihydrocarvone epoxide **2.13**, which, despite bearing the ketonic group exhibited low activity against Jurkat T cells.

Although this study did not embark on the mode of activity, the high activity indicated by carvonoid **2.5** was probably due to the α,β-unsaturated carbonyl system as described above. This system has generally been associated with enhancement of bioactivity. It

has therefore been postulated that the group, being activated for conjugate nucleophilic addition would readily attack cellular nucleophilic sites, thereby inhibiting cell growth or eventually killing the cells. If the cells belong to pathogenic organisms, this postulate proposes the eventual inhibition of the latter's multiplication and hence ultimate extermination of the organism.[310]

Despite the presence of two epoxide units on the *p*-menthane skeleton being noted as a protagonist towards activity enhancement, the substitution pattern of the two epoxides also appeared to be a requirement for enhanced activity against Jurkat T cells. Thus, with one endocyclic epoxide and isopropoxyl group compounds **2.6**, and **2.15** indicated higher activity as compared to the derivatives **2.7** and **2.14**, whose epoxide groups were both present in the cyclohexyl ring. However, the dihydrocarveol derivative (**2.12**) registered dismally low activity towards the two cell lines despite being a diepoxide. It may be inferred that the presence of the hydroxyl group at position 2 of the cyclohexyl ring may have had a negative impact towards apoptotic induction.

Another interesting observation was made for compounds **2.9** and **2.10**, which differed only in the configuration of the hydroxyl groups (**Fig. 2.1**), the former being *cis* while the latter is *trans*. Compound **2.9** exhibited higher activity than **2.10** against both cell lines.

Against CHO cells, a fairly complex possible SAR was observed. For instance the presence of two epoxide groups in a compound was not sufficient enough to raise the

apoptotic potential of the corresponding compound. Thus, the diepoxide **2.6** that possesses a carbonyl group exhibited higher activity than compounds **2.7**, **2.12**, **2.14** and **2.15**, which either lacked any or had a hydroxyl group attached to the *p*-menthane skeleton. Indeed, it was also noted that a carbonyl group seemed to be an apoptosis protagonist against CHO cells, more so when in conjugation with an olefinic group forming an α,β-unsaturated carbonyl system, as demonstrated by compounds **2.13** and **2.5**.

Generally, it was observed that most of the epoxides tested exhibited good to excellent apoptotic activity against the tested cell lines, which was attributed to the alkylating ability of the epoxide moiety.[311] Epoxides have been known to exhibit sub-micromolar *in vitro* cytotoxic activity and promising anti-tumor activity, and appear to exert their effect by the formation of covalent interstrand within the cell DNA. In addition, some epoxide bearing compounds such as NCO-700 and TOP-008, have been shown to exhibit cytotoxicity through induction of apoptosis.[312] Previous studies have shown that compound NCO-700 is tolerable and non toxic to humans, thus suggesting its potential and those of other epoxide bearing compounds as potential chemotherapeutic agents or leads for further research.[312] Nonetheless, there have been instances where the epoxide moiety has been considered not to be a suitable entity in an antitumor agent, since due to its alkylating ability to nucleophilic species, it has the potential of being non-selective between tumorous and healthy cells.

2.5.4 Apoptosis Induction Activity of the Hydroxylated *p*-Menthanes and their Possible Structure Activity Relationships

When the hydroxylated *p*-menthanes were assayed for apoptosis induction generally the results indicated high apoptotic activity against Jurkat T cells as compared to CHO cells, apart from compounds **2.17** and **2.18** whose activity against CHO cells was slightly higher than for Jurkat T cells (**Table 2.6**). The most active compound was mono-hydroxylated limonene derivative **2.16** (92.3 and 79.04 % non-viable cells against Jurkat T and CHO cells respectively). Amongst the diols tested the second most active against Jurkat T cells was compound **2.20**, a derivative of perillaldehyde that exhibited 88.6 % cell death, followed by **2.19** obtained from dihydrocarveol which showed 69.8 % cell death. The triol **2.23** showed above average (68.4 %) apoptotic activity while the tetrol derivative **2.22** exhibited a lower activity of 55.1 %. All the tested compounds showed an activity of above 40 % non-viable cells against CHO, except for the tetrol **2.23** whose activity was 21.5 %. These results suggested the highest level of selective activity of the tested compounds between the two cell lines in this study.

A notable possible SAR characteristic related to the number of hydroxyl groups playing a role in determining the activity of the tested compounds. This was indicated by the high activity recorded for the mono-hydroxylated **2.16** as compared with the activity of the rest of the compounds. While comparing the activity of the diols, it was noted that the position of the hydroxyl groups may have played a crucial role for apoptosis inducing potential of the compounds. Thus, compound **2.20** with a 7,8-substituted diol

exhibited the highest activity against Jurkart T cells among the diols tested followed by the 2,8-substituted derivative **2.19**. It may therefore be presumed that a hydroxyl group at position 8 of the menthane skeleton has a positive impact towards apoptotic induction as corroborated by the most active compound **2.16**, for which despite being mono-hydroxylated, the hydroxyl group is at position 8. Likewise, it may be inferred that a hydroxyl group at position 1 of the cyclohexanyl skeleton exhibited antagonistic characteristics as exemplified by the low activity of compounds **2.17** and **2.18** against the Jurkat T cells. The presence of an olefinic group in the cyclohexyl ring might have been responsible for an increased apoptotic potential for compounds **2.16** and **2.20**, while the high selective activity exhibited by the latter may be attributed to the hydroxymethyl group, hence further supporting the postulation on the positions of the OH group.
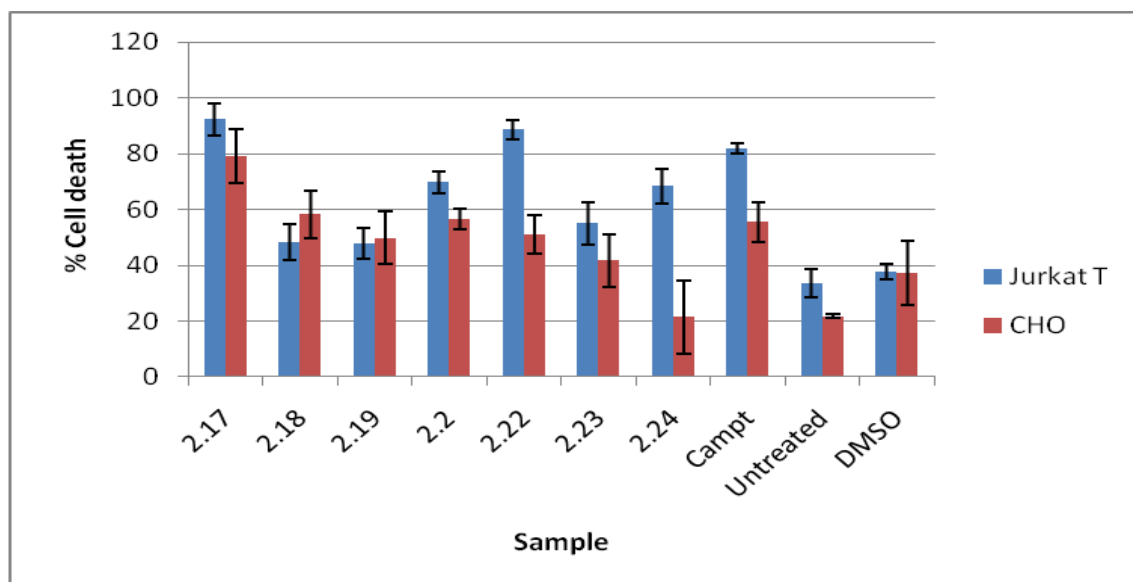
Fig. 2.5   **Apoptosis activity for hydroxylated *p*-manthanes**

Table 2.6   **Flow cytometric results for apoptotic activity of the hydroxylated *p*-menthane against CHO and Jurkat T cells at (1 µM)**

	% Non-viable (apoptotic) cells	
Compound	**Jurkat T**	**CHO**
2.17	92.3 ± 5.63	79.04 ± 9.62
2.18	48.1 ± 6.42	58.18 ± 8.67
2.19	47.9 ± 5.63	49.89 ± 9.34
2.20	69.8 ± 3.85	56.63 ± 3.54
2.22	88.6 ± 3.37	51.14 ± 6.87
2.23	55.1 ± 7.55	41.67 ± 9.42
2.24	68.4 ± 6.11	21.5 ± 13.15
Camptothecin	81.9±1.72	55.5±7.03
-ve control	33.6±5.03	21.7±0.62
DMSO	37.7±2.65	37.3±11.46

2.6   Conclusion

The results discussed in this chapter indicated a good potential of the synthesized compounds both as mosquito repellents and apoptotic agents though in varying degrees. An improvement in repellent activity in comparison to the precursor monoterpenoids was observed for most of the compounds obtained. Four of the tested compounds (**2.13**, **2.15**, **2.20** and **2.21**) exhibited higher repellence than the insect repellent *p*-menthane-3,8-diol (**1.79**), a phenomenon that was also observed when assayed for apoptosis induction against Jurkat T and CHO cells, in which some compounds exhibited higher activity than that of the standard drug Camptothecin.

Despite the low yields that were obtained in a number of reactions described in this chapter, the study forms a basis for further research towards establishing the targeted compounds as potential mosquito repellents and for apoptotic induction.

2.7 Experimental Procedures

2.7.1 General Procedures

These general conditions are applicable to all subsequent chapters.

Glassware: All glassware were soaked in chromic acid overnight before being washed thoroughly in hot water and soap, rinsed with acetone and then oven dried at $110\,^{\circ}C$ overnight.

Solvents: All solvents were of analytical grade from Sigma Aldrich or Merck BDH and were used as supplied.

Chromatography: Analytical thin layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} plates (Merck) of 0.20 mm thickness. The plates were first visualized under UV light on both long (365 nm) and short wave lengths (254 nm), then sprayed with anisaldehyde as a detection reagent followed by charring at 100-140 $^{\circ}C$. Column Chromatography was done using silica gel 60 (0.040 - 0.63 mm, 230 - 400 mesh, Merck), eluting with different solvent systems as indicated in the relevant sections and the resulting chromatographic fractions being analyzed by TLC. Fractions with the same R_f values were combined and the solvent was evaporated *in vacuo*.

Melting points (**m.p.**): These were determined on a Buchi B-545 electronic melting point apparatus and are uncorrected.

Nuclear Magnetic Resonance (**NMR**): Spectra were recorded on Bruker 200, 300 and 600 spectrometers operating at 200, 300 and 600 MHz respectively for ^{1}H NMR and 50, 75 and 150 MHz respectively for ^{13}C NMR, using tetramethyl silane (TMS) and solvent signal as internal standards for ^{1}H and ^{13}C NMR respectively.

Mass Spectra (**MS**): These were recorded under electron ionization at 70eV.

2.7.2 Modified BAP Epoxidation

NH_4HCO_3 and H_2O_2 were dissolved in water, mixed with MeCN, and the substrate added. The mixture was immersed in an ice bath and stirred until the substrate was consumed. The reaction mixture was then diluted with water (100 mL) and extracted with $CHCl_3$ or EtOAc (5 x 200 mL). The filtrate was washed with water (2 x 40 mL), dried, and concentrated by removal of solvent. The crude product was purified by chromatography over SiO_2 (5% EtOAc in n-hexane and then gradient elution in ascending polarity).

8,9-Epoxy-p-menth-6-en-2-one (**2.5**). Carvone (**1.85**, 5.25 g, 35 mmol), NH_4HCO_3 (6.30 g, 79 mmol), H_2O_2 (38 mL, 360 mmol), water (130 mL) and MeCN (240 mL) were mixed and then stirred for 2 h on an ice bath and the epoxide **2.5** (2.78 g, 47.7 %) was obtained as brownish oil, R_f 0.51 (EtOAc/Hex, 2/3 v/v); ^{1}H NMR (300 MHz,

CDCl$_3$), δ 6.82 (1H, m, H-6), 2.67 (1H, t, $J_{H3, H4}$ = 4.2 Hz, H-3), 2.51 (1H, d, J = 6.8 Hz, H-9), 2.38 (1H, d, J = 5.6 Hz, H-8), 2.31 (1H, t, $J_{H3, H4}$ = 4.0 Hz, H-3), 2.26-2.00 (3H, m, H-4, H-5), 1.66 (3H, bs, H-7) and 1.23 (3H, s, H-10); ^{13}C NMR (75 MHz, CDCl$_3$), δ 198.1 (C-2), 144.8 (C-6), 134.1 (C-1), 57.8 (C-8), 51.7 (C-9), 40.5 (C-4), 39.6 (C-3) and 18.3 (C-10); MS, *m/z* (% rel. int.)189 ([M+Na]$^+$, 100), 189 (20), 184 (4) and 107 (4).

1,6:8,9-Diepoxy-p-menthan-2-one (**2.6**). Compound **2.6** was obtained as a thick yellowish oil as a mixture with **2.5** through work up as discussed above; yield, 1.17 g (18.4 %); R$_f$ 0.38 (2/3 v/v EtOAc/Hex,); ^{1}H NMR (300 MHz, CDCl$_3$), δ 3.53 (1H, d, J = 4.4 Hz, H-6), 2.67 (2H, dd, J = 5.1, 4.8 Hz, H-3), 2.51-2.30 (2H, m, H-9), 2.25-1.75 (3H, m, H-4, H-5), 1.27 (3H, s, H-7) and 1.18 (3H, s, H-10); ^{13}C NMR (75 MHz, CDCl$_3$), δ 204.4 (C-2), 60.4 (C-6), 57.6 (C-8), 55.3 (C-1), 51.7 (C-9), 38.3 (C-3), 24.9 (C-5), 18.1 (C-10) and 15.1 (C-7); MS, *m/z* (% rel. int.) 205 ([M+Na]$^+$, 90), 169 (46), 151 (20) and107 (13).

1,2:3,4-Diepoxy-p-menthane (**2.7**). *p*-Mentha-1,3-diene (**2.3**, 2.7 mL, 2.58 g, 19 mmol), NH$_4$HCO$_3$ (3.15 g), H$_2$O$_2$ (19 mL), water (65 mL) and MeCN (120 mL) were stirred for 2 h in an ice bath and **2.7** was obtained as a yellow oil; yield, 1.36 g (42.63 %); R$_f$, 0.44 (EtOAc/Hex, 2/3 v/v); ^{1}HNMR (600 MHz, CDCl$_3$), δ 3.23 (1H, m, H-3), 3.06 (1H, d, J = 3.9 Hz, H-2), 2.45 (1H, m, H-8), 1.77 (2H, m, H-5, H-6), 1.40 (2H, m, H-5, H-6), 1.24 (3H, s, H-7), 0.94 (3H, d, J = 1.8 Hz, H-9) and 0.92 (3H, d, J = 1.5 Hz, H-10).

8,9-Isopropoxy-p-menthan-2-ol, (**2.8**). *p*-Menthan-2-ol (**2.2,** 2.7 mL, 2.7 g, 17.5 mmol), NH_4HCO_3 (3.15 g), H_2O_2 (19 mL), water (65 mL) and MeCN (120 mL) were stirred for 2 h in an ice bath and **2.8** was obtained as a yellowish oil; yield, 0.78 g (26.1 %); R_f 0.38 (EtOAc/Hex, 2/3 v/v); ^{1}H NMR (300 MHz, CDCl$_3$), δ 3.18 (1H, dd, $J_{H2, H3ax}$ = 10.1, $J_{H2, H3eq}$ =4.3 Hz, H-2), 2.67 (1H, d, J = 5.6 Hz, H-9), 2.58 (1H, d, J = 5.3 Hz, H-9), 1.97 (1H, m, H-3), 1.81-1.64 (4H, m, H-1, H-4, H-5), 1.37-1.07 (3H, m, H-3, H-5, H-6), 1.26 (3H, s, H-10) and 1.01 (3H, d, J = 6.3 Hz, H-7); ^{13}C NMR (75 MHz, CDCl$_3$), δ 79.1 (C-2), 63.1 (C-8), 56.9 (C-9), 47.2 (C-1), 43.7 (C-4), 41.2 (C-3), 36.6 (C-6), 31.5 (C-5), 21.4 (C-10) and 20.8 (C-7); MS, *m/z* (% rel. int.) 171 ([M+1]$^+$, 15), 153 (40), 135 (80), 123 (38), 107 (100) and 105 (70).

2.7.3 *m*-CPBA Epoxidation

m-Chloroperoxybenzoic acid (*m*-CPBA) was added to a stirred solution of the substrate in CH_2Cl_2 (25 mL) at RT and the solution stirred further for 8-24 h until TLC indicated completion of the substrate. The reaction mixture was then washed successively with 10% aqueous NaHSO$_3$ (3 x 20 mL) and saturated NaHCO$_3$ (3 x 20 mL) and the organic layer was dried over MgSO$_4$. After filtration and evaporation, the crude product was purified by chromatography over SiO$_2$ (5% EtOAc in n-hexane and then gradient elution in ascending polarity).

Cis-2-hydroxy-1,6-epoxy-p-menthen-8,9-ene (**2.9**). A mixture of carveol (*p*-mentha-1,8-diene-2-ol, **1.84**, 4 ml, 3.78 g, 24 mmol) and *m*-CPBA (8.28 g, 48 mmol) stirred for 18

h at RT afforded **2.9** as a colourless oil; yield, (0.94 g, 23.3%); R_f 0.56 (EtOAc/Hex, 2/3 v/v); ^{1}H NMR (600 MHz, CDCl$_3$), δ 4.69 (1H, d, J = 0.6 Hz, H-9), 4.67 (1H, t, J_{AB} = 1.8 Hz, H-9), 3.84 (1H, m, H-2), 3.15 (1H, d, J = 5.4 Hz, H-6), 2.00 (1H, m, H-4), 1.96 (1H, ddd, $J_{H3, H2}$ = 1.8, $J_{H3, H5}$ = 1.8 and 1.8 Hz, H-3), 1.77 (1H,dd, $J_{H5, H4}$ = 12.6, $J_{H5, H6}$ = 12.6 Hz, H-5), 1.67 (3H, s, H-10), 1.44 (3H, s, H-7) and 1.32 (1H, dd, $J_{H5, H4}$ = 12.6, $J_{H5, H6}$ = 12.6 Hz, H-5); ^{13}C NMR (150 MHz, CDCl$_3$), δ 147.6 (C-8), 109.7 (C-9), 72.2 (C-2), 62.3 (C-6), 60.4 (C-1), 40.4 (C-4), 33.9 (C-3), 29.1 (C-5), 20.1 (C-10) and 19.1 (C-7).

Trans-2-hydroxy-1,6-epoxy-p-menthen-8,9-ene (**2.10**). Compound **2.10** was obtained as a colourless oil from the reaction **1.84** as described above; yield for **2.11**, 1.032 g (25.4 %); R_f 0.64 (EtOAc/Hex, 2/3 v/v); ^{1}H NMR (600 MHz, CDCl$_3$), δ 4.74 (1H, bs, H-9), 4.68 (1H, bs, H-9), 3.92 (1H, m, H-2), 3.29 (1H, dd, $J_{H6, H5}$ = 2.4, 2.4 Hz, H-6), 2.25 (1H, d, J = 10.2 Hz, H-3), 2.18 (1H, m, H-4), 1.70 (3H, bs, H-10), 1.67 (1H, m, H-3), 1.64 (1H, m, H-5), 1.43 (3H, s, H-7) and 1.35 (1H, m, H-5); ^{13}C NMR (150 MHz, CDCl$_3$), δ 148.0 (C-8), 109.5 (C-9), 68.1 (C-2), 63.2 (C-6), 59.9 (C-1), 35.8 (C-4), 31.6 (C-3), 30.1 (C-5) and 21.0 (C-7, C-10).

2-Hydroxy-8,9-isopropoxy-p-mentha-1-ene (**2.11**). Compound **2.11** was obtained as colourless oil from the reaction of **1.84**; yield, 0.312 g (7.8 %); R_f 0.47 (EtOAc/Hex, 2/3 v/v); ^{1}H NMR (200 MHz, CDCl$_3$), δ 5.56 (1H, m, H-6), 4.02 (1H, m, H-2), 2.62 (1H, dd, J_{AB} = 3.4, 3.4 Hz, H-9), 2.31 (1H, d, J = 1.4 Hz, H-9), 2.09 (m, 1H, H-4), 1.96-1.45 (4H, m, H-3, H-5), 1.79 (3H, s, H-7) and 1.28 (3H, s, H-10); ^{13}C NMR (50 MHz, CDCl$_3$), δ

134.5 (C-1), 124.2 (C-6), 67.5 (C-2), 59.1 (C-8), 52.9 (C-9), 33.9 (C-4), 33.6 (C-3), 27.5 (C-5), 20.8 (C-10) and 18.4 (C-7).

1,6:8,9-Diepoxy-p-menthan-2-ol (**2.12**). Compound **2.12** was obtained as colourless oil from the reaction of **1.84**; yield, 0.25 g (5 %); R_f, 0.25 (EtOAc/Hex, 2/3 v/v); ^{1}H NMR (200 MHz, CDCl$_3$), δ 3.95 (1H, m, H-2), 3.28 (1H, m, H-6), 2.63 (1H, d, J = 4.6 Hz, H-9), 2.54 (1H, dd, J_{AB} = 2.6, 2.6 Hz, H-9), 2.04 (1H, m, H-4), 1.96-1.27 (4H, m, H-3, H-5), 1.42 (3H, s, H-7) and 1.25 (3H, s, H-10); ^{13}C NMR (50 MHz, CDCl$_3$), δ 68.7 (C-2), 62.3 (C-6), 60.4 (C-1), 60.0 (C-8), 53.7 (C-9), 38.6 (C-4), 33.9 (C-3), 27.8 (C-5), 19.0 (C-7) and 21.8 (C-10).

8,9-Epoxy-p-menthan-2-one (**2.13**). A mixture of dihydrocarvone (*p*-menth-8-en-2-one, **2.1**, 4 mL, 3.2 g, 21 mmol) and *m*-CPBA (5g, 21 mmol) treated as described above for 8 h afforded **2.13** (1.67 g, 47.3 %) as colourless oil; R_f, 0.56 (EtOAc/Hex, 2/3 v/v); ^{1}H NMR (200 MHz, CDCl$_3$), δ 2.64 (1H, d, J = 4.0 Hz, H-9), 2.55 (1H, t, J_{AB} = 4.2 Hz, H-9), 2.50-1.48 (8H, m, H-1, H-3, H-4, H-5, H-6), 1.24 (3H, m, H-10) and 0.99 (3H, d, J = 6.4 Hz, H-7); ^{13}C NMR (50 MHz, CDCl$_3$), δ 210.7 (C-2), 57.6 (C-8), 52.1 (C-9), 45.0 (C-1), 44.0 (C-3), 43.0 (C-4), 33.7 (C-5), 26.6 (C-6), 17.4 (C-7) and 13.6 (C-10).

1,2:4,5-Diepoxy-p-menthane (**2.14**). γ-Terpinene (*p*-mentha-1,4-diene, **2.4**, 3.6 mL, 3g, 26 mmol) and *m*-CPBA (9.1 g, 53 mmol) stirred for 24 h afforded **2.14** as colourless oil; yield 1.68 g (38.5 %); R_f, 0.47 (EtOAc/Hex, 2/3 v/v); ^{1}HNMR (600 MHz, CDCl$_3$), δ 2.92 (1H, d, J = 3.0 Hz, H-5), 2.84 (1H, d, J = 2.4 Hz, H-2), 2.47 (1H, d, J = 16.8 Hz, H-

3), 2.54 (1H, d, J = 17.4 Hz, H-6), 2.12 (1H, dddd, $J_{H2, H3}$ = 3.0, 3.0, 3.0, 3.0 Hz, H-2), 2.08 (1H, d, J = 16.7 Hz, H-6), 1.27 (3H, s, H-7), 0.95 (3H, d, J = 7.2 Hz, H-10) and 0.92 (3H, d, J = 7.2 Hz, H-9); ^{13}C NMR (150 MHz, CDCl$_3$), δ 60.4 (C-4), 57.3 (C-2), 56.1 (C-5), 54.9 (C-1), 35.0 (C-6), 28.9 (C-3), 23.4 (C-8), 22.8 (C-7), 18.4 (C-9) and 17.2 (C-10).

2.7.4   Epoxidation of Limonene Via Halohydrin Formation

A flask equipped with a magnetic stirrer and a dropping funnel was added N-bromosuccinimide (0.2 mol), 1,4-dioxane (100 ml), water (50 ml) and CaCO$_3$ (10 g). Limonene (**1.83**, 0.1 mol) was added under stirring, and the mixture was stirred further for 6 h, poured into water, and filtered. The filtrate was extracted with diethyl ether, and the combined extracts washed with water and a 5% solution of Na$_2$S$_2$O$_3$, dried over MgSO$_4$, and evaporated to obtain the bromohydrin **2.15a** as a yellow oil. Crude **2.15** was treated with a dropwise addition of a solution of KOH (15.0 g) in water (15 ml) and ethanol (150 ml), and the mixture stirred for 2 h at RT, diluted with water, and then extracted with diethyl ether. The ethereal extract was washed with water (5 x 40 mL) and dried over Na$_2$SO$_4$. The crude product was purified by chromatography over SiO$_2$ (EtOAc/n-hexane gradient) and compound **2.15** was obtained as yellowish oil.

1,2:8,9-Diepoxy-p-menthane (**2.15**). Yield, 714 mg (23 %); R$_f$, 0.73 (EtOAc/Hex, 2/3 v/v); ^{1}H NMR (200 MHz, CDCl$_3$), δ 2.93 (2H, t, $J_{H6, H5}$ = 5.2 Hz, H-6), 2.47 (2H, m, H-9), 2.0 (1H, m, H-4), 1.92-1.49 (3H, m, H-2, H-5), 1.29 (2H, m, H-3), 1.24 (3H, s, H-7)

and 1.15 (3H, d, J = 1.8 Hz, H-10); ^{13}C NMR (50 MHz, CDCl$_3$), δ 59.5 (C-1), 58.1 (C-6), 57.1 (C-8), 52.1 (C-9), 38.8 (C-4), 29.8 (C-2), 26.1 (C-5), 22.6 (C-3), 20.8 (C-7) and 17.2 (C-10).

2.7.5 Oxymercuration-Demercuration

To mercury (II) acetate (5 g, 15.68 mmol) placed in a 100 ml three necked flask fitted with a dropping funnel, magnetic stirrer and a thermometer, water (20 ml) was added and the mixture was stirred until the acetate dissolved, after which THF (20 ml) was rapidly run into the solution and stirring continued for 15 min and then the respective substrate (15.68 mmol) was added followed by 3 M NaOH (20 ml) and a solution of NaBH$_3$ (0.38 g) in 3 M NaOH (20 ml). The rate of addition was controlled to maintain the temperature at about 25 $^{\circ}$C. Reduction occurred readily as it was observed with the separation of elementary mercury.   The mixture was finally stirred at ambient temperatures for 3 h and allowed to stand overnight in a separatory funnel supported over an empty conical flask. The mercury phase at the bottom of the flask was released into a safety bottle followed by the aqueous alkaline phase, retaining the organic layer. The aqueous phase was saturated with NaCl and the additional organic layer removed. The aqueous phase was then extracted with two 30 ml portions of diethyl ether and the extract combined with the organic phase. Most of the solvent was carefully removed under reduced pressure (rotary evaporator) and diethyl ether (30 ml) and water (20 ml) was added to the residue and the ethereal layer separated, washed with four portions of water (20 ml), then dried over anhydrous CaSO$_4$. The ether was finally removed by

flash distillation. The crude product was purified by chromatography over SiO_2 (EtOAc/n-hexane gradient) to yield the hydroxylated products.

p-Menth-1-en-8-ol (**2.16**). Limonene (**1.83**) when treated as above yielded **2.16** as yellow oil; yield 1.25 g (52 %); R_f, 0.66 (Pet ether/EtOAc 1:1 v/v); ^{1}H NMR (300 MHz, CDCl$_3$), δ 5.39 (1H, dd, $J_{H2, H3ax}$ = 1.8, $J_{H2, H3eq}$= 1.5 Hz, H-2), 2.04-1.49 (9H, m, H-3, H-4, H-5, H-6, H-7), 1.16 (3H, s, H-9) and 1.15 (3H, s, H-10); ^{13}C NMR (75 MHz, CDCl$_3$), δ 133.3 (C-1), 120.4 (C-2), 71.8 (C-8), 44.9 (C-4), 30.7 (C-6), 26.6 (C-3), 25.9 (C-10), 24.8 (C-9), 23.7 (C-5) and 22.1 (C-7); MS, m/z (% rel. int.) 155 ([M+1]$^+$, 2), 139 (22), 96 (85), 81 (100), and 59 (68).

p-Menthane-1,8-diol (**2.17**). Compound **2.17** was obtained as white crystals together with **2.16** from limonene; yield, 0.4 g (14.9 %); R_f, 0.60 (pet ether/EtOAc 1:1, v/v); m.p. 112-113 $^{\circ}$C; ^{1}H NMR (300 MHz, CDCl$_3$), δ 1.74 (2H, m, H-2, H-6), 1.63 (2H,m, H-3, H-5), 1.48 (2H, dd, J = 12.9, 3.3 Hz, H-3, H-5), 1.37 (2H, dd, J = 12.9, 3.3 Hz, H-2, H-6), 1.25 (1H, m, H-4), 1.19 (3H, s, H-7), and 1.16 (6H, s, H-9, H-10); ^{13}C NMR (75 MHz, CDCl$_3$), δ 72.0 (C-8), 68.1 (C-1), 48.6 (C-4), 38.4 (C-2, 6), 30.2 (C-7), 25.5 (C-9, 10) and 22.4 (C-3 and C-5); MS, m/z (% rel. int.) 173 ([M+1]$^+$, 1), 139 (20), 96 (73), 81 (100) and 59 (72).

p-Menthane-1,4-diol (**2.18**). Compound **2.18** was obtained as white crystals on treatment of γ-terpinene (**2.3**) as discussed above; yield, 0.26 g (10.7 %); m.p. 152-154 $^{\circ}$C; ^{1}H NMR (300 MHz, CD$_3$OD), δ 1.73 (4H,m, H-2, H-3, H-5, H-6), 1.55 (1H, m, H-

8), 1.42 (4H, m, H-2, H-3, H-5, H-6), 1.18 (3H, s, H-7), 0.92 (3H, bs, H-9) and 0.91 (3H, bs, 10); ^{1}C NMR (75 MHz, CD$_3$OD), δ 73.6 (C-4), 69.6 (C-1), 39.7 (C-8), 34.9 (C-2 and C-6), 31.4 (C-7), 29.9 (C-3 and C-5) and 17.4 (C-9 and C-10); MS, *m/z* (% rel. int.) 171 ([M]$^+$, 1), 139 (8), 129 (35), 111 (100), 71 (25) and 55 (40).

p-Menthane-2,8-diol (**2.19**). Treatment of dihydrocarveol (*p*-menth-8-en-2-ol, **2.2**) as described above yielded **2.19** as white crystals; yield, 1.36 g, (50.7 %); m.p. 107-108 $^°$C; ^{1}H NMR (300 MHz, CD$_3$OD), δ 3.06 (1H, dd, $J_{H2, H1}$ = 4.2, $J_{H2, H3}$ = 3.6 Hz, H-2), 2.07 (1H, d, *J* = 12.0 Hz, H-3), 1.77 (2H, m, H-5, H-6), 1.39 (1H, t, *J* = 3.0 Hz, H-4), 1.16 (6H, s, H-9, H-10), 1.14 (1H, m, H-1) and 1.01 (3H, d, *J* = 6.3 Hz, H-7); ^{13}C NMR (75 MHz, CD$_3$OD), δ 76.0 (C-2), 71.5 (C-8), 48.0 (C-4), 40.0 (C-1), 36.4 (C-3), 33.2 (C-5), 26.6 (C-6), 25.6 (C-9) and 25.6 (C-10); MS, *m/z* (% rel. int.) 173 ([M+1]$^+$, 1), 139 (10), 96 (60), 81 (100) and 59 (90).

p-Menthane-7,8-diol (**2.20**). Treatment of perillaldehyde (**1.106**) as described above afforded **2.20** as white crystals; yield, 158 mg (6 %); m.p. 87-90 $^°$C; ^{1}H NMR (300 MHz, CD$_3$OD), δ 5.67 (1H, s, H-2), 3.93 (2H, s, H-7), 2.18-1.96 (5H, m, H-3, H-4, H-6), 1.85 (1H, m, H-5), 1.18 (3H, s, H-10) and 1.17 (3H, s, H-9); ^{13}C NMR (75 MHz, CD$_3$OD), δ 137.3 (C-1), 121.9 (C-2), 71.9 (C-8), 65.8 (C-7), 45.2 (C-4), 26.3 (C-3, 6), 25.8 (C-10), 24.9 (C-9) and 23.5 (C-5).

2.7.6   Dihydroxylation by OsO$_4$

To a mixture of *t*-BuOH (100 ml) and 30% H$_2$O$_2$ (25 ml), was added anhydrous Na$_2$SO$_4$/MgSO$_4$ in small portions and the alcohol layer containing most of the hydrogen peroxide was removed and dried with MgSO$_4$ followed by anhydrous CaSO$_4$ giving a stable solution of 6.3% H$_2$O$_2$ in *t*-BuOH.   The corresponding substrate (25 mmol) mixed with the reagent (55 ml, 0.1 mol) and a 0.5% solution of OsO$_4$ (3 ml) in anhydrous *t*-BuOH was added and the mixture cooled to 0 $^\circ$C, and then allowed to stand overnight. Recrystalization from EtOAc yielded the desired products.

p-Menthane-1,2,3,8-tetrol (**2.22**). Treatment of *trans-p*-menth-6-ene-2,8-diol (**2.21**, 4.256 g (25 mmol) as described above afforded **2.22** as white crystals; yield, 1.42 g (27.8%); m.p. 127-129 $^\circ$C; ^{1}H NMR (300 MHz, DMSO), δ 4.36 (OH, d, *J* = 4.5 Hz, 6-OH), 3.89 (OH, d, *J* = 7.2 Hz, 2-OH), 3.86 (OH, s, 1-OH), 3.63 (OH, d, *J* = 4.5 Hz, 8-OH), 3.44 (1H, dd, $J_{H2, H3ax}$ = 6.6, $J_{H2, H3eq}$ = 6.6, 2.7 Hz, H-2), 3.36 (1H, dddd, $J_{H6, H5}$ = 4.5, 4.2, 4.2, 4.5 Hz, H-6), 1.67-1.17 (5H, m, H-3, H-4, H-5), 1.12 (3H, s, H-7), 1.00 (3H, s, H-9) and 0.99 (3H, s, H-10); ^{1}C NMR (75 MHz, DMSO), δ 73.7 (C-2), 72.2 (C-1), 70.9 (C-3), 70.3 (C-8), 40.6 (C-4), 31.2 (C-6), 29.5 (C-5), 27.4 (C-9), 26.8 (C-10) and 23.6 (C-7); MS, *m/z* (% rel. int.) 227 ([M+Na]$^+$, 73), 205 ([M+1]$^+$, 90), 169 (48), 151 (20) and 107 (13).

p-Menthan-1,2,8-triol (**2.23**). Treatment of α-terpeneol (**1.112**, 4.126 ml, 3.85 g, 25 mmol) as above afforded compound **2.23** as white crystals; yield, 302 mg (6.93 %); m.p.

105-107 $^{\circ}$C; ^{1}H NMR (300 MHz, CD$_3$OD), δ 3.29 (1H, m, H-2), 1.79 (3H, m, H-3, H-5, H-6), 1.54 (1H, m, H-4), 1.41 (3H, m, H-3, H-5, H-6), 1.23 (3H, s, H-7) and 1.16 (6H, s, H-9 and H-10); ^{13}C NMR (75 MHz, CD$_3$OD), δ 75.2 (C-2), 71.6 (C-8), 70.4 (C-1), 47.8 (C-8), 37.3 (C-6), 31.0 (C-3), 25.8 (C-10), 25.6 (C-9), 25.5 (C-7) and 21.7 (C-5); MS, m/z (% rel. int.) 190 ([M+1]$^+$,1 %), 126 (40), 112 (65), 71 (100), 59 (55), 43 (80).

2.7.7 Mosquito Repellence Bioassay

Female adult *Anopheles gambiae* s.s. Giles mosquitoes were used in all experiments as supplied by the International Center of Insect Physiology and Ecology (ICIPE) insectary unit. The mosquito colony was obtained from Ifakara in Tanzania and reared under standard insectary conditions at ICIPE. The adult mosquitoes were maintained on a 6 % glucose solution and females fed on human blood thrice a week. Mosquitoes used in the experiment were 5-7 days old, initially fed on glucose (6 % solution) and then starved for 18 h before carrying out the assays.

The repellency assays were performed in a dark room with deem red light as the only source of illumination. The room temperature and humidity were controlled at 28±2 $^{\circ}$C and 70±5 % respectively to mimic the feeding conditions for female *An. gambiae* mosquitoes. Cages (50 x 50 x 50 cm) made with aluminium sheet bottom, window screen (mesh size 256) on top and back, and a cotton sleeve for access on the front were used to study the dose responses. Different concentrations of samples (10^{-5}-10^{-1} g/ml) were prepared by dissolving the samples (1 g) in analytical grade acetone (10 ml) followed by ten-fold dilution to obtain the subsequent concentrations.

Acetone acted as a control in all experiments. Fifty test mosquitoes and six adult human volunteers who did not apply any lotion, perfume, oil or perfumed soap on the day of the experiment were used. The forearm of each volunteer from the elbow was washed with water, left to dry and then covered by a glove in order to be unattractive to mosquitoes.

The test sample (1 ml) was spread evenly over the treatment area and presented one after the other to the same caged mosquitoes for a particular sample and person. Sequential exposure of the arms to cages with zero and then progressively high doses of the repellent for 3 min was done. After a test of each concentration, the hands were washed using a non-perfumed soap and tap water, and allowed to dry naturally for at least 20 min before dispensing the subsequent concentration.

The number of mosquitoes landing on or probing the arm were counted and recorded for each volunteer. Mosquitoes were shaken off the arm before they took a blood meal. The right arm was used for the treatment while the left arm was the control. Exposure of the treatment and control arms was alternated to provide a standard for comparing the avidity of biting.

Percentage protective efficacy was calculated using the formula PE = (C-T/T) x 100%, where C and T are the mean numbers of mosquitoes that landed on the control and test hand, respectively. The repellency concentration at 50% (RC_{50}) was obtained using POLO PLUS computer program.

2.7.8   Apoptosis Assay

Apoptosis assays was carried out at the University of Western Cape, South Africa according to APOPercentage™ Assay standard method against CHO and Jurkat T cell lines, while using Camptothecin as the standard drug.

CHAPTER THREE

HYDROXY-*p*-MENTHAMINES: SYNTHESIS AND APOPTOTIC ACTIVITY

Abstract

This chapter reports results from aminolysis of the epoxides 8,9-epoxy-*p*-menth-6-en-2-one, *cis*-2-hydroxy-1,6-epoxy-*p*-menthen-8-ene, 8,9-epoxy-*p*-menthan-2-one and 1,2:4,5-diepoxy-*p*-menthane using benzyl amine, aniline and piperidine in a 30 % MeCN:H_2O solvent mixture and triethylamine as catalyst, or in water in the absence of a catalyst and this yielded 9 *β*-amino alcohols. The compounds thus synthesized were assayed for apoptotic inducing potential and some of them showed activity thus indicating their potential as candidates for further investigations.

3.1   Introduction

As discussed in Chapter One, the considerable importance of *β*-amino alcohols as potential medicinal compounds cannot be over emphasized. Thus, the synthesis of these compounds is generally considered to be of great significance. One of the most widely used routes for the synthesis of *β*-amino alcohols is the direct aminolysis of oxiranes at elevated temperatures and in the presence of an excess of amines. However, in efforts to search for better synthetic conditions for amino alcohols, improvements in synthetic procedures have been accomplished, among them being the involvement of promoters such as metal salts like lithium perchlorate using different organic solvents. Unfortunately, lithium perchlorate has been responsible for many laboratory accidents,

sometimes lethal,[313,314] thus making other metal promoters including Lewis acids such as tin (II) salts, lanthanide chlorides, and cobalt (III) chloride among others, become desirable.[315-318]

Despite the good number of accomplishments made in search for aminolysis catalysts, there are still some limitations associated with the existing methods. For example, deactivated amines like aromatic amines tend to fail in opening epoxides or require high reaction temperatures. Furthermore, many of the catalysts used are either corrosive or expensive. Further still, some of the synthetic methodologies require the use of stoichiometric or moisture sensitive catalyst, and hazardous organic solvents. Moreover, there is no general procedure that is suitable for aminolysis of various epoxides since the nucleophilic amines used possess different reactivities.[319] Therefore, in order to accomplish aminolysis reactions successfully, there has always been the need to identify the existing synthetic methodologies or to establish new ones, or to use a variety of substrates suitable for accomplishing the reactions using the presently available methodologies. These approaches are discussed hereunder.

Ever since Breslow[320] studied Diels-Alder reactions in aqueous media, many organic reactions that were traditionally carried out exclusively in organic solvents have also been performed in aqueous media. The potential advantages of using water as a solvent are its low cost, safety, ease of use and also being environmentally benign.[319] Therefore, efforts have been made to carry out aminolysis and other organic reactions in water and in an environment free of organic solvents. For instance $Bi(OTf)_3$ has been used as a

promoter for oxirane ring opening while $ZrCl_4$ has been investigated as a catalyst for epoxide opening by amines in solvent free conditions.[321,322] However, these catalysts are relatively expensive. Therefore, there is need to establish alternative aminolysis reactions that are facile, easy to handle and involve cheap reactants and catalysts, in the presence of water as the solvent.

Another example in which water has been used as a solvent involves a reaction that was described by Fan and Hou,[323] which involved ring opening of epoxides and aziridines with various nucleophiles in the presence of catalytic amount of Bu_3P.[323] In such cases it was discovered that some reactions which did not take place in organic solvents proceeded smoothly in aqueous media.[323] It is also noteworthy mentioning that Wu and Hong-Guang[324] accomplished epoxide ring opening reactions with amines in water catalysed by the relatively cheap and safe reagent, namely Et_3N (TEA). Another interesting discovery that has attracted attention was by Najmodin and Mohamed, who achieved aminolysis in water without the use of any catalyst.[319]

The last mentioned synthetic route was reinvestigated in these studies with the overall aim of providing a more economic and practical approach for the preparation of the targeted *p*-menthane amino alcohols that would be available for bioassays as potential apoptosis inducers.

3.2   Results

3.2.1   *β*-Amino Alcohol Derivatives from Various Menthane Epoxides

Initially, the oxirane ring opening process was investigated by treating dihydrocarvone epoxide (**2.9**) with benzylamine in water according to the procedure reported in the literature.[319] The epoxide was treated with a molar equivalent of benzylamine at room temperature while vigorously stirring the reaction mixture. However, after 8 h no reaction was observed to have taken place (TLC). The epoxide ring opening failed even after elevating the reaction temperature to 60 °C for another 6 h and after 12 h at 60 °C some reaction was noted to have taken place, yielding 27 % of the expected amino alcohol 9-benzylamino-8-hydroxy-*p*-menth-2-one (**3.1**) whose structure was established on the basis of analysis of spectroscopic data.

In order to speed up the reaction, Et$_3$N was employed as a catalyst as reported by Fan and Hou.[323] Thus, the reaction when carried out for 24 h indicated slight improvement in the yield of the amino alcohol formed to 42 %. Nonetheless, considering the length of the reaction time and the low yield, it was considered desirable to change the reaction conditions.   Hence, the reaction was conducted in water while slowly adding acetonitrile. This indicated the optimum amount of acetonitrile to be 30 % in water, with 0.1 molar equivalent of TEA, which furnished 97.2 % yield of the amino alcohol (**3.1**) from benzylamine after 12 h (**Scheme 3.1**).
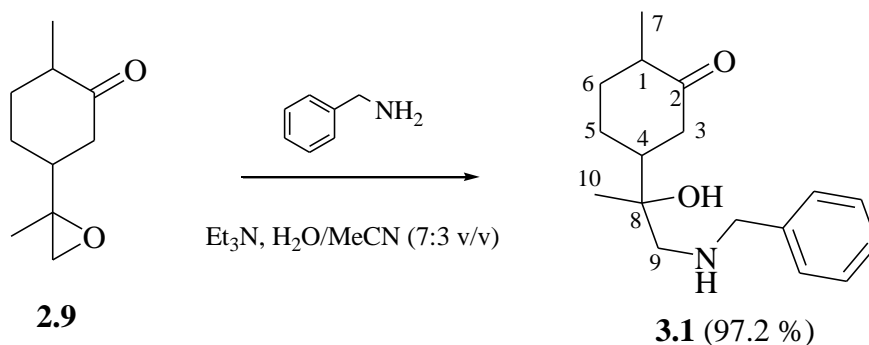
Scheme 3.1   Epoxide ring opening of 2.9 with benzylamine

Analysis of the NMR spectra indicated that the product was obtained as a mixture of two diastereomers, which was attributed to the presence of stereogenic centers at C-1, C-4 and C-8.

Compound **3.1** exhibited high (88.5%) apoptotic induction against Jurkat T and CHO (88.2 %) cell lines at the tested concentration of 1 μM, which as shown in the representative flow cytometric graphs (**Fig. 3.1**), implied non-selective activity amongst the two cell lines.
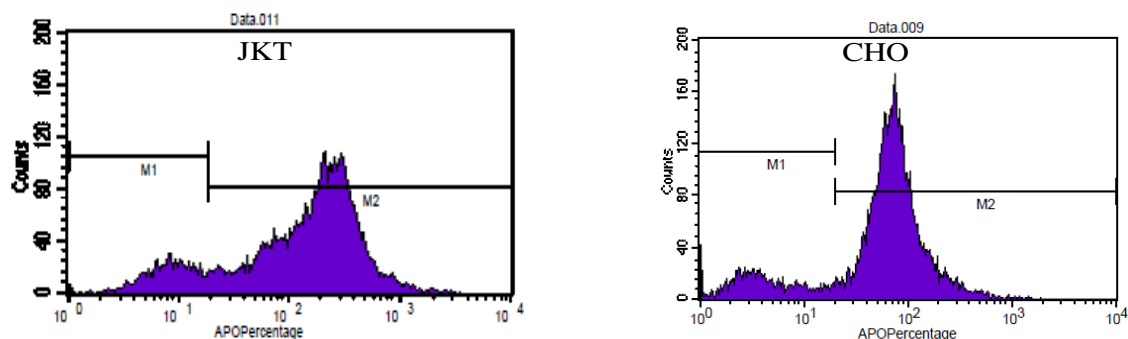
Fig. 3.1   Flow cytometric graphs showing the activity of compound 3.1 against Jurkat T and CHO cells

The reaction conditions stated above were replicated for the far less reactive (electron deficient) substrate, i.e. redistilled aniline which furnished 42 % yield of the amino alcohol 9-phenylamino-8-hydroxy-*p*-menth-2-one (**3.2**) after 24 h whose structure was confirmed from analysis of NMR spectroscopic data. The low yield of the aniline derivative was attributed to its poor nucleophilicity. However, the reaction with piperidine in water gave a higher yield (69 %) of the amino alcohol 9-piperidinyl-8-hydroxy-*p*-menth-2-one (**3.3**) after 24 h, which was presumed to be due to high solubility in water.
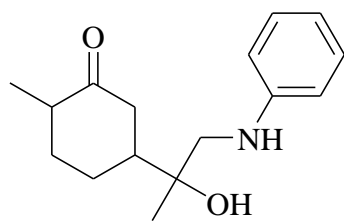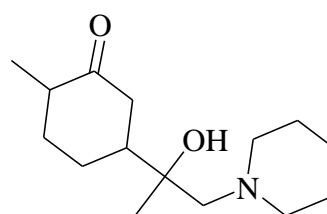
3. 2 (42 %)   **3.3** (69 %)

Compound **3.2** exhibited slightly higher apoptotic induction activity against CHO as compared to Jurkat T cells, leading to the formation of non-viable cells at 58.0 % and 49.8 % respectively for the two cell lines, as shown in the representative flow cytometric graphs in **Fig. 3.2**.
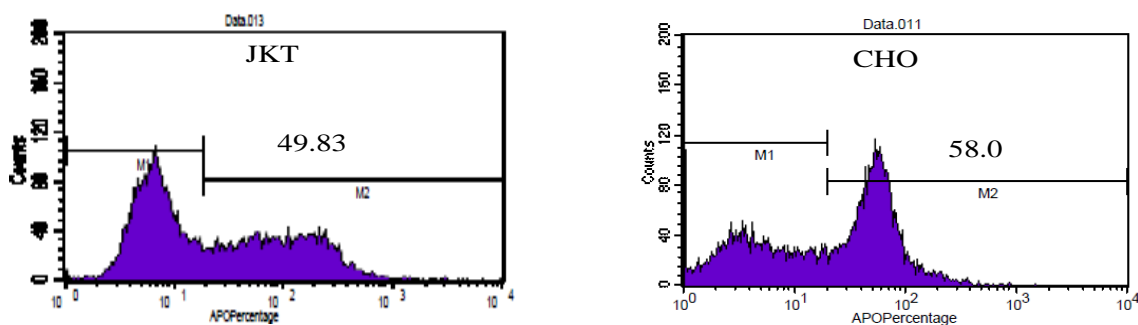
Fig. 3.2   **Flow cytometric graphs showing the activity of compound 3.2 against Jurkat T and CHO cells**

Compound **3.3** exhibited fairly good activity against CHO as compared to Jurkat T cells, with 57.42 % and 33.72 % non-viable cells respectively as indicated by the flow cytometry graphs (**Fig. 3.3**), thus showing a good level of selective activity against the two cell lines.
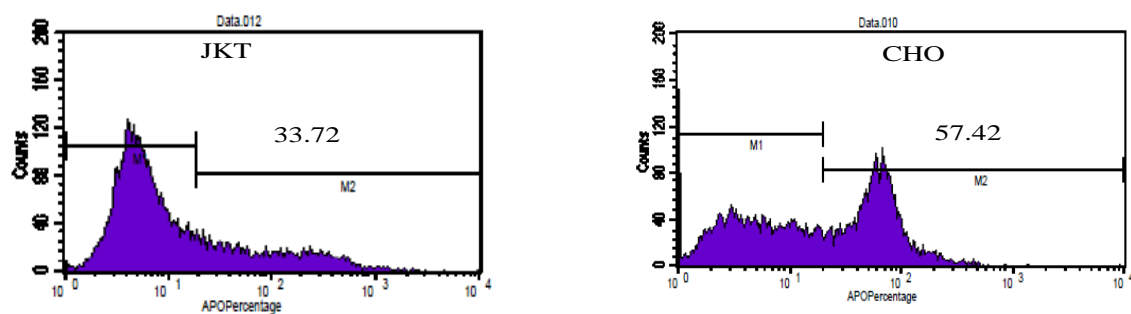
Fig. 3.3   **Flow cytometric graphs showing the activity of compound 3.3 against Jurkat T and CHO cells**

3.2.1.1 Carvone Derivatives

Having successfully obtained the targeted products from the dihydrocarveol epoxide (**2.5**), which has an isopropoxyl group, the isoproxylated carvone derivative **2.13** was also treated with similar nucleophiles. The reaction with both benzylamine and aniline did not yield the targeted amino alcohols attributed to their low nucleophilicity, but it did when treated with piperidine where the amino alcohol 8-hydroxy-9-piperidinyl-*p*-menth-2-enone (**3.4**) was obtained in 70 % yield after 12 h of reaction time (**Scheme 3.2**). Structure **3.4** was established upon analysis of NMR spectral data.
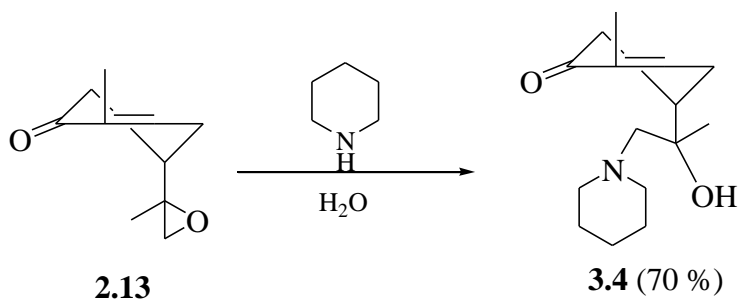
Scheme 3.2   Epoxide ring opening of compound 2.13 with piperidine

3.2.1.2 Carveol Derivatives

The other epoxide substrate in these studies was *cis*-2-hydroxy-1,6-epoxy-*p*-menthen-ene (**2.5**), which upon treating with an equimolar amount of benzylamine afforded 33 % of 6-benzylamino-*p*-menth-8-ene-1,2-diol (**3.5**) after 72 h (**Scheme 3.3**), whose structure was established based on analysis of spectral data.
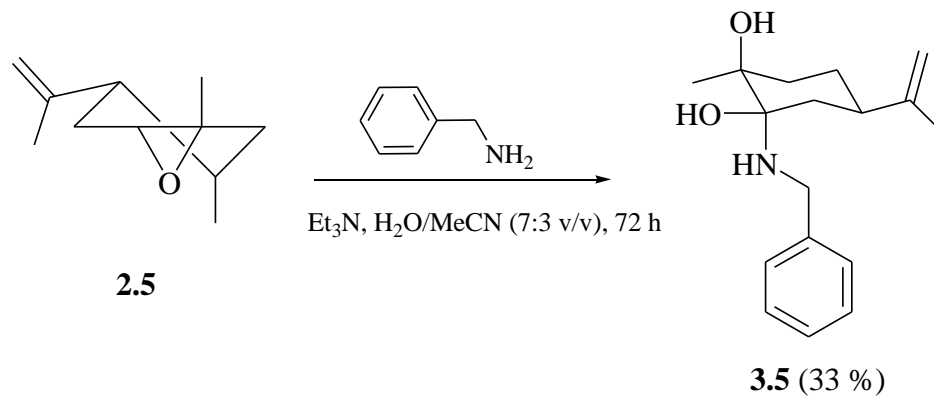
Scheme 3.3   Epoxide ring opening of compound 2.5 with benzyl amine

The low yield and long time this reaction took in comparison to the yield obtained for a similar reaction with compound **2.9** as the substrate was attributed to steric hindrance by the epoxymethyl substituent that hindered the approach of the nucleophile to the oxirane ring.

Compound **3.5** showed high apoptotic activity against CHO cells, showing 69.12 % of non-viable cells in comparison with 37.63 % against Jurkat T cells, thus indicating a good level of selectivity (**Fig 3.4**).
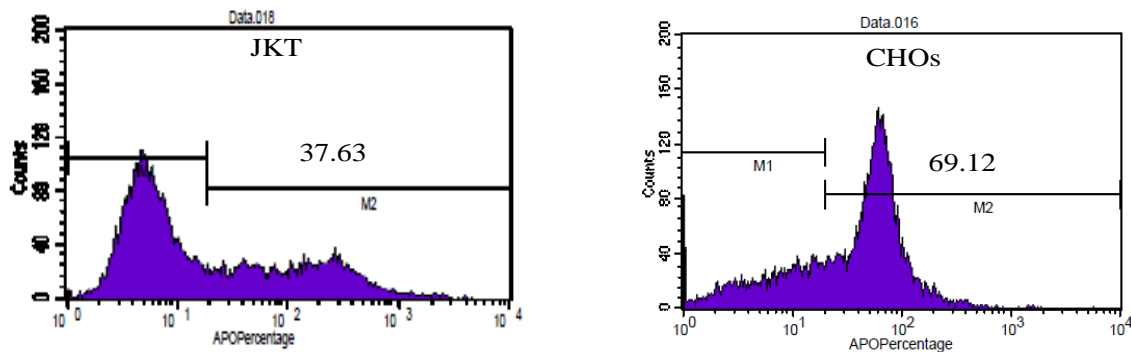
Fig. 3.4 **Flow cytometric graphs showing the activity of compound 3.5 against Jurkat T and CHO cells**

Cis-2-hydroxy-1,6-epoxy-*p*-menthen-ene (**2.5**) was also treated in a similar fashion with an equimolar amount of aniline affording only 16 % of the amino alcohol 6-phenylamino-*p*-menth-8,9-ene-1,2-diol (**3.6**) after 96 h. Upon treatment of **2.5** with piperidine in water for 48 h, the reaction yielded 48 % of the amino alcohol 6-piperidinyl-*p*-menth-8,9-ene-1,2 diol (**3.7**). The structures of both compounds were established upon analysis of their spectral data.
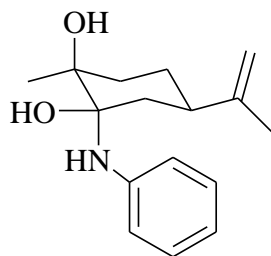
3.6
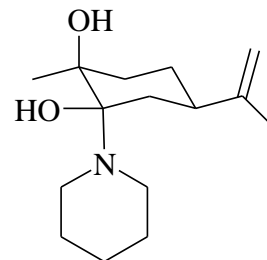
3.7

Compound **3.6** exhibited above average activity against both Jurkat T and CHO cell lines, with higher activity being recorded against Jurkat T (70.93 % non-viable cells) in comparison to CHO cells (62.37 % non-viable cells) as shown in **Fig 3.5**.
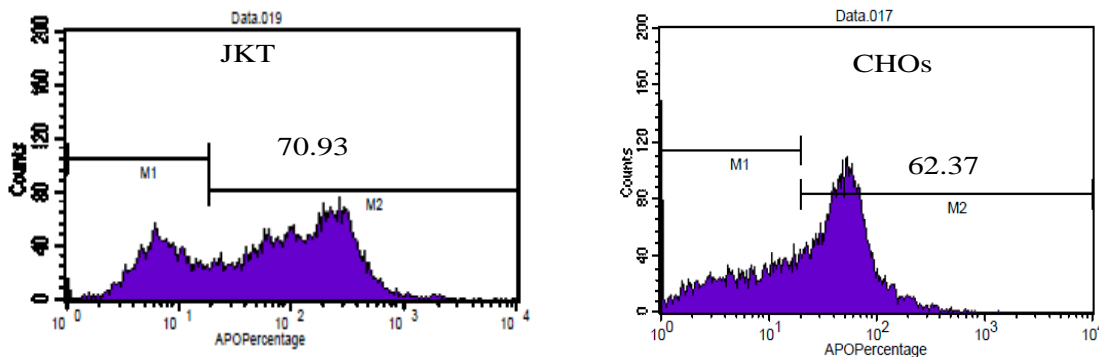
Fig. 3.5   **Flow cytometric graphs showing the activity of compound 3.6 against Jurkat T and CHO cells**

The highest selectivity against the two cell lines was observed for compound **3.7** with 92.68 % non-viable cells being recorded for Jurkat T cells as compared to 39.99 % non-viable for CHO cells as indicated by flow cytometric graphs in **Fig 3.6**.
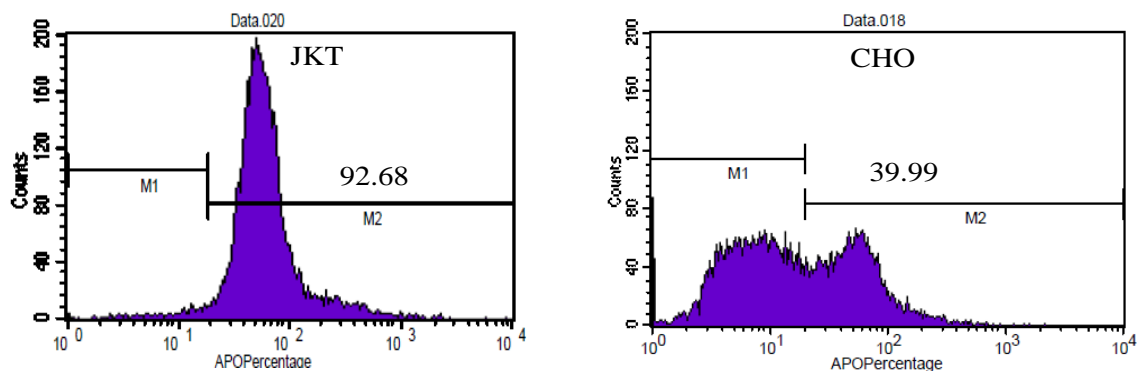
Fig. 3.6   **Flow cytometric graphs showing the activity of compound 3.7 against Jurkat T and CHO cells**

3.2.1.3 γ-Terpinene Derivatives

In the first attempted synthesis of compound **3.8**, 1,2:4,5-diepoxy-*p*-menthane (**2.10**) was treated for 36 h with 2 molar equivalent of benzylamine in 30 % MeCN in H_2O and TEA as the catalyst and the reaction monitored by TLC, but only an inextricable mixture was obtained even after addition of an excess of TEA and benzylamine. The reaction was repeated in a solvent consisting of 50 % MeCN in H_2O, in the presence of an excess of benzylamine and the catalyst for 72 h at 60 $^{\circ}$C (**Scheme 3.4**). However, yet again the reaction product only consisted of a complex mixture. Work up of the reaction mixture followed by chromatography furnished the amino alcohol 2-benzylamino-4,5-epoxy-*p*-menthane-1-ol (**3.8**) in 29 % yield, whose structure was established upon analysis of spectroscopic data.
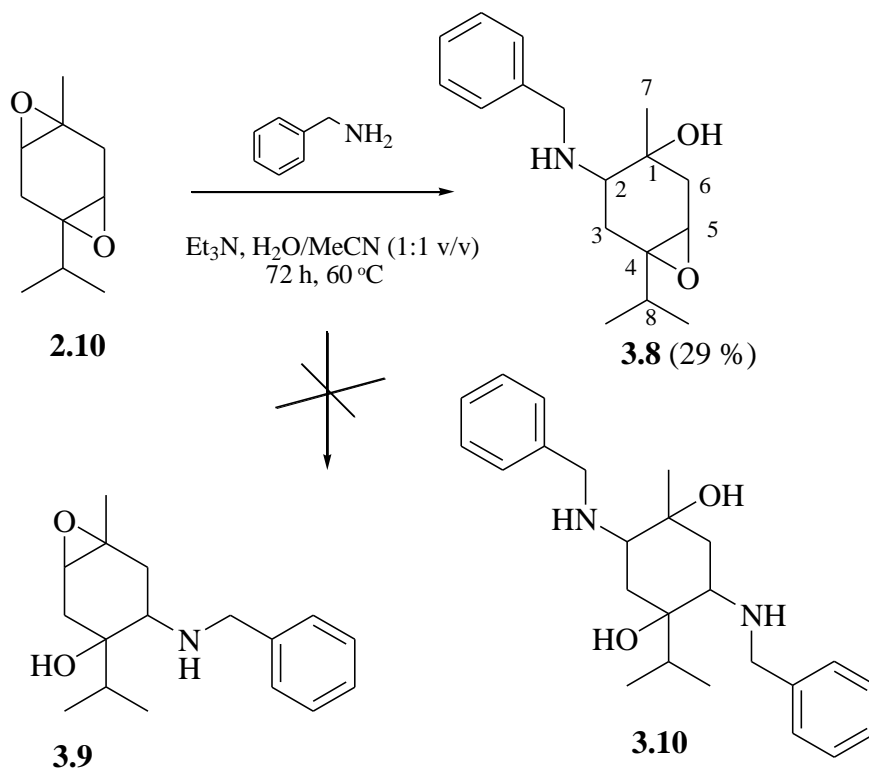
Scheme 3.4 Expected products of epoxide ring opening of compound 2.10 with benzylamine

The other possible products **3.9** and **3.10** were not obtained (**Scheme 3.8**). The spectral data distinguished the product **3.8** from **3.9** in that the ^{13}C NMR signals for the two quaternary carbons C-1 and C-4 appeared at 70.4 and 63.6 respectively for the former, which in the latter the C-1 chemical shift would have appeared more upfield and for C-4 more downfield.

The regioselectivity portrayed by the reaction leading to the formation of only one product **3.8** could be explained by considering steric effects of the two allyl substituents in the substrate. Thus, while the back side attack on the diepoxide was expected to

proceed simultaneously on both C-2 and C-5 of the oxirane rings, steric effects favoured

nucleophilic attack at C-1 rather than C-4. The stereochemical implications could be

derived from the initial stages of the reaction, whereby the 1,2-epoxide ring is opened to

the β-amino alcohol leading to an inversion of the near planar structure to a half-chair

conformation (**Scheme 3.5**), in which the isopropyl substituent is directly shielding the

remaining site of attack at C-5.   In effect, this crowding effect would sterically hinder

further attack, therefore leading to the formation of 2-benzylamino-4,5-epoxy-*p*-

menthane-1-ol (**3.8**) as the only reaction product.
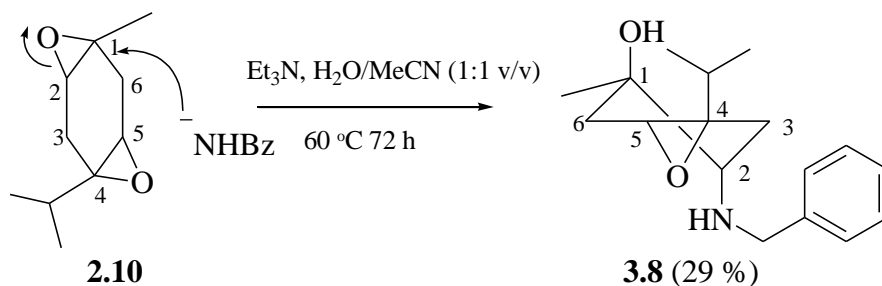
Scheme 3.5   Mechanism of nucleophilic epoxide ring opening and conformational

preference of 2-benzylamino-4,5-epoxy-*p*-menthane-1-ol (3.8)

The attempted reaction of 1,2:4,5-diepoxy-*p*-menthane (**2.10**) with aniline failed to yield

the target amino alcohol even after carrying out the reaction for 96 h. This was attributed

to the low nucleophilicity of aniline, in addition to severe steric effects that would hinder

nucleophilic attack by aniline. Unlike benzylamine whose amino group is two bonds

away from the benzene ring, in aniline it is one bond away, thereby in close proximity to

a bulky benzene ring that worsens the steric effect by shielding the reactive site from approaching the oxirane rings that are also sterically hindered by the alkyl groups.

In the reaction between **2.10** and piperidine, there was a possibility of obtaining three products as a mixture or one of them preferentially being formed over the others. Therefore, in order to explore the reaction, the di-epoxide **2.10** in water was treated with an excess of piperidine and the amino alcohol 2-piperidinyl-4,5-epoxy-*p*-menthane-1-ol (**3.12**) was obtained after 96 h in a mere 10.9 % yield (**Scheme 3.6**).
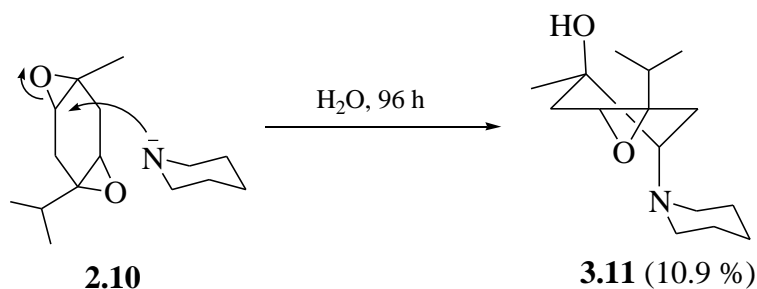
Scheme 3.6   Epoxide ring opening of compound 2.10 with piperidine

Regarding the observed regioselectivity, the factors discussed for the formation of **3.8** would also apply in this case, leading to the amino alcohol 2-piperidinyl-4,5-epoxy-*p*-menthane-1-ol (**3.11**), in which both OH and piperidinyl groups are *pseudo*-axial (**Scheme 3.6**). The low yield could be attributed to the nature of the amino group in the ring, which as described for the reaction with aniline, is due to steric reasons.

Compound **3.11** showed high activity in both cell lines as indicted by the flow cytometric graphs in **Fig. 3.7**, with over 90 % cell deaths in both cases. However the activity was slightly higher against Jurkat T cells (96.51 % non-viable cells).
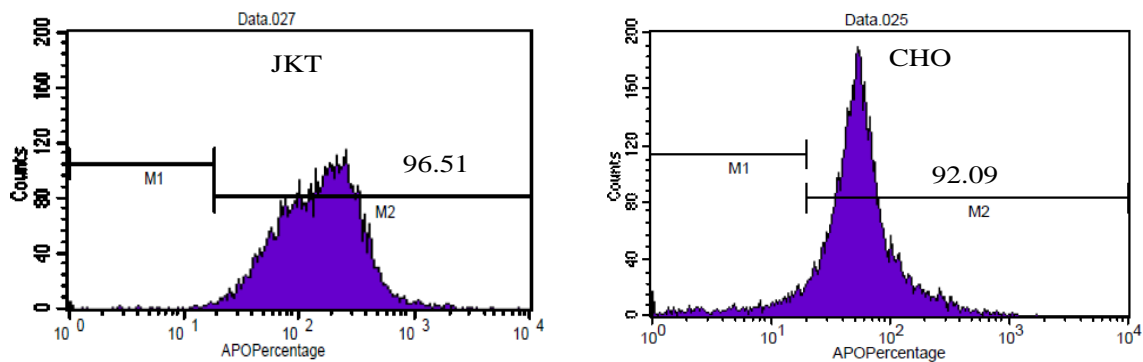
Fig. 3.7 **Flow cytometric graphs showing the activity of compound 3.12 against Jurkat T and CHO cells**

3.3 Discussion

The general procedure employed for the formation of the amino alcohols through nucleophilic oxirane ring opening was based on the method described by Najmodin,[319] but with some modification of the solvent system, which was a mixture of MeCN and water in the ratio 3:7 v/v. The essence in selecting such a solvent system in which water was predominant related to the latter's ready availability, cheapness and being environmentally benign, while solubility of the substrates is also enhanced. However, some of the reactions took a long duration to furnish the expected products and the yields were rather low (**Table 3.10**). This could be attributed to the low nucleophilicity

of the amines, the structural nature of the epoxides and, particularly for the aromatic amines, their solubility.

On the other hand, piperidine being a more facile nucleophilic compound reacted readily, affording the corresponding amino alcohols in water in relatively high yields. However, this depended on the substitution profile of the substrates, in which the terminal epoxides took a relatively shorter duration and gave better yields as compared to compounds in which the epoxide group was attached to the cyclic part of the substrate (**Table 3.1**). This chemoselectivity was also reported for the reaction of cyclohexene oxide with a mixture of aniline and piperidine in water, whereby only piperidine reacted with the oxide to form the corresponding amino alcohol (**Scheme 3.7**).[320]

0%   100%

Scheme 3.7   Chemoselective nucleophilic addition of aliphatic amine to cyclohexane oxide presence of aniline[319]

It was also noted that at the same catalytic concentration of TEA the degree of epoxide ring opening was notably low when water was used as the only solvent, but higher for the finally adopted solvent system incorporating acetonitrile.   This observation

suggested that the catalytic activity of TEA is the result of synergistic action between the catalyst and the solvent.

Being a base catalyzed S_N2 reaction, the epoxide ring opening process occurred *via* a back side attack on the least hindered carbon, contrary to an acid catalyzed epoxide ring opening where the attack would take place on the more substituted carbon.[325] Also noted was the fact that the cyclohexene epoxides underwent epoxide ring opening with amino nucleophiles in accordance with Furst-Plattners' Rule to give diaxial products.[326] This observation was indeed ascertained by the coupling constants and the downfield chemical shifts of C-7 in ^{1}H and ^{13}C NMR spectra. For instance compound **3.5** the *J* value of H-6 was 3.2 Hz and 3.8 Hz arising from coupling with both equatorial and axial protons on H-5. The methyl substituent was confirmed to be equatorial by the relatively downfield chemical shift in ^{13}C NMR spectra for compounds **3.5**, **3.6**, **3.8** and **3.11**.

In the reactions higher yields of the products were obtained from the isopropoxyl derivatives as compared to the cyclohexene oxides. This was attributed to the ease with which the nucleophiles could access the terminal epoxides that were less sterically hindered in comparison with the cyclohexene epoxides. All cyclohexene epoxides investigated had alkyl substituents neighbouring the oxirane ring and this limited their accessibility due to steric hindrance. This could also be the reason for the longer duration taken to obtain the target products and as observed from the ring opening of γ-terpinene diepoxide (**2.10**), leading to the selective opening of the less sterically hindered oxirane ring neighbouring a methyl group preferentially over that neighbouring

the isopropyl group.   Otherwise, in general, the reactions exhibited complete regioselectivity with attack of nucleophiles taking place on the less substituted epoxide carbon.

The reaction of compound **2.13** with benzylamine and aniline yielded a complex mixture of products that could not be separated by column chromatography, probably due to the presence of closely related isomeric products. On the other hand treatment of aniline with compound **2.10** for more than four days gave no reaction, which would probably be due to less solubility and poor nucleophilicity of aniline.

The results from the nucleophilic oxirane ring opening with amines are summarized in **Table 3.1**.

Table 3.1   **Ring opening of various epoxide substrates with different amines**

Amine	Substrate	Solvent	Time (H)	Product	Yield (%)
Benzylamine	**2.9**	A	12	**3.1**	97.7
	2.5	A	72	**3.4**	33
	2.13	A	---	**---**	---
	2.10	A	72	**3.8**	29
Aniline	**2.9**	A	24	**3.2**	20
	2.5	A	72	**3.5**	15.7
	2.13	A	---	**---**	---
	2.10	A	---	**---**	---
Piperidine	**2.9**	B	24	**3.3**	69
	2.5	B	72	**3.6**	48
	2.13	B	12	**3.7**	70
	2.10	B	96	**3.11**	11

A = 30 % (MeCN: H_2O)   B = H_2O --- = no product

3.4   Apoptosis Induction Results of the Synthesized p*P*-Menthane Amino Alcohols and their Possible Structure Activity Relationships

The amino alcohols obtained as shown in **Table 3.1** were subjected to bioassays to determine their efficacy as apoptosis inducers against Jurkat T and CHO cell lines. Results are summarized in **Table 3.2**.

Table 3.2 **Results of flow cytometric assays of the synthesized *p*-menthane amino alcohols indicating activity against CHO and Jurkat T cells at 1 μM**

	% Non-viable (apoptotic) cells	
Compound	CHO	Jurkat T
3.1	88.2 ± 2.41	88.5 ± 1.71
3.2	58.0 ± 1.40	49.8 ± 2.58
3.3	57.4 ± 3.66	33.7 ± 4.31
3.5	69.1 ± 1.97	37.6 ± 3.06
3.6	62.4 ± 1.72	70.9 ± 4.48
3.7	39.9 ± 1.79	92.7 ± 2.34
3.11	92.1 ± 1.88	95.5 ± 2.04
Camptothecin	81.9±1.72	55.5±7.03
-Ve control	33.6±5.03	21.7±0.62
DMSO	37.7±2.65	37.3±11.46

The results indicated that compound **3.11** exhibited the highest apoptotic induction against both cell lines among all the synthesized amino alcohols. It induced 92.09 and 95.51 % cell death against CHO and Jurkat T cells respectively, thus indicating low level of selective activity between the two cell lines at 1 μM concentration. Compound **3.1** also indicated high apoptotic activity for both cell lines, exhibiting >85% cell death for CHO and Jurkat T cells, which also indicated minimal selective activity. The other two dihydrocarvone derivatives **3.2** and **3.3** showed some level of selective activity

against the two cell lines, with both showing higher activity against CHO than Jurkat T cells (**Table 3.2**).

Amongst the carveol derived amino alcohols, the piperidinyl derivative **3.6** exhibited very high apoptotic activity against Jurkat T cells (92.7% non-viable cells) but less activity against CHO cells (39.9 % non-viable cells), thus indicating the highest level of selectivity amongst all the tested amino alcohols (**Fig. 3.8**).

The results shown in **Table 3.2** and **Fig. 3.8** would lead to some type of SAR, in which it would be concluded that for the case of Jurkat T cells, the peperidinyl group on the cyclohexane skeleton was considered to have induced high apoptotic activity than an isopropenyl peperidinyl group, as shown by the high activity of compounds **3.11** and **3.7**. On the other hand, the latter group influenced the lowest activity against CHO cells.

On comparing compounds **3.1**, **3.2** and **3.3**, which were all amino alcohol derivatives of dihydrocarvone, it was generally observed that the benzyl group was more protagonistic for both cell lines, while the isopropenyl piperidinyl group exhibited the least apoptotic potential.

From the results in **Table 3.2** only two compounds (**3.1** and **3.11**) exhibited higher apoptotic activity against the CHO cells than Camptothecin that was used as the positive control. However, against the Jurkart T Cells a higher activity was observed for four compounds showing, above 55.5 % cell death that was exhibited by Camptothecin.
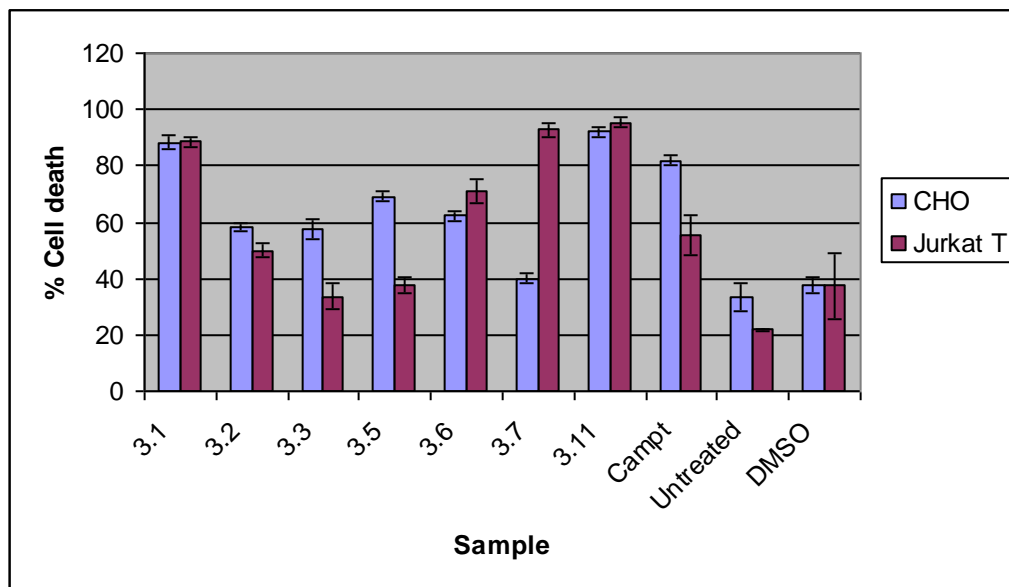
Fig. 3.8 **Comparative apoptotic activity of synthesized terpenyl amino alcohols**

3.5 Conclusion

The above results indicate the great potential of the *β*-amino alcohols derived in this study as apoptosis inducers. Most of the compounds tested exhibited higher activity than Camptothecin which is a drug of choice for a number of tumorigenic diseases. The ease with which the compounds were obtained also indicated the usefulness of the established solvent and catalyst system, which were deemed to enhance the reaction synergistically.

3.6   Experimental Procedures

3.6.1   General Experimental Procedures

These were the same as described in **Chapter Two**.

3.6.2   General Reaction Procedure for the Synthesis of *β*-Amino Alcohols

The nucleophilic epoxide ring opening reactions for the formation of amino alcohols were carried out by treating the corresponding epoxides placed in a test tube equipped with a magnetic stirrer containing TEA and MeCN/H_2O (30 % v/v, 2 mL), with the respective amine added in one portion while stirring. The reaction mixture was left running at room temperature while being monitored by TLC. After a specified reaction time, water (2 mL) was added, and the organic materials were extracted with diethyl ether (2 x 10 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure followed by column chromatography of the crude product on silica gel (EtOAc/hexane gradient) and the pure *β*-amino alcohols were obtained, purified by recrystalization or repeated chromatography after which they were analyzed by IR and NMR spectroscopy. Respective molar concentrations of the reagents are mentioned in the specific sections below.

Piperidine derivatives were obtained *via* a similar method in water without the catalyst (TEA).

9-Benzylamino-8-hydroxy-p-menth-2-one (**3.1**). This was obtained as a white solid by treating 8,9-epoxy-p-menthan-2-one (**2.9**, 150 mg, 0.9 mM), benzylamine (96.3 mg, 0.9

mM) and Et$_3$N (112 mg, 0.1 mM); yield, 212 mg (97.7 %); R$_f$, 0.32 (1/1 v/v hexane/EtOAc); IR, ν_{max} (CHCl$_3$) cm^{-1} 3444, 2928, 1720, 1495, 1253 and 1027; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.18 (5H, m, Ph), 4.03 (1H, q, J = 14.2 Hz, NH), 3.74 (2H, d, J = 1.4 Hz, H-7'), 2.60 (1H, d, J = 5.2 Hz, H-9), 2.47 (1H, d, J = 5.2 Hz, H-9), 2.41 (1H, m, H-1), 2.25- 2.07 (2H, m, H-3), 2.12-1.65 (5H, m, H-4, H-5, H-6), 1.01 (3H, s, H-10) and 0.92 (3H, d, J = 6.2 Hz, H-7); ^{13}C NMR (50 MHz, CDCl$_3$), δ 212.8 (C-2), 139.4 (C-1'), 128.6 (C-2', 6'), 128.2 (C-3', 5'), 127.4 (C-4'), 72.0 (C-8), 56.2, 56.0 (C-9), 54.4 (C-7'), 47.8 (C-1), 45.0, 42.5 (C-4), 34.7 (C-3), 30.9, 29.7 (C-6), 25.7 (C-10) and 14.3 (C-7); ESIMS, m/z (% rel. int.) 275 ([M]$^+$, 2), 265 (10), 249 (80) and 198 (100).

9-Phenylamino-8-hydroxy-p-menth-2-one **(3.2).** Treatment of 8,9-epoxy-p-menthan-2-one (**2.9**, 150 mg, 0.9 mM) with aniline (84 mg, 0.9 mM) and Et$_3$N (112 mg, 0.1 mM), gave **3.2** as a thick yellowish oil ; yield, 42 mg (20 %); R$_f$, 0.33 (1:1 v/v hexane/EtOAc); IR, ν_{max} (CHCl$_3$) cm^{-1} 3460, 1743, 1372, 1235 and 1045; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.28 (2H, m, H-3', H-5'), 6.80 (3H, m, H-2', H-4', H-6'), 4.22 (1H, m, NH), 3.22 (2H, dd, J_{AB} = 12.8, 12.8 Hz H-9), 2.50 (1H, m, H-1), 2.39 (1H, m, H-1), 2.27 (2H, m, H-3), 2.08 (2H, m, H-3), 2.08-1.61 (5H, m, H-4, H-5, H-6), 1.31 (3H, bs, H-10), 1.12 (1H, dd, J = 1.4, 1.4 Hz, H-7) and 1.10 (1H, dd, $J_{H7, H1}$ = 1.4, 1.4 Hz, H-7); ESIMS, m/z (% rel. int.) 261 ([M]$^+$, 15), 245 (100), 219 (20), 201 (60) and 189 (20).

9-Piperidinyl-8-hydroxy-p-menth-2-one **(3.3).** Treatment of 8,9-epoxy-p-menthan-2-one (**2.9**, 150 mg, 0.9 mM), piperidine (76.5 mg, 0.9 mM) and water (2 mL), yielded compound **3.3** as colourles crystals; yield, 158 mg (69 %); R$_f$, 0.29 (1:1 v/v

hexane:EtOAc); IR, ν_{max} (CHCl$_3$) cm^{-1} 3407, 1709, 1455, 1376, 1220 and 1039. ^{1}H NMR (200 MHz, CDCl$_3$), δ 2.61 (2H, m, H-9), 2.55 (4H, d, J = 2.4 Hz, H-1', H-5'), 2.41 (1H, m, H-1), 2.35 (1H, m, H-1), 2.19-1.23 (6H, m, H-2, H-5, H-6), 1.74 (1H, m, H-4), 1.54 (4H, m, H-2', H-4'), 1.42 (2H, m, H-3'), 1.08 (3H, s, H-10) and 1.02 (3H, s, H-7); ^{13}C NMR (50 MHz, CDCl$_3$), δ 213.3 and 212.7 (C-2), 71.5 and 71.4 (C-8), 65.6 and 65.4 (C-9), 57.1 (C-2', C-5'), 48.7 and 48.3 (C-1), 4.9 and 44.8 (C-4), 43.4 and 42.5 (C-3), 34.7 and 34.6 (C-6), 26.5 and 26.2 (C-1', C-3'), 25.7 (C-4'), 23.6 (C-10) and 22.9 (C-7) and 22.5 (C-5); ESIMS, m/z (% rel. int.) 253 ([M]$^+$, 5), 249 (48), 198 (100) and 165 (40).

8-Hydroxy-9-piperidinyl-p-menth-2-enone (**3.4**). Compound **3.4** was obtained as a yellow syrup on treatment of 8,9-epoxy-p-menth-6-en-2-one (**2.13**, 200 mg, 1.2 mM) with piperidine (102 mg, 1.2 mM) and water (2 mL); yield, 212 mg (70 %); R$_f$, 0.42 (1:1 v/v hexane: EtOAc); ^{1}H NMR (200 MHz, CDCl$_3$), δ 6.79 (1H, dd, $J_{H6, H5ax}$ = 1.8 Hz, $J_{H6, H5eq}$ = 1.2 Hz, H-6) , 6.71 (1H, dd, $J_{H6, H5ax}$ = 1.8 Hz, $J_{H6, H5eq}$ = 1.2 Hz, H-6), 2.66 (2H, d, J = 2.2 Hz, H-9), 2.53 (4H, m, H-1', H-5'), 2.45-2.02 (5H, m, H-3, H-4, H-5), 1.76, (3H, s, H-7), 1.55 (6H, m, H-2', H-3', H-4'); ^{13}C NMR (50 MHz, CDCl$_3$), δ 200.1 and 199.4 (C-2), 145.5 and 144.4 (C-6), 134.8 and 134.6 (C-1), 71.2 and 71.0 (C-8), 65.1 and 64.7 (C-9), 56.8 and 56.5 (C-1', C-5'), 43.5 (C-4), 39.5 and 38.7 (C-3), 27.1 and 26.5 (C-2', C-4'), 26.0 (C-3'), 22.4 (C-10) and 15.2 (C-7); ESIMS, m/z 251 ([M]$^+$, 5), 249 (85) and 198 (100).

6-Benzylamino-p-menthe-8,9-ene-1,2-diol (3.5). Compound **3.5** was obtained as white crystals upon treatment of 1,6-epoxy-p-menth-8-en-2-ol (**2.5**, 150 mg, 0.9 mM) with benzylamine (96.3 mg, 0.9 mM) and Et_3N (112 mg, 0.1 mM); yield, 82 mg (33 %); R_f, 0.35 (1:1 v/v hexane:EtOAc); m.p. 63 °C; IR, ν_{max} (CHCl$_3$) cm^{-1} 1643, 1496, 1456, 1376 and 1244; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.22 (5H, m, Ph), 4.46 (2H, bs, H-9), 3.82 (1H, d, J = 13.2 Hz, H-7'), 3.75(1H, d, J = 5.2 Hz, H-2), 3.70 (1H, d, J = 4.8 Hz, H-2), 3.60 (1H, d, J = 13.2 Hz, H-7'), 2.80 (1H, d, J = 3.2 Hz, H-6), 2.78 (1H, d, J = 3.8 Hz, H-6), 2.30 (1H, m, H-4), 1.90-1.45 (4H, m, H-3, H-5), 1.67 (3H, s, H-10) and 1.27 (3H, s, H-7), ^{13}C NMR (50 MHz, CDCl$_3$), δ 149.0 (C-8), 140.6 (C-1'), 128.4 (C-2', 6'), 128.1 (C-3', 5'), 127.0 (C-4'), 109.0 (C-9), 73.7 (C-1), 72.4 (C-2), 61.8 (C-6), 52.4 (C-7'), 37.1 (C-4), 35.1 (C-3), 29.2 (C-5), 23.7 (C-10) and 21.0 (C-7); ESIMS, m/z (% rel. int.) 275 ([M]$^+$, 5), 249 (65), 198 (100) and 165 (10).

6-Phenylamino-p-menth-8,9-ene-1,2-diol (3.6). Compound **3.6** was obtained as white powder upon treatment of 1,6-epoxy-p-menth-8-en-2-ol (**2.5**, 150 mg, 0.9 mM), aniline (85 mg, 0.9 mM) and Et_3N (112 mg, 0.1 mM); yield 37 mg (15.7 %); R_f, 0.47 (1:1 v/v hexane:EtOAc); m.p. 65 °C; IR, ν_{max} (CHCl$_3$) cm^{-1} 3544, 3393, 1507, 1450 and 1310. ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.18-6.70 (5H, m, Ph), 4.72 (2H, bs, H-9), 3.70 (2H, m, H-2, H-6), 2.19 (1H, m, H-4), 2.00-1.45 (4H, m, H-3, H-5), 1.71 (3H, s, H-10) and 1.39 (3H, s, H-7); ^{13}C NMR (50 MHz, CDCl$_3$), δ 148.1 (C-8), 147.4 (C-1'), 129.3 (C-3', C-5'), 117.6 (C-4'), 113.3 (C-2', C-6'), 109.4 (C-9), 73.7 (C-1), 72.3 (C-2), 57.8 (C-6), 37.7 (C-4), 34.9 (C-3), 30.3 (C-5), 23.9 (C-10) and 20.9 (C-7); ESIMS, m/z (% rel. int.) 261 ([M]$^+$, 2), 249 (75), 198 (100) and 163 (18).

6-Piperidinyl-p-menth-2-ene-1,2-diol (**3.7**). Compound **3.7** was obtained as yellow syrup after 1,6-epoxy-p-menth-8-en-2-ol (**2.5**, 150 mg, 0.9 mM) was treated with piperidine (80 mg, 0.9 mM) and water (2 mL); yield, 110 mg (48 %); R_f, 0.41 (1:1 v/v hexane:EtOAc); IR, ν_{max} (CHCl$_3$) cm^{-1} 1647, 1548, 1456, 1376, 1156, and 1108. ^{1}H NMR (200 MHz, CDCl$_3$), δ 4.83 (2H, d, J = 6.2 Hz, H-9), 3.70 (1H, dd, $J_{H2, H3ax}$ = 2.8, $J_{H2, H3eq}$ = 1.2 Hz, H-2), 2.85 (1H, m, H-6), 2.42 (4H, m, H-1', H-5'), 2.18 (1H, m, H-4), 1.84 (3H, s, H-10),1.70-1.43 (4H, m, H-3, H-5), 1.58 (6H, m, H-2',H-3', H-4') and 1.22 (3H, s, H-7); ^{13}C NMR (50 MHz, CDCl$_3$), δ 148.5 (C-8), 107.9 (C-9), 75.0 (C-1), 74.0 (C-2), 63.2 (C-6), 53.2 (C-1', 5'), 38.0 (C-4), 30.0 (C-3), 26.6 (C-2', C-4'), 24.4 (C-3'), 23.8 (C-5), 22.6 (C-10) and 21.6 (C-7); ESIMS, *m/z* (% rel. int.) 253 ([M]$^+$, 5), 249 (72) and 198 (100).

2-Benzylamino-4,5-epoxy-p-menthane-1-ol (**3.8**). The amino alcohol **3.8** was obtained as yellow syrup from treatment of 1,2:4,5-diepoxy-p-menthane (**2.10**, 150 mg, 0.9 mM) with excess benzylamine and Et$_3$N (224 mg, 0.2 mM); yield, 62 mg (29%); R_f, 0.37 (1:1 v/v hexane:EtOAc); IR, ν_{max} (CHCl$_3$) cm^{-1} 3086-3027, 1495, 1455, 1366, 1115, 1028, 1009, 744 and 699; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.22 (5H, m, Ph), 4.37 (1H, d, J = 5.6 Hz, H-5), 3.91 (2H, d, J = 12.8, Hz, H-7'), 3.66 (2H, d, J = 12.8, Hz, H-7'), 2.87 (1H, d, J = 1.8 Hz, H-2), 2.16 (1H, m, H-8), 1.96-1.23 (4H, m, H-3, H-6), 1.10 (3H, s, H-7), 0.87 (3H, s, H-9 or H-10) and 0.84 (3H, s, H-9 or H-10); ^{13}C NMR (50 MHz, CDCl$_3$), δ 140.5 (C-1'), 128.3 (C-2', C-6'), 128.2 (C-3', C-5'), 127.0 (C-4'), 70.4 (C-1), 60.7 (C-2), 63.6 (C-4), 57.8 (C-5), 37.6 (C-3), 35.1 (C-6), 26.8 (C-8), 24.4 (C-7), 18.0 (C-9, C-10), ESIMS, *m/z* (% rel. int.) 275 ([M]$^+$, 2), 249 (65) and 198 (100).

2-Piperidinyl-4,5-epoxy-p-menthane-1-ol (**3.11**). Compound **3.11** was obtained as orange crystals upon treatment of 1,2:4,5-diepoxy-*p*-menthane (**2.10**, 150 mg, 0.9 mM) with excess piperidine and water (2 mL); yield, 25 mg (11 %); m.p. 79-82 °C; R_f, 0.22 (1:1 v/v hexane:EtOAc); IR, ν_{max} (CHCl$_3$) cm^{-1} 1647, 1548, 1456, 1376, 1156 and 1108; ^{1}H NMR (200 MHz, CDCl$_3$), δ 4.35 (1H, s, H-5), 2.89 (1H, d, J = 5.8 Hz, H-2), 2.69-2.47 (4H, m, H-1', H-5'), 2.08 (1H, m, H-8), 1.95-1.25 (5H, m, H-3, H-4, H-6), 1.11 (3H, s, H-7), 1.01 (3H, d, J = 5.8 Hz, H-9 or H-10) and 0.98 (3H, d, J = 5.8 Hz, H-9 or H-10); ^{13}C NMR (50 MHz, CDCl$_3$), δ 68.4 (C-4), 64.8 (C-2), 65.4 (C-1), 56.9 (C-1', C-5'), 53.4 (C-5), 40.5 (C-6), 35.0 (C-8), 29.6 (C-3), 26.9 (C-2', C-4'), 24.6 (C-3'), 20.6 (C-7), 18.4 (C-9 or C-10), and 17.7 (C-9 or C-10); ESIMS, *m/z* (% rel. int.) 253 ([M]$^+$, 2), 249 (70), 198 (100) and 163 (18).

CHAPTER FOUR

SYNTHESIS OF HYDROXY-*p*-MENTHANEBENZYL MERCAPTANS AND THEIR APOPTOTIC ACTIVITIES

Abstract

Eight *β*-hydroxy-*p*-menthane benzyl mercaptans were synthesized through epoxide ring opening with benzyl thiol as the nucleophile and the apoptotic induction potential of the mercaptans evaluated against CHO and Jurkat T cells. In all cases the activity against Jurkat T cells was higher than that against CHO cells, which was an opposite trend as compared to the standard drug Camptothecin. The structures of the synthetic products were established based on analysis of spectroscopic data.

4.1   Introduction

The treatment of common tumourous diseases using the available anti-tumour agents is still challenging, due to the high toxicity levels of a number of the chemotherapeutic agents currently in use. Therefore, there is still need to establish curative and non-toxic chemotherapeutics for the treatment of cancer. Together with improved treatment strategies, new anti-tumour agents are clearly needed and one of the approaches to get such compounds involves the improvement of molecular structures of the already available clinically useful, as well as the search for new tumour curative agents from natural sources. Therefore, this study was inspired by the fact that there are some

clinically tested isoprenoids that have exhibited high degree of chemopreventive and chemotherapeutic potential against tumour cells,[327] but have not been explored further for drug development.

As indicated in Chapter One, sulfur containing compounds have been known to exhibit anti-cancer activities and this has offered inspiration to study hydroxysulfides as potential apoptosis inducers. In the literature, β-hydroxysulfides (hydroxymercaptans) are reported to serve as important intermediates for the synthesis of several bioactive and medicinally important natural products.[328] Most often, these compounds can be synthesized by nucleophilic ring opening of epoxides with thiols as the nucleophiles in the presence of a base [329] or an acidic catalyst [330] and organic solvents.

A number of special conditions for the above reactions have also been reported in the literature, which include microwave irradiation [331] and the use of complex Lewis acids.[332] Nucleophilic opening of epoxide rings with thiols has also been reported to take place in water, in the presence of some catalysts.[333] Despite their synthetic utilities most of the reaction conditions suffer from a number of drawbacks, which include the use of obnoxious thiols, strong and non-selective acidic catalysts, expensive and toxic reagents, organic solvents, low yields, and long reaction time.

Because of the importance of β-hydroxysulfides in drug development, the establishment of environmentally friendly, high yielding, and clean approaches for the synthesis of these compounds are being pursued. Thus, there has been an upsurge of research for

carrying out organic reactions in aqueous media, because water is the most abundant, cheapest, and an environmentally friendly solvent in comparison with other conventional organic solvents. This was the essence of conceiving the investigations whose results are reported in this chapter, aimed at preparing *β*-hydroxysulfides through nucleophilic ring opening of *p*-menthane based epoxides.

4.2   Results and Discussion

Tandem with this study, an economical way of obtaining the target hydroxysulfides for biological evaluation was sought. Initially, the study was performed using the terminal epoxide, namely dihydrocarvone epoxide (**2.9**), in a reaction with benzylmercaptan in water at room temperature, but the corresponding product was formed in rather low yield (21 %). Thus, the low yield for the less hindered epoxide group led to the assumption that reactions involving the more functionalized cyclohexanyl epoxides could take even longer duration, while giving dismally low yields. This necessitated the need to incorporate a catalyst.

In this regard, therefore, like for the amino alcohols discussed in Chapter Three, triethylamine (TEA) was employed for the reactions according to the method reported by Wu and Hong-Guang.[324] This approach was found to be highly efficient. Therefore, in preparing the reported hydroxysulfides the respective monoterpene epoxides were treated with benzylmercaptan in water and the reaction catalyzed by TEA. The reactions

went on fairly smoothly, furnishing the respective *β*-hydroxysulfides in good yields as discussed in the following sections.

4.2.1   8-Hydroxy-9-mercaptobenzyl-*p*-menthan-2-one (4.1)

Dihydrocarvone epoxide (8,9-epoxy-*p*-menthan-2-one, **2.9**) when treated with 1.2 molar equivalent of benzyl mercaptan afforded *β*-hydroxylsulfide (**4.1**) as the only product and in excellent yield (**Scheme 4.1**), whose structure was confirmed through analysis of NMR spectroscopic data.

2.9

Et$_3$N, H$_2$O, 24 h, RT

4.1 (96 %)
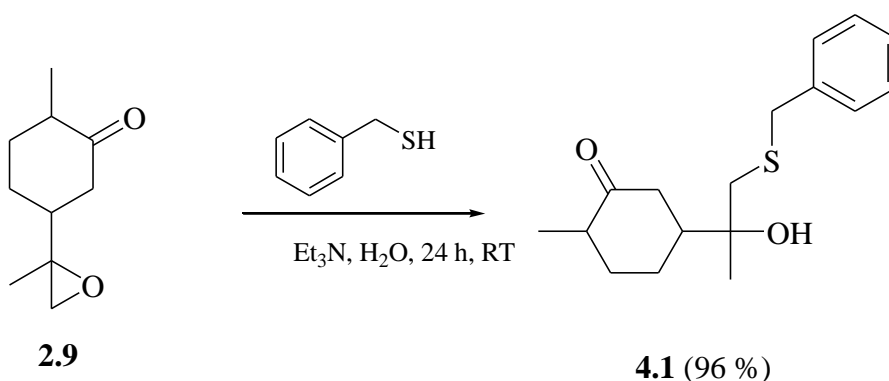
Scheme 4.1   Epoxide ring opening of compound 2.9 with benzyl mercaptan

8-Hydroxy-9-mercaptobenzyl-*p*-menthan-2-one (**4.1**) was evaluated for apoptotic induction potential at a concentration of 1 µM against CHO and Jurkat T cells. The flow cytometry charts (**Fig 4.1**) indicated the compound to have exhibited 85.41 and 95.95 % apoptotic activity against CHO and Jurkat T cells respectively. This clearly indicated high apoptotic activity of 8-hydroxy-9-mercaptobenzyl-*p*-menthan-2-one (**4.1**), despite

showing some degree of selective activity the difference was rather small, thereby implying that the level of cytotoxicity against the two cell lines is almost similar.

Fig. 4.1   Flow cytometry histograms showing apoptotic activity for compound 4.1 against CHO and Jurkat T cells

4.2.2   1,2-Eepoxy-9-benzylthio-*p*-menthan-8-ol (4.2) and 2,9-Di-mercaptobenzyl-*p*-menthan-1,8-diol (4.3)

The diepoxide derivative of limonene, namely 1,2:8,9-diepoxy-*p*-menthane (**2.16**), was treated with 3 molar equivalents of benzyl mercaptan in anticipation that both oxirane rings would open up, either exclusively yielding the di-substituted derivative or as the major product together with one or two mono-epoxide derivatives. Among the three possibilities above, the reaction upon work up, yielded a mixture of mono- and di-substituted products 1,2-epoxy-9-benzylthio-*p*-menthan-8-ol (**4.2**) and 2,9-di-mercaptobenzyl-*p*-menthan-1,8-diol (**4.3**) respectively, the later as the minor product constituting 0.2 % and the former as the major product (77 %, **Scheme 4.2**). Structures

of the two compounds **4.2** and **4.3** were identified based on analysis of ^{1}H and ^{13}C NMR spectral data. However, the ^{13}C NMR resonances for both compounds appeared as twin signals, which indicated that each of the compounds was a mixture of stereoisomers, formed as the result of non-stereoselectivity of the epoxide ring opening reaction. Unfortunately, the respective stereoisomers could not be separated by chromatography in their pure forms.

2.16   **4.2** (77 %)   **4.3** (0.2 %)
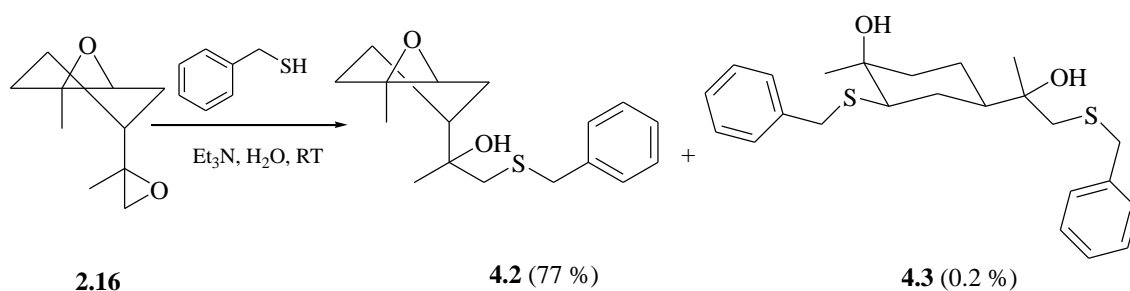
Scheme 4.2   Epoxide ring opening of 2.16 with benzyl mercaptan

Contrary to expectations, the mono-benzyl mercaptan **4.2** was obtained as the major product even after allowing the reaction to proceed for 36 h. This could be attributed to compound **2.16** in its half chair conformation, which, with a newly introduced bulky and *pseudo*-axially oriented hydroxysulfide moiety at the isopropoxyl end in **4.2** could be responsible for the regioselectivity because a back side attack on the epoxide group would be more restricted due to steric hindrance by the methyl group that is also *pseudo*-axial, thereby contributing to the low yield of the di-mercaptan **4.3**.

The apoptotic activity results for **4.2** and **4.3** as shown by flow cytometric analysis indicated the two compounds to be active, showing 76.3 and 71.1 % death against CHO

cells while against Jurkat T cells the effected death was 99.17 and 99.63 % respectively, at 1 μM concentration (**Fig. 4.3** and **Fig 4.4**). These results indicated slightly higher selective activity between the two cell lines in comparison to compound **4.1**, hence implying that the structural differences could have spurred the selectivity.
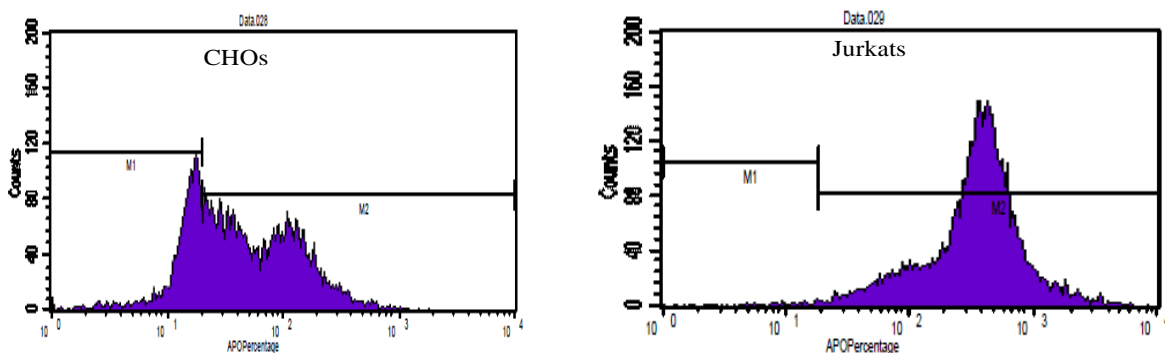
Fig. 4.3   **Flow cytometry histograms showing apoptotic activity for compound 4.2 against CHO and Jurkat T cells**
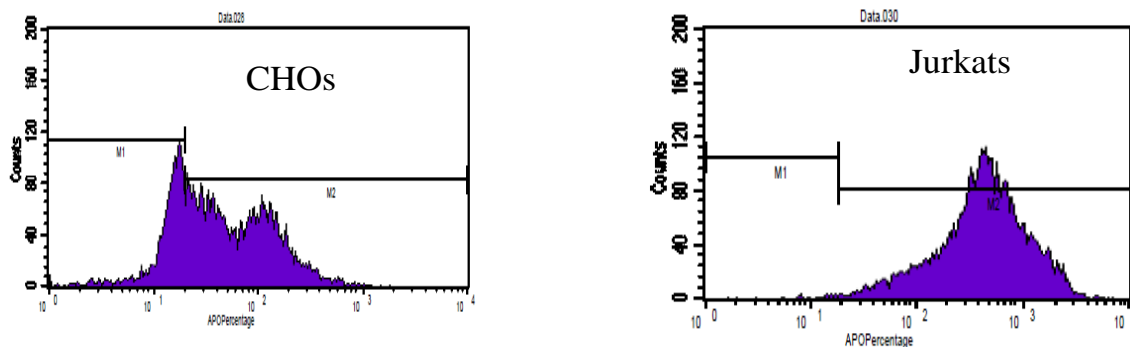
Fig. 4.4   **Flow cytometry histograms showing apoptotic activity for compound 4.3 against CHO and Jurkat T cells**

4.2.3   6-Mercaptobenzyl-1,2-*p*-menth-8,9-ene-1,2-diol (**4.4**)

Treatment of 2-hydroxy-1,6-epoxy-*p*-menthen-8,9-ene (**2.5**) with 1.2 molar equivalent of benzyl mercaptan afforded the hydroxylbenzyl mercaptan **4.4** (6-mercaptobenzyl-1,2-p-menth-8,9-ene-1,2-diol) as the only product and in excellent yield (99 %) after 24 h of stirring at room temperature in the presence of TEA as the catalyst. Structure **4.4** for the product was established based on analysis of spectroscopic data (IR, NMR and MS). Unlike for the reaction discussed in the previous section in which the 1,2-substituted site was deemed more hindered and less reactive than the 8,9-isopropoxyl site thus yielding the minor product, this reaction yielded excellent amount of the target product (**4.4**). However, in contrast to the previous reaction, in this case there was only one epoxide group; hence as such there were no competing reactive sites. In addition, the isopropenyl group in compound **2.5** is less bulky than the benzylmercaptan in the former compound. Therefore, steric hindrance is much lower in **2.5**, hence contributing to higher accessibility and reactivity of compound **2.5** (**Scheme 4.3**).
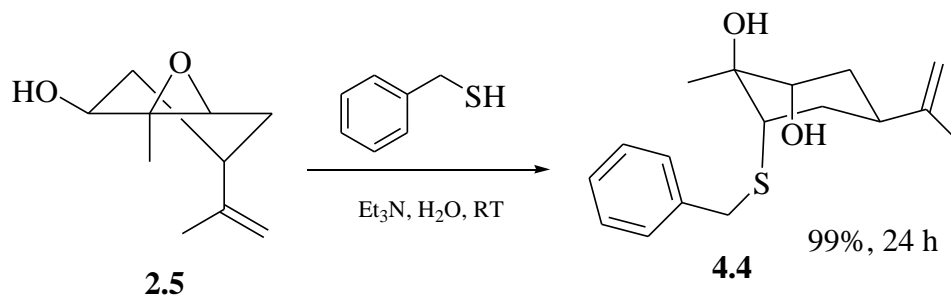
Scheme 4.3   Epoxide ring opening of compound 2.5 with benzyl mercaptan

When assayed for apoptosis induction compound **4.4** exhibited activity (**Fig. 4.5**) at 69.73 and 97.84 % death of the CHO and Jurkat T cell populations respectively, hence indicating a relatively good level of selective activity for this compound. This suggested that the presence of a 1,2-diol substitution in presence of the mercaptobenzyl group might have prompted selective cytotoxicity against the two cell lines.
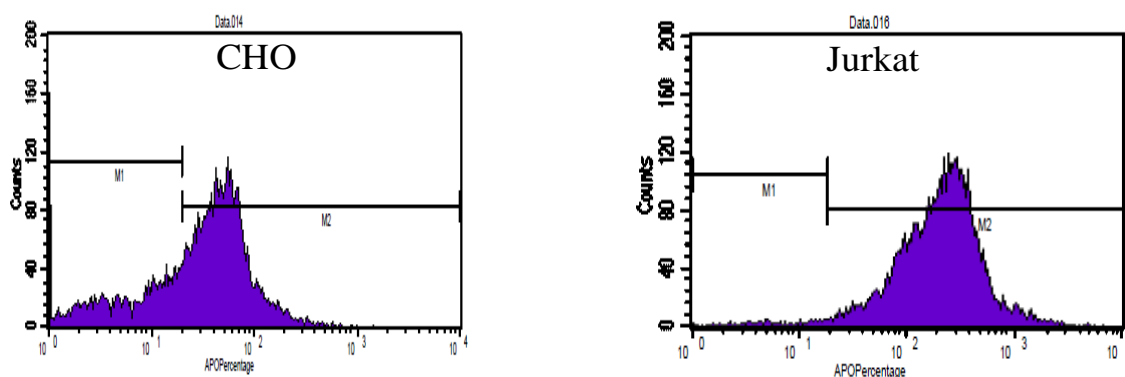
Fig. 4.5   **Flow cytometry histograms showing apoptotic activity for compound 4.4 against CHO and Jurkat T cells**

4.2.4   9-Mercaptobenzyl-*p*-menth-1,6-ene-2,8-diol (**4.5**)

The epoxide ring opening reaction for 2-hydroxy-8,9-isopropoxy-*p*-mentha-1-ene (**2.7**) with 1.2 molar equivalent of benzyl mercaptan afforded 9-mercaptobenzyl-*p*-menth-1,6-ene-2,8-diol (**4.5**) as the only product and in excellent yield (81.2 %) after 24 h of stirring at room temperature in the presence of TEA as the catalyst (**Scheme 4.5**). The structure of the synthesized compound **4.5** was established upon analysis of spectroscopic data.

2.7

Et$_3$N, H$_2$O, 24 h, RT

4.5 (82 %)
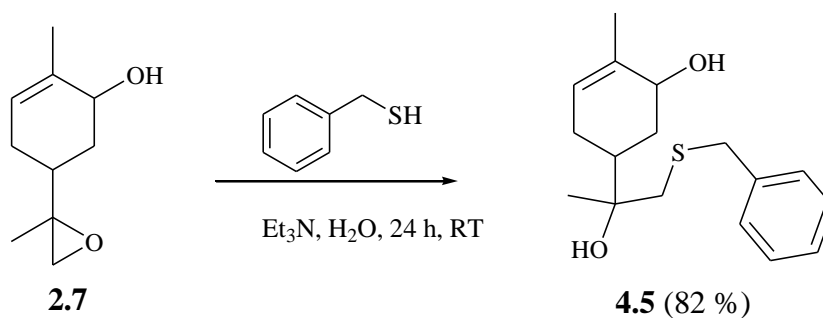
Scheme 4.5   Epoxide ring opening of compound 2.7 with benzyl mercaptan

Analysis of apoptotic induction by flow cytometry for compound **4.5** indicated higher potency against Jurkat T than CHO cells, producing 97.82 and 65.01 % non-viable cells respectively. The results also indicated a relatively good degree of selective activity against the two cell lines (**Fig. 4.6**). Hence like compound **4.5** it was presumed that the presence of hydroxyl groups in the molecule may be responsible for the selectivity.
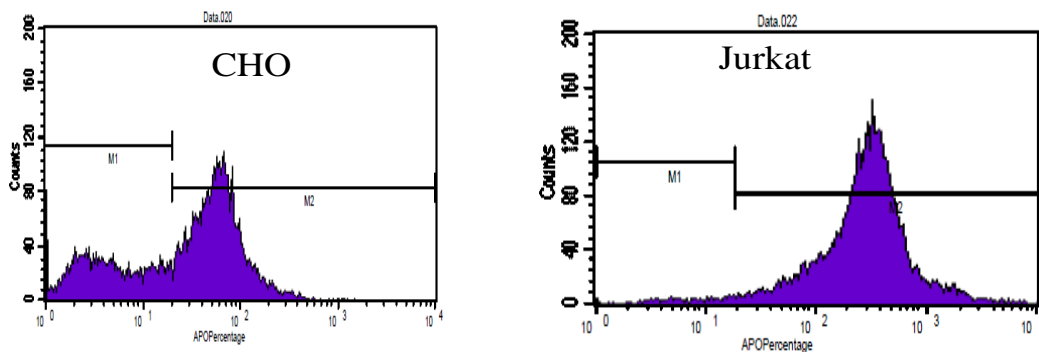
Fig. 4.6   **Flow cytometry histograms showing apoptotic activity for compound 4.5 against CHO and Jurkat T cells**

4.2.5   6,9-Dimercaptobenzyl-*p*-menthane-1,2,8-triol (**4.6**)

Treatment of carveol diepoxide (**2.8**) with 2.5 molar equivalent of benzyl mercaptan afforded the di-benzyl mercaptan hydroxysulfide **4.6** in low yield (24%) after 24 h of stirring at room temperature and TEA as the catalyst (**Scheme 4.5**). The structure of the product **4.6** was established upon analysis of its spectroscopic data. No compound was formed as a mono-epoxide ring opening product, indicating non-regioselectivity of the reaction.   This was contrary to what was observed in the reaction involving the diepoxide **2.16** (**Section 4.2.2**). This indicated the importance of the hydroxyl group α to the more substituted epoxide carbon on the cyclohexanyl ring in directing the approaching nucleophilic species to the reaction site. However, overall the reaction of

2.8 was less facile than that involving the diepoxide **2.16** as the substrate, as the overall yield was much higher in the reaction involving compound **2.16** than with **2.8**.
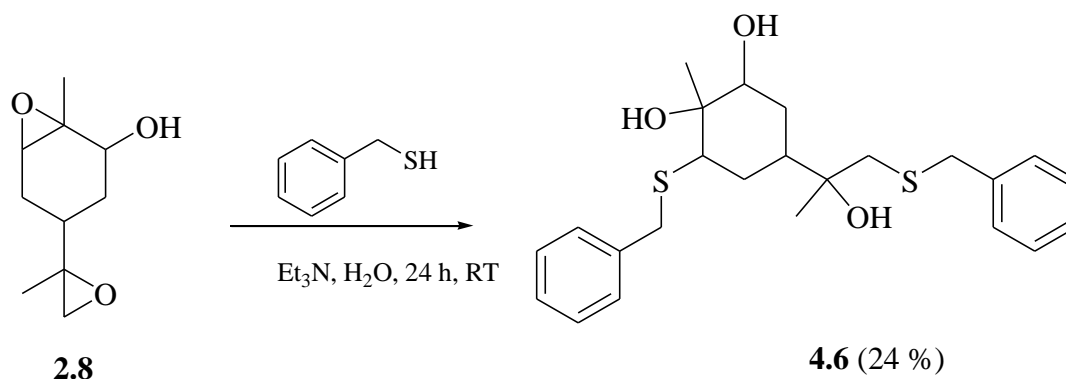
Scheme 4.5   **Epoxide ring opening of compound 2.9 with benzyl mercaptan**

The results from flow cytometric analysis of compound **4.6** indicated higher activity of the compound against Jurkat T as compared with CHO cells (99.04 % non-viable cells for the former as compared to 88.89 % for the latter, **Fig. 4.7**). Hence, despite showing a high potency against the two cell lines, the level of selective activity was rather low, probably due to the presence of two mercaptobenzyl groups and a highly hydroxylated *p*-menthane skeleton, thereby heightening the cytotoxicity level.
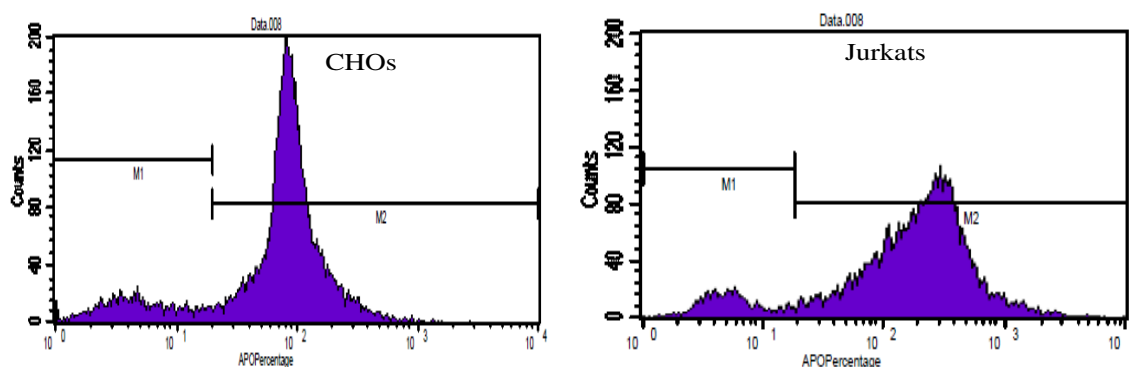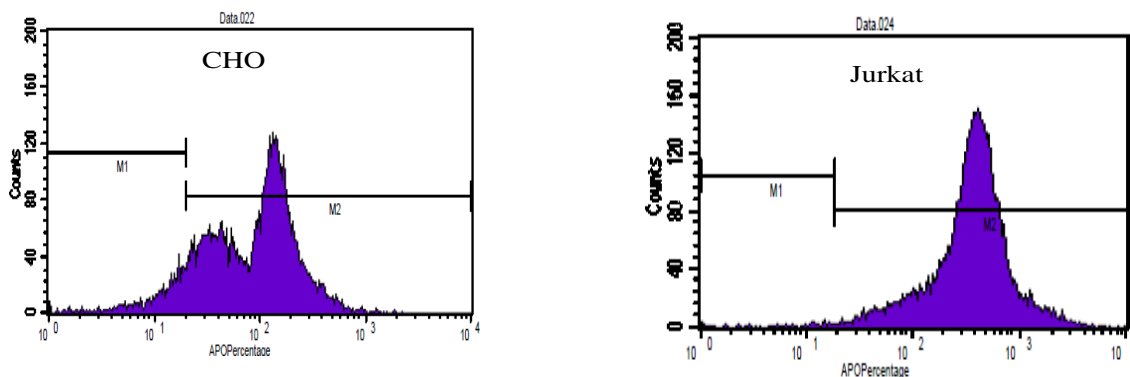
Fig. 4.7   **Flow cytometry histograms showing apoptotic activity for compound 4.6 against CHO and Jurkat T cells**

4.2.6   4,5-Epoxy-2-mercaptobenzyl-*p*-menthane-1-ol (**4.7**)

Treatment of γ-terpinene diepoxide (**2.10**) with 2.5 molar equivalent of benzyl mercaptan yielded 4,5-epoxy-2-mercaptobenzyl-*p*-menthane-1-ol (**4.7**) in 62 % yield after stirring the reaction mixture for 24 h (**Scheme 4.6**).   The structure **4.7** for the synthesized compound was established based on analysis of spectroscopic data.

2.10   **4.7** (62 %)
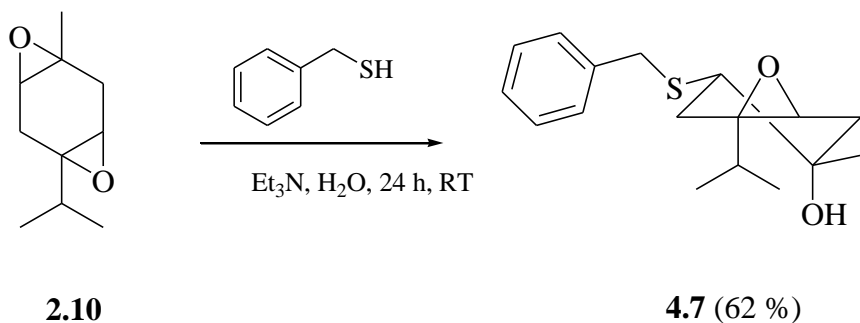
Scheme 4.6   Epoxide ring opening of compound 2.10 with benzyl mercaptan

The nearly symmetric structure of the diepoxide raised the question as to which between the two epoxide rings would preferentially undergo opening by the benzyl mercaptan. However, based on the crowded environment for the 4,5-epoxide ring due to the presence of the sterically bulky isopropyl group in comparison with the methyl substituent neighbouring the 1,2-epoxide, the latter was shown to be more accessible, and therefore preferentially reacted with the nucleophile.

Apoptosis was established for compound **4.7** as indicated by the flow cytometry histograms (**Fig. 4.8**), being higher against Jurkat T (98.93 % non-viable cells) as compared to CHO cells (87.41 %). The comparatively high results could be attributed to the presence of the epoxide group due to its highly alkylating ability in cells [311] and from the observed high activity trend that was presumed to be enhanced by the mercaptobenzyl group present in the compounds already discussed.
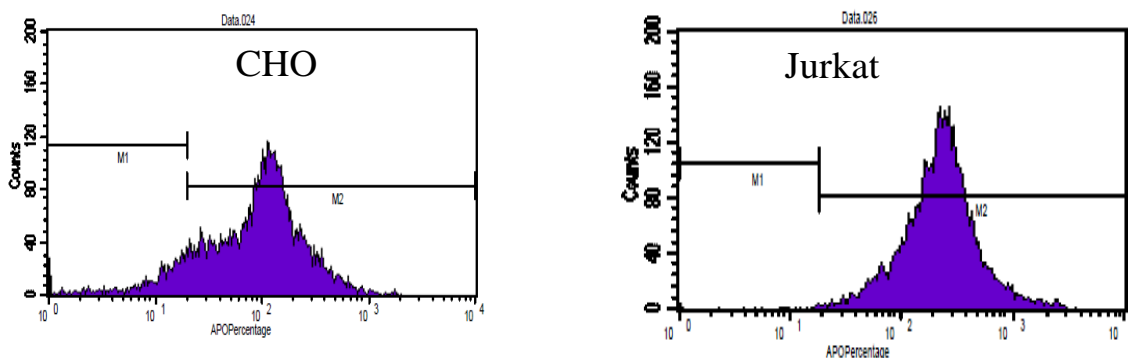
Fig. 4.8   **Flow cytometry histograms showing apoptotic activity for compound 4.7 against CHO and Jurkat T cells**

4.2.7 **9-Mercaptobenzyl-*p*-menthen-2-one** (**4.8**)

When 8,9-carvone epoxide (**2.13**) was treated with 1.2 molar equivalent of benzyl mercaptan it afforded 9-mercaptobenzyl-*p*-menthen-2-one (**4.8**) as the only product and in very good yield (88.8 %) after 24 h of stirring at room temperature with TEA as the catalyst (**Scheme 4.7**).
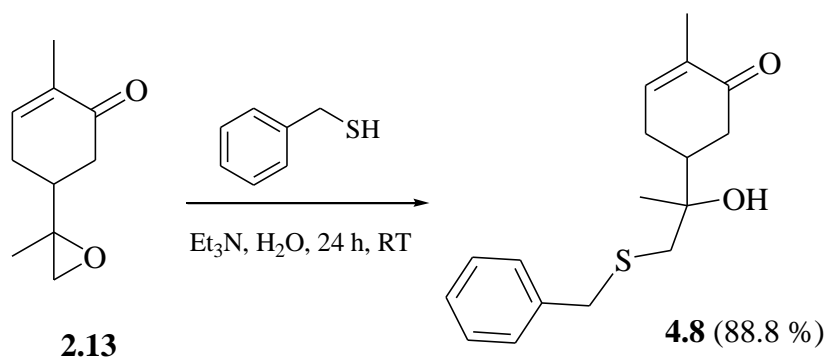
Scheme 4.7 **Epoxide ring opening of compound 2.13 with benzylmercaptan**

Compound **4.8** exhibited a good level of apoptotic activity against both cell line, with a higher potency against the Jurkat T as compared to CHO cells (89.22 % non-viable cells for the former and 73.76 % for the latter), thus implying some level of selective activity against the two cell types (**Fig. 4.9**). The results indicate comparably high activity against both cell lines. Inference was made that, the presence of α,β-unsaturation in the *p*-menthane skeleton may have stimulated additional influence upon the activity induced by the mercaptobenzyl moiety. This was based on the fact that α,β-unsaturation has been reported to enhance bioactivities.[310]
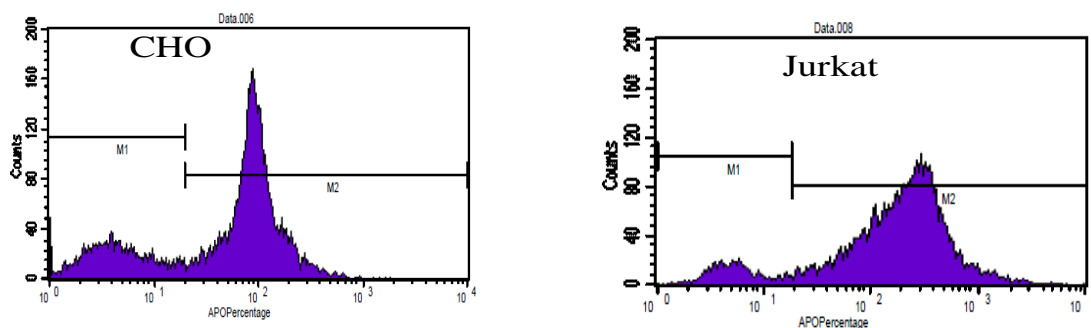
Fig. 4.9   **Flow cytometry histograms showing apoptotic activity for compound 4.8 against CHO and Jurkat T cells**

4.3   Structure Activity Relationship Analysis of the *β*-Hydroxysulphides

The apoptosis induction results obtained from the flow cytometry analysis of the hydroxysulfides prepared in these studies to determine the efficacy of the compounds

that were synthesized as described in this chapter against Jurkat T and CHO cells indicated some structure-activity relationships for both cell lines tested. Thus, as shown in **Table 4.9** among the compounds tested, derivative **4.6** exhibited the highest apoptotic activity against CHO cells, followed closely by **4.7** and **4.1**, all of which produced > 85 % non-viable cells. Compound **4.6** is composed of two mercaptobenzyl substituents and three hydroxyl groups while the other two compounds **4.7** and **4.1** have only one mercaptobenzyl and one hydroxyl group.   Therefore, it may be inferred that the additional mercaptobenzyl group in **4.6** was responsible for the high activity than the olefinic and ketonic groups that are present in compounds **4.7** and **4.1** respectively.

A similar activity trend among the three compounds was observed against Jurkat T cells. However, on average the most potent compound against Jurkat T cells was **4.3**, inducing 99.63 % cell death. The compound also contains two mercaptobenzyl substituents but with two hydroxyl groups. Comparatively, compound **4.3** exhibited a slightly lower activity, producing 71.07 % non-viable CHO cells. This, in comparison to compound **4.6**, may be attributed to the presence of only two hydroxyl groups in **4.3** in contrast to three in **4.6**. Therefore, that implied that an additional hydroxyl group conferred higher apoptotic activity against the CHO cells in the latter compound. This indicated that amongst the hydroxysulfides tested some level of selective activity between the two cell lines was generally achieved. In most cases the compounds demonstrated better apoptotic activity than Camptothecin that was used as the standard drug.

Compounds **4.4** and **4.5** (both of which contain a differently substituted hydroxyl and mercaptobenzyl group) exhibited the lowest activities against CHO cells. This observation might have implied that the relative position of the hydroxyl and mercaptobenzyl group was not a crucial factor for the activity of the two compounds, or that the presence of differently positioned epoxide groups did not have a positive effect towards their apoptotic potential.

An interesting observation was made for the two limonene derivatives **4.2** which has an epoxide moiety and the di-benzylated mercaptan **4.3** that lacked an epoxide functional group. Both compounds exhibited similar activities against the two cell lines. This probably implied that both the epoxide moieties in compound **4.2** and the additional benzyl mercaptan group in compound **4.3** expressed similar influence towards cell death. However, this possibility was not fully substantiated in these studies, for which further studies are needed.

Compound **4.6**, which also had both functional groups as stated above, exhibited high level of activity against both cell lines, while exhibiting the highest activity (88.9 %) against CHO cells. Likewise, this raised the question as to which, between the epoxide and the mercaptan group was a better protagonist site or could the two groups be acting synergistically? This is an area that requires further research.

The presence of the carbonyl group in compounds **4.1** and **4.8** also seemed to be essential in the enhancement of apoptotic activity, more so for a saturated cyclohexyl

moiety as indicated by **4.1** in comparison to **4.8** that has an α,β-unsaturated carbonyl group. Both compounds were highly active against the two cell lines, with both indicating higher potency against Jurkat T than against CHO cells.

Gauging by the activity of compound **4.7**, it could be inferred that the presence of a cyclohexanyl epoxide group served to enhance the apoptotic potential of the compound coupled to the cyclohexanyl mercaptobenzyl moiety.

Table 4.1 **Summary of flow cytometric results showing the activity of the synthesized terpenyl benzyl hydroxymercaptans against CHO and Jurkat T cells**

	% Non-viable (apoptotic) cells	
Compound	**CHO**	**Jurkat T**
4.1	85.41 ± 8.22	**95.95 ± 1.25**
4.2	76.26 ± 0.93	**99.17 ± 1.97**
4.3	71.07 ± 6.12	**99.63 ± 2.43**
4.4	69.73 ± 7.46	**97.84 ± 0.64**
4.5	65.01 ± 12.18	**97.82 ± 0.62**
4.6	88.89 ± 11.7	**99.04 ± 1.84**
4.7	87.41 ± 10.22	**98.93 ± 1.73**

4.8	73.76 ± 3.43	**89.22 ± 7.98**
Camptothecin	81.9 ± 1.72	**55.5 ± 7.03**
-ve control	33.6 ± 5.03	**21.7 ± 0.62**
DMSO	**37.7 ± 2.65**	**37.3 ± 11.46**
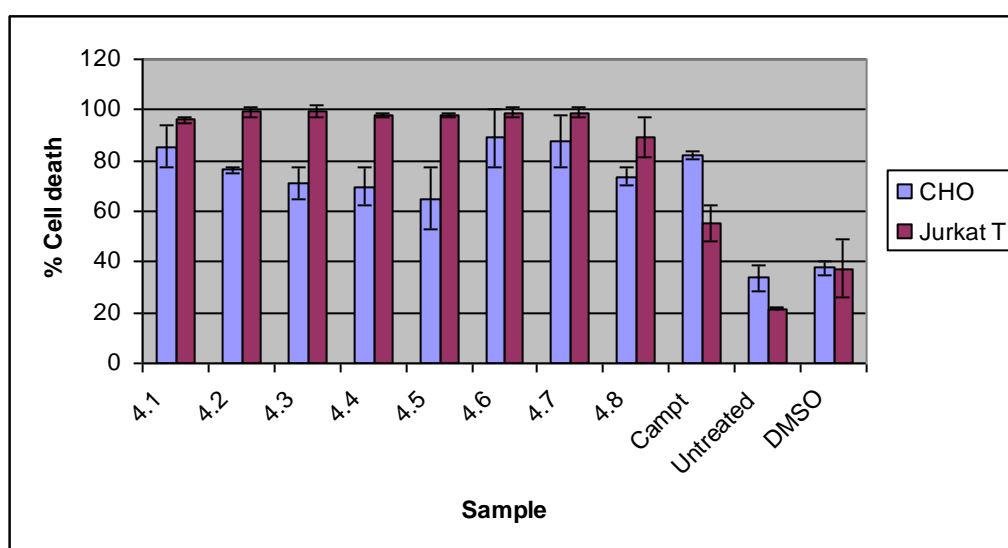
Fig. 4.10 **Comparative apoptotic activity of synthesized terpenyl benzyl hydroxymercaptans**

4.4 Conclusion

The results presented in this chapter showed that the nucleophilic epoxide ring opening with benzylmercaptan was facile by furnishing the target compounds in good to excellent yields except for the di-mercaptan derivative **4.6**, which was obtained in a rather low yield of 24 %. This also confirmed that the aqueous solvent system and Et_3N

catalyst was suitable for the reaction, thereby making it possible to obtain the compounds cheaply available in an environmentally safe condition. The apoptosis inducing potential exhibited by compounds **4.1-4.8** was relatively high and in most cases higher than that of the standard drug Camptothecin, implying that they posses a high level of cytotoxicity.

4.5   Experimental Procedures

4.5.1   General Experimental Procedures

These were the same as described in Chapter Two.

4.5.2   General Procedure for the Epoxides Ring Opening Reactions with Benzyl Mercaptan

To a suspension of the corresponding epoxide in water was added benzyl mercaptan in equimolar amounts or as otherwise indicated, followed by the addition of Et_3N and the reaction mixture was stirred briskly at room temperature for the indicated time. After completion of the reaction (TLC), the reaction mixture was extracted with EtOAc. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The pure product was obtained by silica gel column chromatography.

8-Hydroxy-9-mercaptobenzyl-p-menthan-2-one (**4.1**). This was obtained as a white oil by treating 8,9-epoxy-*p*-menthan-2-one (**2.9**, 155 mg, 0.92 mM) with benzyl mercaptan (111 mg, 0.92 mM) in the presence of Et_3N (101 mg, 1 mM) for 24 h; yield, 211 mg (99

%); ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.27 (5H, m, Ph), 3.70 (2H, bs, H-7'), 2.67-2.01 (5H, m, H-1, H-3, H-9), 1.98-1.18 (5H, m, H-4, H-5, H-6), 1.11 and 1.12 (s and s, H-10) and 0.97 (3H, d, J = 6.2 Hz, H-7); ^{13}C NMR (50 MHz, CDCl$_3$), δ 212.8 and 212.8 (C-2), 137.9 (C-1'), 128.7 (C-2' and C-6'), 128.4 (C-3' and C-5'), 127.0 (C-4'), 73.1 and 73.0 (C-8), 48.1 (C-9), 44.6 (C-1), 43.0 and 42.9 (C-4), 42.2 and 42.3 (C-7'), 37.8 (C-3), 34.5 and 34.2 (C-6), 26.3 and 25.5 (C-10), 23.5 and 23.4 (C-5) and 14.1 (C-7).

1,2-Epoxy-9-benzylthio-p-menthan-8-ol (**4.2**). Treatment of a mixture of limonene di-epoxide **2.16** with benzyl mercaptan (298 mg, 2.4 mmol) and Et$_3$N (132 mg, 1.3 mmol) afforded **4.2** as a colourles oil; yield, 198 mg (77%); R$_f$, 0.73 (1:1 v/v hexane/EtOAc); ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.31 (5H, m, Ph), 3.74 (2H, m, H-7'), 2.97 (1H, dd, J = 5.4, 5.4 Hz, H-2), 2.63 (1H, dd, J_{AB} = 13.2, 13.2 Hz, H-9), 2.53 (1H, dd, J_{AB} = 8.4, 8.4 Hz, H-9), 2.11-1.05 (7H, m, H-3, H-4, H-5, H-6) and 1.30 (6H, s, H-7 and H-10); ^{13}C NMR (50 MHz, CDCl$_3$), δ 138.1 (C-1'), 128.9 (C-2' and C-6'), 128.6 (C-3' and C-5'), 127.2 (C-4'), 73.6 (C-8), 59.2 and 59.0 (C-2), 57.7 and 57.6 (C-1), 42.9 and 42.3 (C-9), 41.9 (C-4), 38.1 and 38.0 (C-7'), 30.7 (C-6), 25.9 and 25.2 (C-10), 23.5 and 22.6 (C-3), 20.4 (C-7) and 19.4 (C-5).

2,9-Di-mercaptobenzyl-p-menthan-1,8-diol (**4.3**). Compound **4.3** was obtained together with **4.2** as described above as a thick white oil; yield, 85 mg (0.2 %); R$_f$, 0.26 (7:3 v/v hexane/EtOAc); ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.34-7.21 (10H, m, Ph), 3.74 (m, 4H, H-7' and H-7''), 2.70 (1H, m, H-9), 2.67 (1H, m, H-2), 2.54 (1H, dd, J_{AB}= 10.2, 10.2 Hz, H-9), 1.92-1.26 (5H, m, H-3, H-4, H-5), 1.30 and 1.29 (3H, s and s, H-7) and 1.11 (3H,

d, *J* = 2.4 Hz, H-10); ^{13}C NMR (50 MHz, CDCl$_3$), δ 138.3 (C-1'), 138.2 (C-1"), 129.0

(C-2' and C-6'), 128.9 (C-2" and C-6"), 128.6 (C-3' and C-5'), 128.5 (C-3" and C-5"),

127.2 (C-4'), 127.0 (C-4"), 73.8 and 73.7 (C-8), 72.2 (C-1), 51.9 and 51.8 (C-2), 43.2

and 43.0 (C-9), 40.2 and 40.1 (C-4), 38.2 (C-7"), 37.2 and 37.1 (C-6), 34.8 (C-7'), 29.5

(C-10), 28.2 and 27.2 (C-7), 22.4 and 21.4 (C-5) and 23.6 and 23.4 (C-3).

6-Mercaptobenzyl-p-menth-8,9-ene-1,2-diol (**4.4**). Treatment of *trans*-2-hydroxy-1,6-

epoxy-*p*-menthen-8,9-ene (**2.6**, 200 mg, 1.2 mM), with benzyl mercaptan (149 mg, 1.2

mM) and Et$_3$N for 24 h with stirring yielded compound **4.4** as thick white oil; yield, 313

mg (99 %); R$_f$, 0.47 (1:1 v/v hexane/EtOAc); IR, ν$_{max}$ (CHCl$_3$) cm^{-1} 3350, 3050, 1492,

1452, 2928, 1640 and 694; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.24 (5H, m, Ph), 4.67 (2H,

bs, H-9), 3.70 (2H, bs, H-7'), 3.62 (1H, m, H-2), 2.89 (1H, bs, H-6), 2.39 (1H, m, H-6),

1.93 (1H, m, H-4), 1.80-1.45 (4H, m, H-3, H-5), 1.67 (3H, s, H-10) and 1.33 (3H, s, H-

7); ^{13}C NMR (50 MHz, CDCl$_3$), δ 148.6 (C-8), 137.9 (C-1'), 129.0 (C-2' and C-6'), 128.5

(C-3 and C-5'), 127.1 (C-4'), 109.1 (C-9), 74.2 (C-1), 72.3 (C-2), 51.4 (C-6), 38.2 (C-7'),

37.4 (C-4), 35.1 (C-5), 31.5 and 30.9 (C-3), 25.2 (C-10) and 20.9 (C-7); MS, *m/z* (% rel.

int.) 293 ([M+1]$^+$, 5), 292 (20), 249 (60) 150 (60) and 91 (100).

9-Mercaptobenzyl-p-menth-1,6-ene-2,8-diol (**4.5**). Compound **4.5** was obtained as thick

white oil upon treatment of 2-hydroxy-8,9-isopropoxy-*p*-menth-1-ene (**2.7**, 150 mg, 0.9

mM) with benzyl mercaptan (112 mg, 0.9 mM) and Et$_3$N (90 mg) for 24 h; yield, 160

mg (81.2 %); R$_f$, 0.43 (1:1 v/v hexane/EtOAc); IR, ν$_{max}$ (CHCl$_3$) cm^{-1} 3392, 3055, 2940,

1601 and 701; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.24 (5H, m, Ph), 5.49 (1H, m, H-6), 3.96

(1H, m, H-2), 3.71 (2H, d, J = 2.2 Hz, H-7'), 2.65 (1H, m, H-9), 2.54 (1H, dd, J_{AB} = 6.6, 6.6 Hz, H-9), 2.00-1.22 (5H, m, H-3, H-4, H-5), 1.73 3H, (s, H-7) and 1.09 (3H, s, H-10); ^{13}C (50 MHz, CDCl$_3$), δ 138.2 (C-1'), 134.6 and 134.3 (C-6), 128.9 (C-2' and C-6'), 128.5 (C-3' and C-5'), 127.1 (C-4'), 125.2 and 124.7 (C-1), 73.6 and 73.5 (C-8), 68.5 and 68.4 (C-2), 43.1 and 42.1 (C-4), 38.1 (C-9), 36.8 and 36.7 (C-7'), 32.8 and 32.0 (C-3), 27.2 and 26.4 (C-5), 23.9 and 23.0 (C-10), and 20.8 and 20.7 (C-7); MS, m/z (% rel. int.) 292 ([M]$^+$, 5), 249 (60) and 198 (100).

6,9-Dimercaptobenzyl-p-menthane-1,2,8-triol (**4.6**). Treatment of 1,6:8,9-diepoxy-*p*-menthan-2-ol (**2.8**, 200 mg, 1 mM) with benzyl mercaptan (292 mg, 2 mM) and Et$_3$N after 24 h yielded compound **4.6** as white oil; yield, 95 mg (24 %); R$_f$, 0.64 (2:8 v/v hexane/EtOAc); IR, ν$_{max}$ (CHCl$_3$) cm^{-1} 3430, 3055, 2929, 1494, 1454 and 702; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.23 (10H, m, Ph), 3.72 (4H, m, H-7' and H-7''), 3.27 (1H, m, H-2), 2.85 (2H, m, H-6), 2.60 (2H, m, H-9), 2.33-1.50 (5H, m, H-3, H-4, H-5), 1.33 (3H, s, H-7) and 1.08 (3H, s, H-10); ^{13}C (50 MHz, CDCl$_3$), δ 138.1 (C-1' and C-1''), 128.9 (C-2', C-2'', C-6' and C-6''), 128.6 (C-3', C-3'', C-5' and C-5''), 127.2 (C-4' and C-4''), 73.6 (C-8), 59.2, 59.0 (C-2), 57.7 and 57.6 (C-1), 42.9 and 42.3 (C-9), 41.9 (C-4), 38.1 and 38.0 (C-7' and C-7''), 30.7 (C-6), 25.9 and 25.2 (C-10), 23.5 and 22.6 (C-3), 20.4 (C-7) and 19.4 (C-5).

4,5-Epoxy-2-mercaptobenzyl-p-menthane-1-ol (**4.7**). 1,2:4,5-Diepoxy-*p*-menthane (**2.10**, 200 mg, 1.26 mM) upon stirring with benzyl mercaptan (298 mg, 2.5 mM) and Et$_3$N (130 mg) for 24 h yielded compound **4.7** as thick colourles oil; yield, 239 mg (61.6

%); R_f, 0.48 (1:1 v/v hexane/EtOAc); IR, ν_{max} (CHCl$_3$) cm^{-1} 3468, 3045, 2964, 2930, 1495, 1454, 1428 and 702; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.24 (5H, m, Ph), 3.73 (2H, dd, J_{AB} = 13.2, 13.2 Hz, H-7'), 2.66 (1H, dd, $J_{H6,H5}$ = 5.6 and 5.4 Hz, H-6), 2.21 (2H, dd, $J_{H2, H3}$ = 5.2, 5.2 Hz, H-2), 1.95 (1H, d, J = 4.4 Hz, H-6), 1.90 (1H, d, J = 4.4 Hz, H-5), 1.14 (3H, s, H-7) and 0.92 (6H, d, J = 6.6 Hz, H-9 and H-10); ^{13}C (50 MHz, CDCl$_3$), δ 138.0 (C-1'), 128.8 (C-2' and C-6'), 128.6 (C-3' and C-5'), 127.2 (C-4'), 70.8 (C-4), 63.7 (C-1), 59.3 (C-5), 49.5 (C-2), 37.3 (C-7'), 35.8 (C-6), 34.9 (C-8), 29.1 (C-3), 25.4 (C-7), 18.0 (C-9 or C-10) and 17.2 (C-9 or C-10); MS, m/z (% rel. int.) 294 ([M+2]$^+$, 5), 251 (40), 249 (15) and 219 (100).

9-Mercaptobenzyl-p-menthen-2-one (**4.8**). 8,9-Epoxy-*p*-menth-6-en-2-one (**2.13**, 150 mg, 0.0009 M) was treated with benzyl mercaptan (112 mg, 0.9 mM) and Et$_3$N (90 mg) for 24 h yielding compound **4.8** as thick colourles oil; yield, 155 mg (88.8 %); R_f, 0.43 (7:3 v/v hexane/EtOAc); IR, ν_{max} (CHCl$_3$) cm^{-1} 3446, 3061, 3027, 2972, 2922, 1661, 1494, 1432 and 701; ^{1}H NMR (200 MHz, CDCl$_3$), δ 7.24 (5H, m, Ph), 6.69 (1H, m, H-6), 3.71 (2H, s, H-7'), 2.66 - 2.04 (2H, m, H-9), 2.66 – 1.58 (5H, m, H-3, H-4, H-5), 1.72 (3H, s, H-7) and 1.13 (3H, s, H-10); ^{13}C (50 MHz, CDCl$_3$), δ 199.8 and 199.5 (C-2), 145.1 and 144.4 (C-6), 137.9 (C-1'), 135.3 and 135.1 (C-1), 128.8 (C-2' and C-6'), 128.4 (C-3' and C-5'), 127.2 (C-4'), 72.8 and 72.7 (C-8), 43.8 and 43.7 (C-9), 42.2 and 42.1 (C-4), 39.5 (C-7'), 38.8 and 38.0 (C-3), 27.3 and 26.5 (C-10), 23.8 and 23.7 (C-5) and 15.5 (C-7); MS, m/z (% rel. int.) 291 ([M]$^+$, 20), 199 (5), 181 (100), 110 (98) and 109 (25).

CHAPTER FIVE

SYNTHESIS OF MONOTERPENE GLYCALS AND THEIR APOPTOTIC ACTIVITIES

Abstract

Six chlorinated 2,3-unsaturated *C*-glycals of dihydrocarvone, carvone, carveol and limonene were synthesized *via* a TiCl$_4$ catalyzed condensation reaction and their structures established based on analysis of spectroscopic data. The results indicated regioselectivity by targeting the terminally disposed olefinic groups for all the precursors, furnishing 2,3-unsaturated chloro-*C*-gycosides in varying yields, from excellent to poor. The apoptotic induction potential of the glycals against CHO and Jurkat T cell lines was evaluated and showed varying activity levels, with the highly hydroxylated derivatives exhibiting lower activity in comparison with their acetylated precursors.

5.1   Introduction

Carbohydrates formed in plants through the process of photosynthesis comprise most of the biomass present on earth. They exist in the form of free sugars or monosaccharides, e.g. D-glucose and D-fructose; disaccharides, e.g. sucrose (domestic sugar), lactose (the principle carbohydrate in mammalian milk); and polysaccharides, e.g. starch which is one of the skeletal structures of plant, bacteria and animal cells, and the hard shells of insects and crustecea.[334]

Carbohydrates also referred to as saccharides, were for a long time under-appreciated in the sciences because they were assumed to possess only structural support and energy-storing functions. However, over the last three decades, carbohydrate-containing compounds have been established to possess many interesting and useful biological activities. For example, carbohydrate units are found in many antibiotics and anticancer agents, such as the macrolide antibiotics, the anthracyclins and the enediyne classes of compounds.[335]

Carbohydrates mediate many essential biological processes. For example, the saccharide-containing macromolecules that decorate cell surfaces are vital to a variety of cellular functions, including cell–cell recognition, apoptosis, and differentiation.[336,337] In a similar fashion, glycosylated natural products contain sugar attachments essential for their activity and (continue to) serves as reliable platforms for the development of many of the existing front-line drugs. The diverse chemical space accessible by carbohydrates contributes to a remarkably vast array of biological functions.[338,339]

Carbohydrates are major constituents of numerous other biological systems. Consequently, they play a vital role there in. Any malfunction in their metabolism can lead to several diseases and medical disorders. Cancer, a frequently fatal disease, is often a result of defects in sugar processing enzymes leading to uncontrolled proliferation of tumour cells. The transformation of normal cells to cancerous cells is also catalysed, or processed, by normal sugar-processing glycosidase enzymes. The uncontrolled proliferation of tumor cells can be caused by an elevated level of

glycosidases at the sites of tumors, resulting in aberrant glycosylation, which leads to accumulation of precursors or *neo*-structures. The overall effects cause dramatic changes in the cell-surface carbohydrates and progress to malignancy.[340-343]

It is noteworthy that many clinically important medicines are derived from glycosylated natural products. This is found throughout the anticancer and antiinfective arenas with representative clinical examples, including polyketides e.g. doxorubicin (**5.1**), indolocarbazoles e.g. staurosporine (**5.2**), polyenes e.g. nystatin (**5.3**), coumarins, novobiocin (**5.4**), and cardiac glycosides e.g. digitoxin (**5.5**).[344-346]

5.1

5.2

5.3

5.4
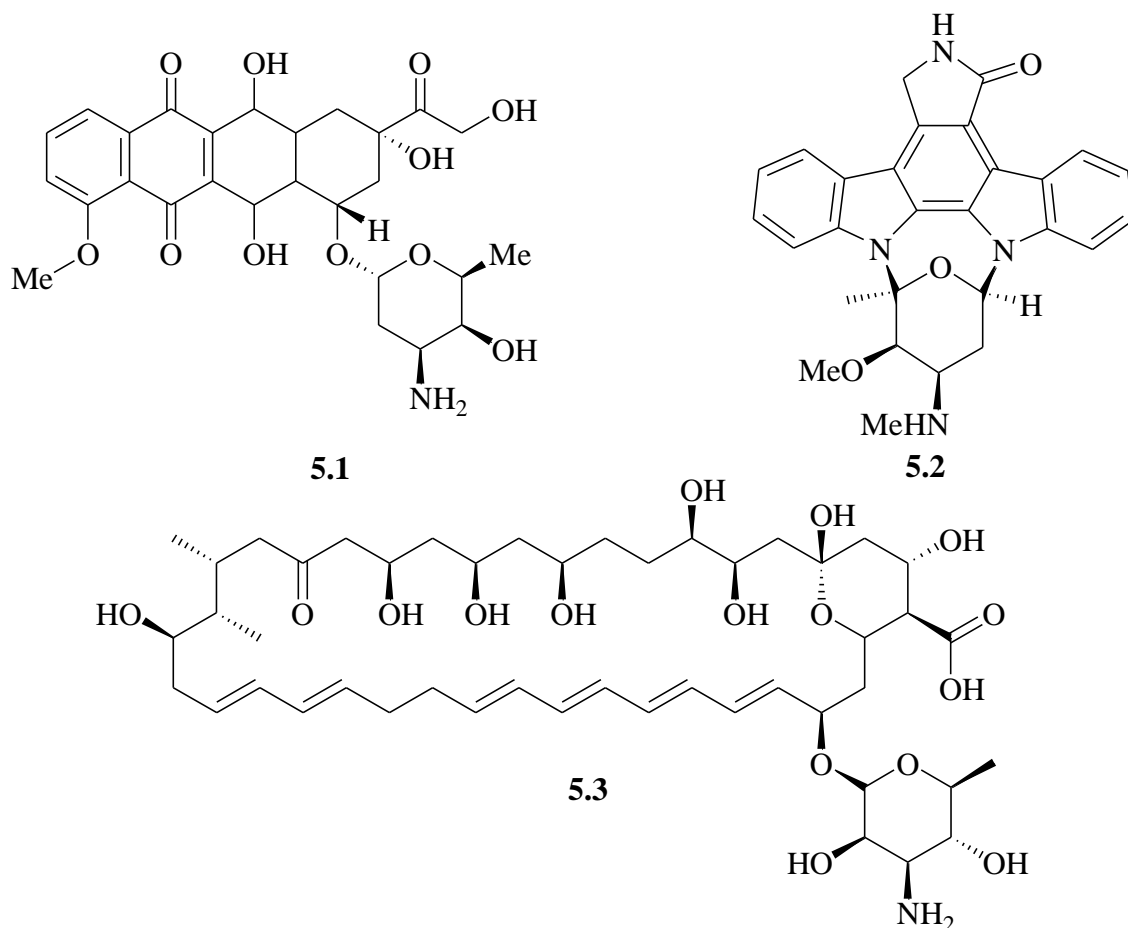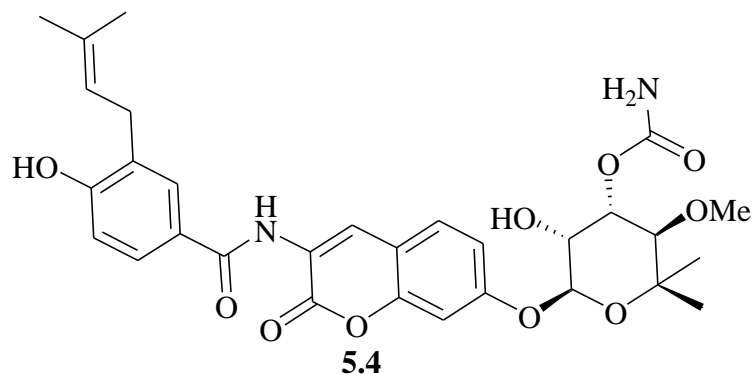
Since the inhibition of malignant cells by cardiac glycosides *in vitro* was reported in the 1960s other anticancer effects of cardiac glycosides have been observed. For instance, the therapeutic effect of cardiac glycosides in breast cancer has been known from 1979.[347,348] These glycosides have traditionally been used to increase cardiac contractile forces in patients with congestive heart failure and cardiac arrhythmias and are found as secondary metabolites in a diverse group of plants including *Digitalis lanata* and *D. purpurea* (i.e. foxglove), e.g. digitoxin (**5.5**) and digoxin (**5.6**), *Scilla maritime* (i.e. proscillaridin A, **5.7**) and *Nerium oleander* (oleandrin, **5.8**). They are also found in frogs, such as *Bufa rubescens* and *B. marinas* (marinobufagenin, **5.9**).

5.5: R = H
5.6: R = OH

5.7

5.8

5.9
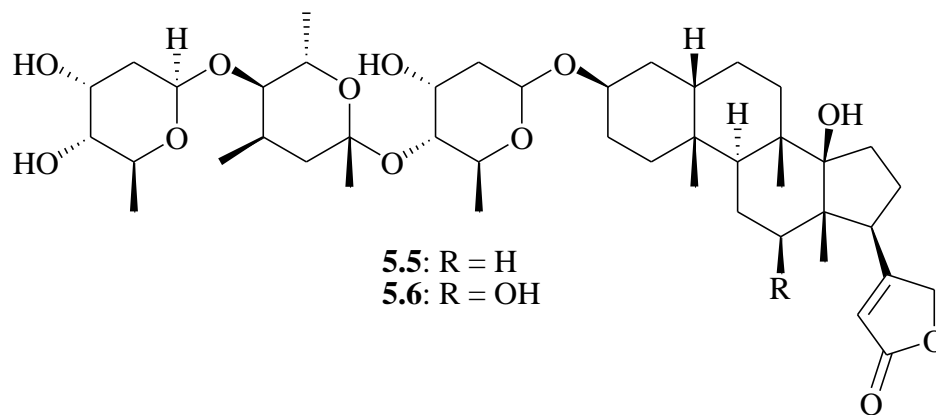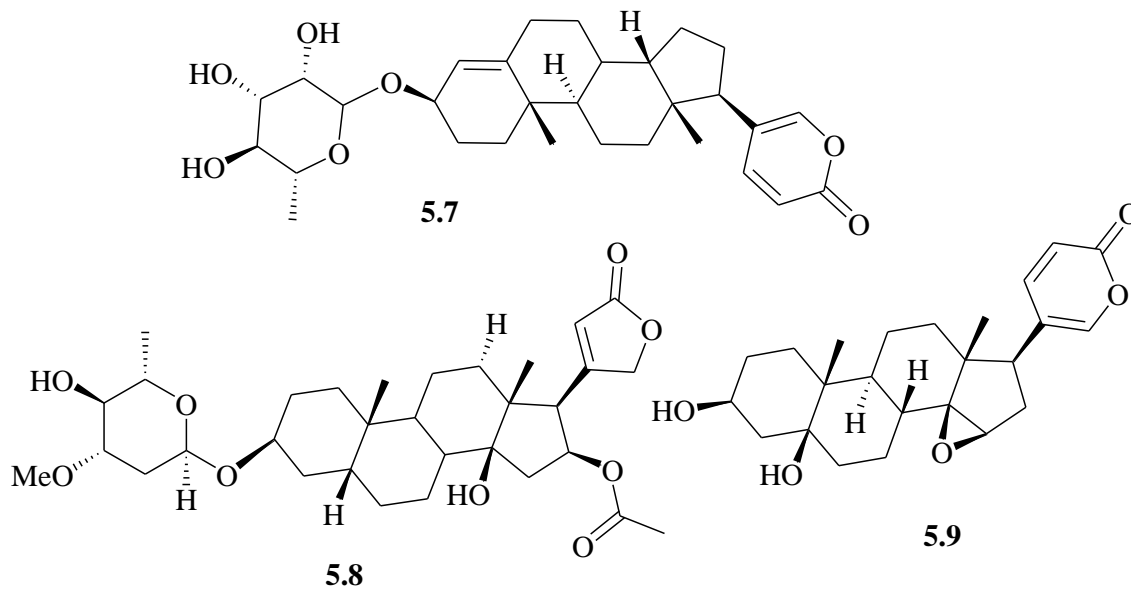
In addition to its well known cardiac activity, digitoxin (**5.5**) has been established to demonstrate *in vitro* anticancer properties.[348-352] Patient profiling suggests the survival rate of cancer patients taking digitoxin to be statistically enhanced.[353] Cardiac glycosides have also been noted to inhibit the expression of genes that are over expressed in prostate cancer cells, in addition to inhibiting activation of the signaling pathway in cystic fibrosis (CF) cells.[349] They also suppress hypersecretion of the protein implicated in lung inflammation, from CF lung epithelial cells.[354] Digitoxin and other cardiac glycosides in non-toxic concentrations have also been shown to induce apoptosis in different malignant cell lines *in vitro*.[349,355]

Due to the metabolic instability in biological systems of naturally occurring carbohydrates, e.g. *O*-glycosidically-linked oligosaccharides, much effort has been directed towards the development of feasible pathways for carbohydrate mimetics,

including *C*-glycosidic sugars,[356-358] which may compete with their *O*-glycosidic counterparts in cell surface adhesion, inhibit carbohydrate processing enzymes, and interfere in the biosynthesis of specific cell surface carbohydrates.[359-361] These carbohydrate mimetics are potential therapeutic agents against cancer, diabetes, HIV and other metabolic diseases.[362]

Increasing attention is currently addressing the synthesis of abiological carbohydrate derivatives (artificial sugars) that contain a carbon–carbon bond at the anomeric center.[356,357] This substitution endows the mimic with the ability to withstand enzymatic hydrolysis and thus serve as a stable substitute for the *O*-glycoside.[363] These isosteres of natural carbohydrates are considered to be tools of great value for the study at molecular level of the role that carbohydrate moieties of glycoproteins and glycolipids play in biological processes.[364] A related field of research includes the identification of lead compounds for drug discovery and the development of carbohydrate-based therapeutics against numerous diseases of high social relevance.[365-366]

From the preceding review, it is evident that the abundance of carbohydrates in nature is not without any purpose. These compounds are increasingly being recognized for their biological importance, more so for their pharmaceutical potential. Since a good number of monocyclic monoterpenes of menthane type have already been reported to show anti-cancer activity, and it has also been established in this study that variously functionalized *p*-menthanes may trigger induction of apoptosis, it was therefore a logical progression to incorporate sugar substructures into core skeletons of the studied

monoterpenoid compounds and, in particular *C*-glycosides *via* glycal addition. Thus, this study focused on incorporating a glycal moiety onto some known anticancer monoterpenes with the view of enhancing their apoptotic potential.

Glycal is a generic name that was adopted for all sugars with a double bond between carbon atoms 1 and 2.[367] These compounds are versatile synthetic intermediates, owing to the variety of transformations associated with their heterocyclic enol ether functionality, and have found ample use in the preparation of *C*-glycosides, carbohydrate mimics, and natural products.[368] Thus, the Ferrier [369] reaction between triacetyl-D-glucal (**5.10**) with respective *p*-menthane monoterpenes was pursued while employing the Lewis acid TiCL$_4$ and chlorinated 2,3-unsaturated *C*-glycals were obtained. The Ferrier reaction involves nucleophilic substitution combined with an allylic shift in a glycal.[369-371]

5.10
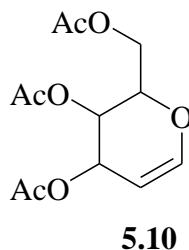
5.2   Results and Discussion

Derivatization of the selected monoterpene precursors, namely dihydrocarvone (**2.1**), carvone (**1.85**), *p*-mentha-1,8-diene-2-ol (**1.84**) and *p*-mentha-1,8-diene (**1.83**) were treated according to **Scheme 5.2** and yielded the corresponding C-glycosides **5.11**, **5.12**,

5.14 and **5.15** in yields ranging from excellent to very poor as discussed in the following section.

The method, which is a one pot, three component reaction utilized TiCl$_4$ as the catalyst in CH$_2$Cl$_2$ at 0°C as it had earlier been investigated by Herscovici *et al.*[372] and found to be effective on different forms of alkenes. The approach provides direct access to *C*-glycosides by avoiding protection and deprotection processes which require multi-step reactions. The reactions went to completion in less than 15 min generally leading to the formation of the unsaturated chloro-*C*-glycosides as shown in the representative reaction in **Scheme 5.1**.

Initial investigations were directed towards the reaction of **5.10** with a terminal olefinic double bond of the *p*-menthane skeleton. Thus, treatment of dihydrocarvone (**2.1**) with 1.5 molar equivalent of triacetyl-D-glucal (**5.10**) in dichloromethane in the presence of TiCl$_4$ led to exclusive alkylation at C-8 of the monoterpenes, affording the chloro-*C*-glycoside di-(4,6-*C*-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol-8-chloro-*p*-menthan-2-one, (**5.11**) in 90.7 % yield after flash chromatography. The structure **5.11** of the synthesized compound was characterized upon analysis of spectroscopic data, which showed that the product existed as a mixture of stereoisomers that were presumed to be α and β anomers of compound **5.11**.
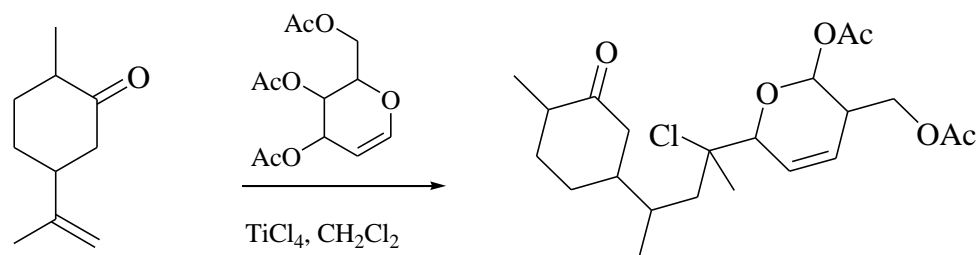
2.1
(Also representing the other precursors)

5.11, 90.7 %
(Also representing the other products)

Scheme 5.1   Synthesis   of   di-(4,6-*C*-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol-8-chloro-*p*-menthan-2-one (5.10)

Successful alkylation of the terminally predisposed olefinic group in dihydrocarvone prompted further exploration focusing on polyfunctional olefin precursors **1.83**, **1.84**, and **1.85** following the method depicted by the representative reaction in **Scheme 5.1** to determine the generality of this methodology. The reactions with these precursors under the catalytic influence of TiCl$_4$ provided the corresponding chloro-*C*-glycosides, 9-[di-(4,6-*C*-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol]-8-chloro-*p*-menthen-2-one (**5.12**, 62 %), 9-[di-(4,6-*C*-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol]-8-chloro-*p*-menthen-2-ol (**5.13**, 7 %) and 9-[di-(4,6-*C*-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol]-8-chloro-*p*-menthe-1-ne (**5.14**, 32 %) after work-up and purification by silica gel column chromatography. The structures of these compounds were confirmed upon analysis of spectroscopic data.

5.12

5.13

5.14
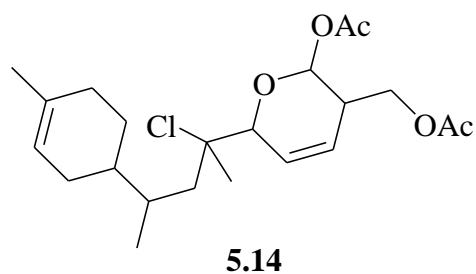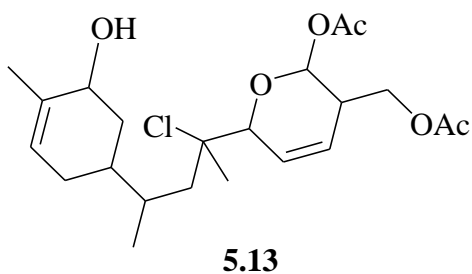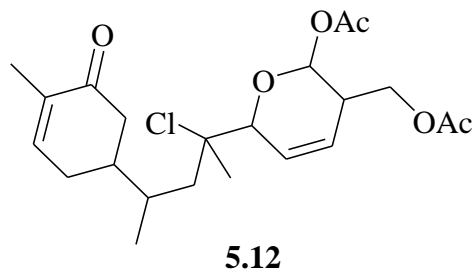
Table 5.1   **Summary of results from the syntheses of 2,3 unsaturated *C*-glycosides 5.11-5.14**

Substrate	Product	Yield (%)
2.1	**5.11**	90.7
1.85	**5.12**	62.3
1.84	**5.13**	7
1.83	**5.14**	32

A plausible reaction mechanism for the formation of the chloro-*C*-glycosides is one in which there is an initial chelation of TiCl$_4$ with the heterocyclic oxygen in **5.10** followed by an attack on the isopropenyl olefinic double bond by one of the Cl atoms from TiCl$_4$. This is then followed by an allylic shift in which one of the acetyl groups is displaced. (**Scheme 5.2**).
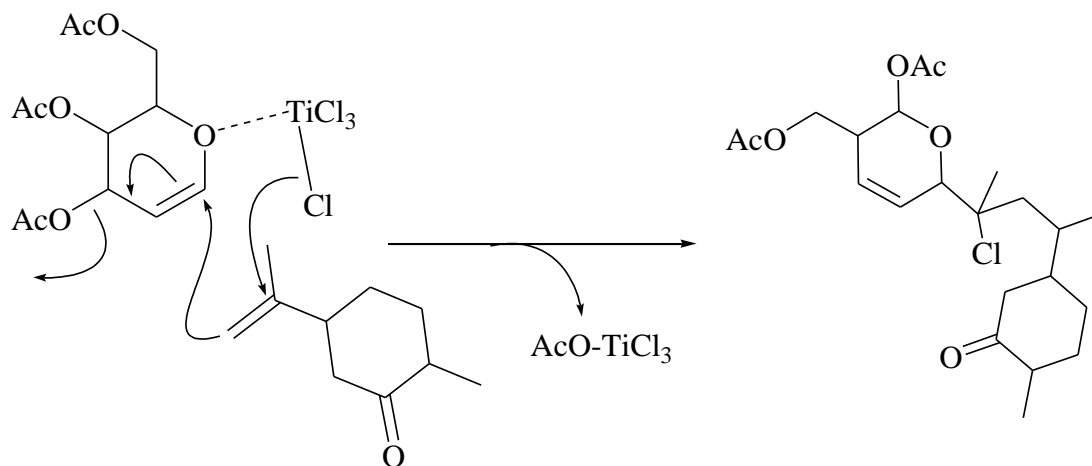
Scheme 5.2   Possible reaction mechanism for the formation of the chloro-*C*-glycals

The reaction with the other three polyfunctional *p*-menthanes investigated in this study provided lower yields than expected and this was attributed to a number of factors. For carvone (**1.85**) a yield of 62.3 % was obtained (**Table 5.1**) for the chloro-*C*-glycoside **5.12**.   The comparatively lower yield could be attributed to the possibility of the formation of an intermediate that is initially generated from the reaction of the carbonyl group with TiCl$_4$ forming a titanium enolate as a result of TiCl$_4$-mediated α,β-unsaturated addition (**Scheme 5.3**).   Thus, this process would bring in a competing reaction that resulted to the decreased yield.
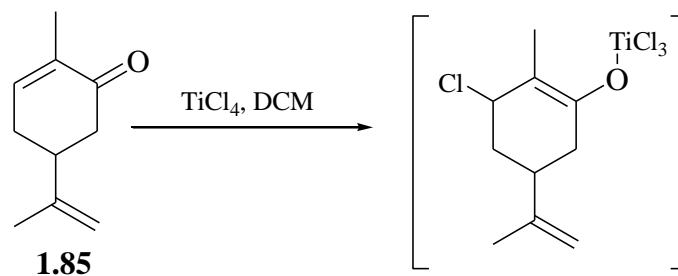
Scheme 5.3   Possible transition state in the reaction of 1.85 with TiCl₄

As for carveol (**1.84**), the presence of the hydroxyl susbstituent might have been the cause for the poor yield (7 %) of the *C*-glycoside **5.13** obtained. This could have been due to the competing sites of chelation for TiCl₄, whereby the heterocyclic glycal oxygen and the carveol hydroxyl groups could be competing for chelation with TiCl₄ catalyst.

The reaction of **5.10** with **1.83** (which has no heterogenous substituent but comprising two olefinic groups) recorded a higher yield of the *C*-glycoside (**5.15**) than that obtained from **1.84**. This could imply that since there were no competing chelation sites for the catalyst, then the main factor to the diminished reactivity could have been purely steric and competition between the two olefinic groups that would have led to a complex reaction mixture as visualized on TLC.

Since most naturally occurring carbohydrates occur in nature as hydroxylated compounds it was considered worthwhile to deacetylate the isomeric mixture **5.11** to obtain a diol so as to determine the apoptosis induction of the latter. Thus, treatment of the acetylated dihydrocarvone glycoside with NaOMe in methanol under nitrogen at

room temperature afforded a stereoisomeric mixture of α and β *C*-glycoside 9-[2,3-dideoxy-α,β-D-hex-2-enitol-8-chloro-*p*-menthan-2-one (**5.15**) in a yield of 86 % after column chromatography (**Scheme 5.4**), whose structure was established upon analysis of spectroscopic data.
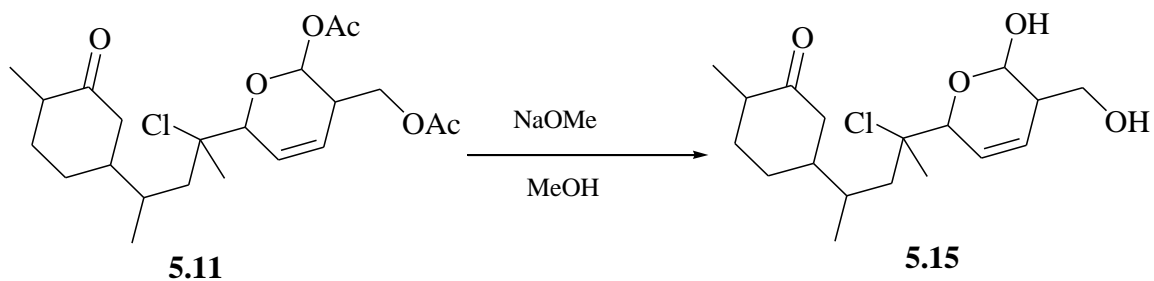
Scheme 5.4   **Synthesis of 9-(2,3-dideoxy-α,β-D-hex-2-enitol)-8-chloro-*p*-menthan-2-one (5.15)**

The acetylated carvone derivative **5.12** on treatment with NaOMe afforded the deacetylated product 9-(2,3-dideoxy-α,β-D-hex-2-enitol)-8-chloro-*p*-menthen-2-one (**5.16**) as white crystals in 85 % yield after recrystalization from ethanol and its structure was also established on the basis of its spectroscopic data. Again the ^{1}H NMR indicated that **5.16** was a stereoisomeric mixture consisting of α and β anomers of the *pseudo*-sugar moiety.

5.16
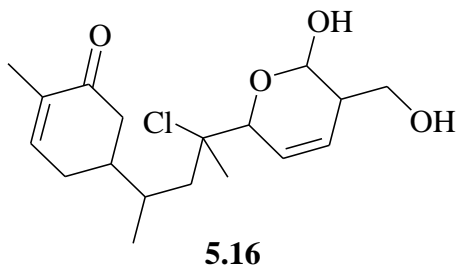
5.3 Apoptosis Induction Assay Results

The isomeric mixture **5.11** which was inseparable was tested against the two cell lines for its apoptotic induction potential at a concentration of 1 μM and was found to exhibit higher activity against Jurkat T cells (93.61 % cell death) in comparison to CHO cells (52.25 % cell death, **Fig. 5.1**).
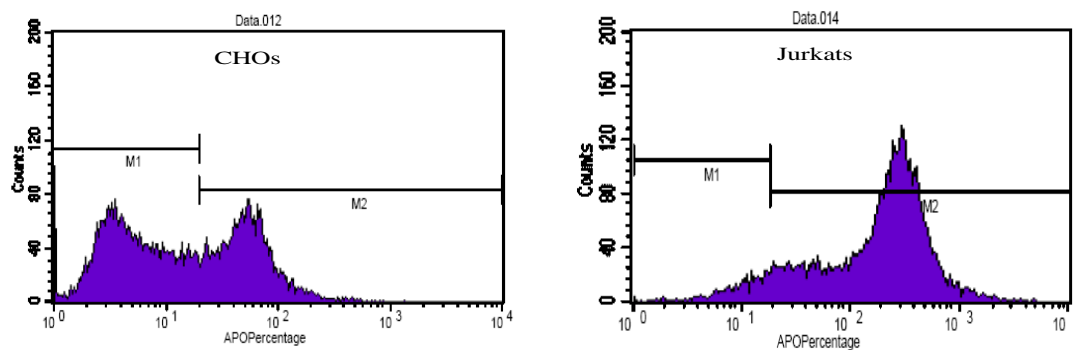
Fig. 5.1   **Flow cytometry histograms showing apoptotic activity for compound 5.11 against CHO and Jurkat T cells**

When tested against CHO and Jukart T cells compound **5.12** exhibited apoptotic activity of 65.70 % and 91.74 % as depicted in **Fig. 5.2**.
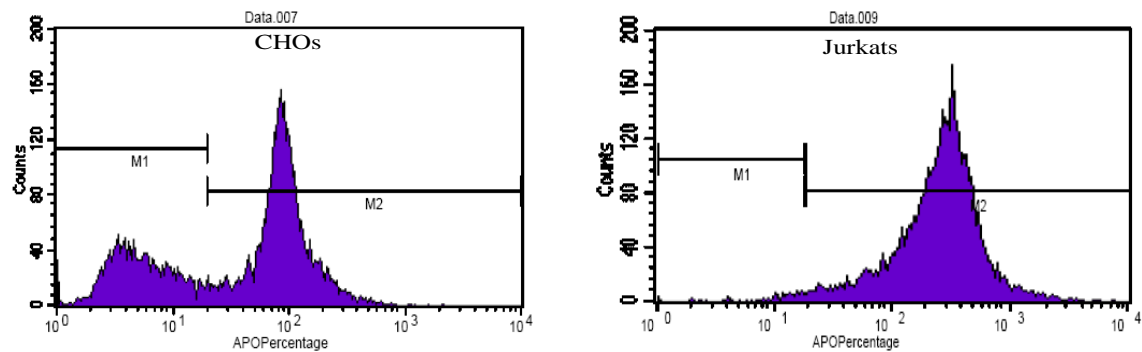
Fig. 5.2   **Flow cytometry histograms showing apoptotic activity for compound 5.12 against CHO and Jurkat T cells**

The flow cytometric results indicated higher activity of compound **5.13** against the CHO than on the Jurkat T cells, with 62.46 and 29.18 % of non-viable cells respectively (**Fig. 5.3**), however, indicating a high level of selectivity of the activity.
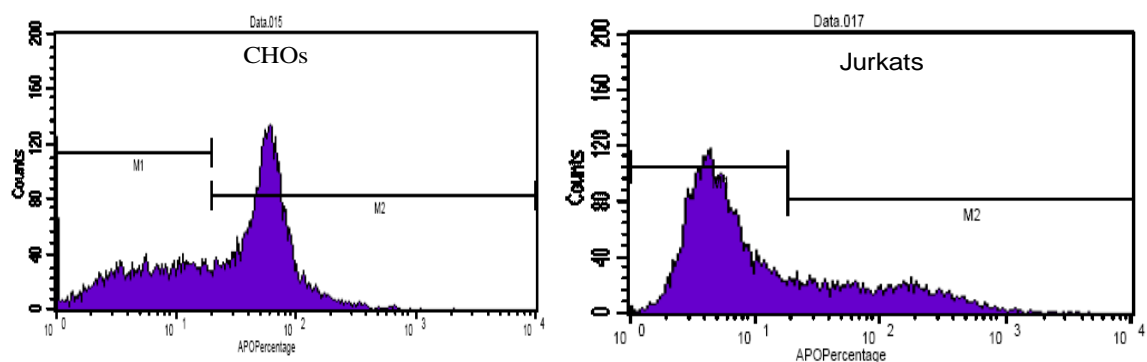
Fig. 5.3   **Flow cytometry histograms showing apoptotic activity for compound 5.13 against CHO and Jurkat T cells**

Compound **5.14** exhibited a good level of apoptotic induction for both cell lines tested against. Thus 85.5 and 89.1 % cell death was recorded against CHO and Jurkat T cells respectively as shown by their flow cytometry histograms (**Fig. 5.4**).
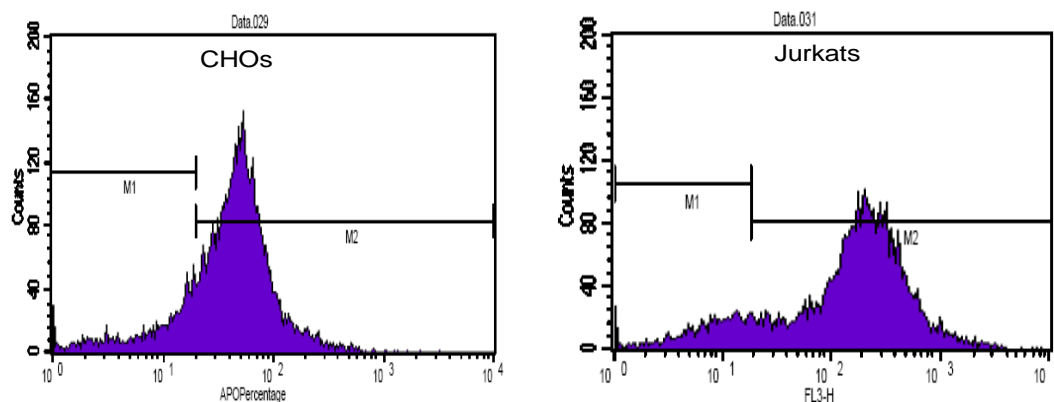
Fig. 5.4   **Flow cytometry histograms showing apoptotic activity for compound 5.14 against CHO and Jurkat T cells**

Against CHO and Jurkat T cells compound **5.15** exhibited apoptotic activity by inducing 42.84 and 35.92 % respectively of cell deaths, which was a lower activity as compared to that of its acetylated precursor (**Fig. 5.5**). This may be attributed to the high hydrophilicity and reduced lipophilicity, which may have eventually reduced its availability for absorption into the cells as compared to it precursor, compound **5.11**.
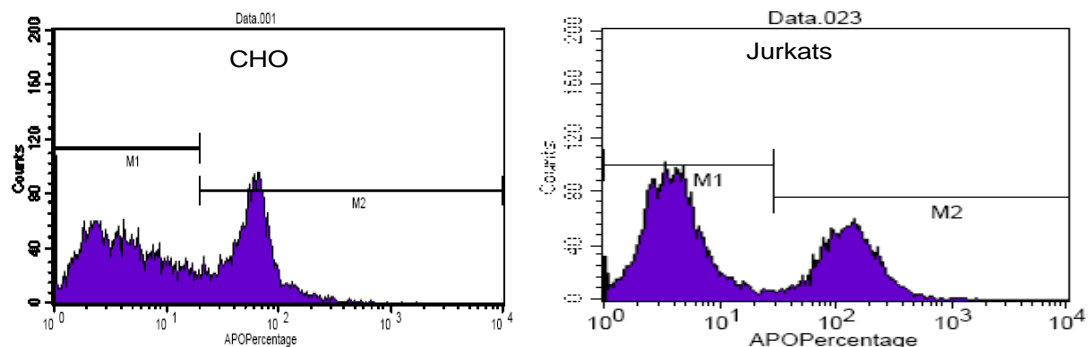
Fig. 5.5 **Flow cytometry histograms showing apoptotic activity for compound 5.15 against CHO and Jurkat T cells**

This deacetylated compound **5.16** showed slightly differing apoptotic activity against the two cell lines (60.78 and 69.13 % cell death against CHO and Jurkat T cells respectively, **Fig. 5.6**), which as for **5.15** was a lower activity than the acetylated compounds (**5.11** and **5.12** respectively) from which they were derived. This can be explained by the same reason as for **5.15** discussed above.
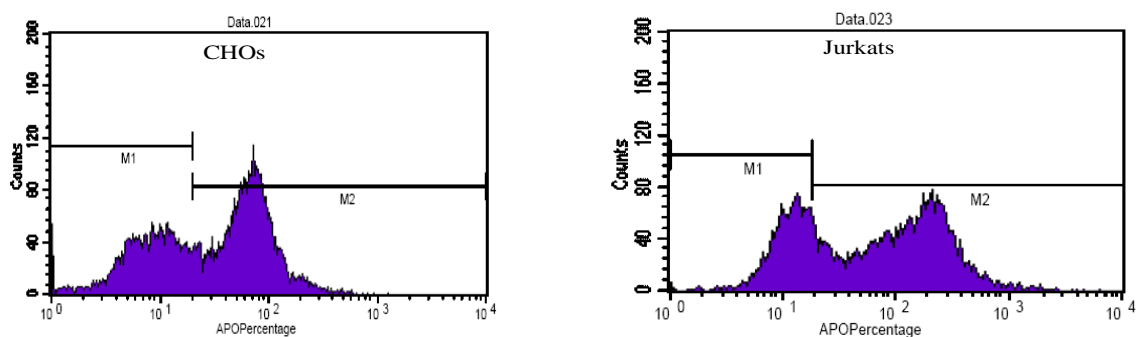
Fig. 5.6 **Flow cytometry histograms showing apoptotic activity for compound 5.13 against CHO and Jurkat T cells**

5.4 Structure Activity Relationship Analysis of the Synthesized Benzyl Mercaptan 4.1-4.8

The results discussed in this chapter related to the reactions of *p*-menthanoids with **5.10** in the presence of TiCl$_4$ yielding chlorinated *C*-glycosides whose apoptosis induction activity was then determined. It was noted that in all the reactions, a high degree of regioselectivity was observed, whereby the terminal olefin group was preferentially alkylated. The configuration of the anomeric centre for the compounds described were not established neither were their ratios determined. Deacetylation of the two derivatives proceeded smoothly, affording the hydroxylated compounds which were intended for further manipulation, such us Sharpless-dihydroxylation reactions to obtain glycosidic principles or further still for epoxidation at the glycal olefinic group for more synthetic transformations to afford more substrates for biological tests.

The potential of the reported compounds as apoptosis inducers were preliminarily tested *in vitro* against CHO and Jurkat T cell lines at a single concentration of 1 μM. The results as summarized in **Table 5.2** and **Graph 5.1** indicate that the compounds exhibited weak to very strong apoptotic effect. Generally, the compounds elicited higher apoptotic activity against Jurkat T cells as compared to the CHO cells. The most potent compound against the Jurkat T cells was **5.12**, which recorded an activity of 93.61 % while showing just above 50 % against the CHO cells, indicating the best selectivity amongst all compounds tested.

Table 5.2   Apoptotic activity of the synthesized *C*-glycosides

Compounds	% Non-viable cells	
	CHO	**Jurkat T**
5.11	52.2±5.15	93.6±1.45
5.12	70.7±3.70	95.7±1.82
5.13	62.8±2.56	37.1±4.01
5.14	85.5±3.04	89.1±1.77
5.15	40.6±1.56	49.1±10.1
5.16	56.1±3.31	69.6±1.48
Camptothecin	55. 5±7.03	81.9±4.92
-Ve control	33.6±5.03	21.7±0.62
DMSO	37.7±2.65	37.3±11.46

Five compounds except **5.15** exhibited higher apoptotic activity against the CHO cells as compared to the anticancer drug Camptothecin that induced 55.45% death of CHO cells. However, only four compounds exhibited higher activity against Jurkat T cells than Camptothecin. Camptothecin on the other hand elicited selective activity by being more potent against Jurkat T than on CHO cells. A good level of selectivity was also recorded for two synthesized *C*-glycosides namely, **5.11** and **5.12** as shown in **Graph 5.1**. These observations generally indicate potential of these compounds as apoptosis inducers.
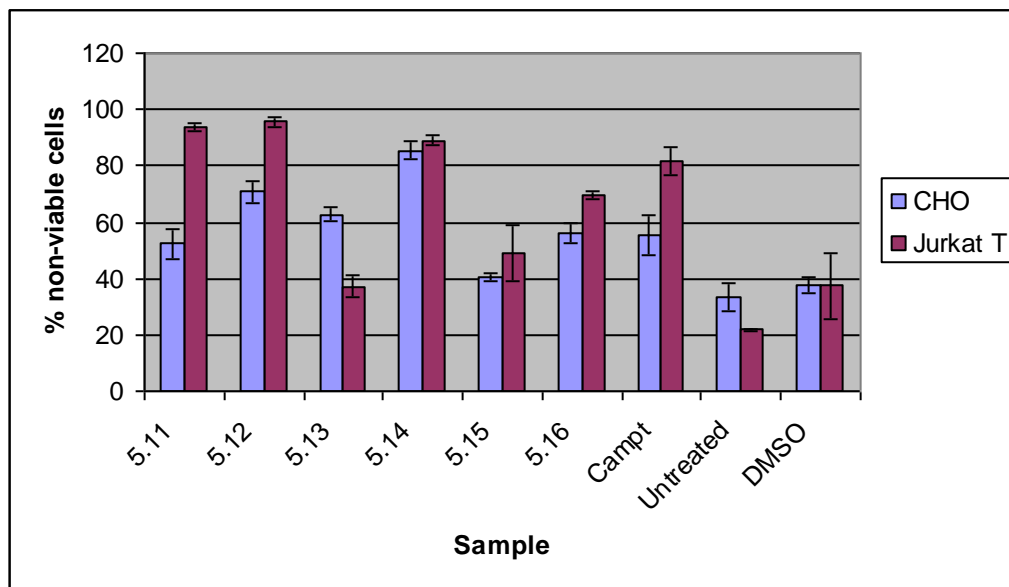
Graph 5.1 **Comparative apoptotic activity of the synthesized *C*-glycosides 5.11-5.16**

An interesting observation was noted, that the deacetylated derivatives **5.15** and **5.16** exhibited lower apoptotic activity as compared to their precursors **5.11** and **5.12**, thus implying the acetyl groups to be structural units that provide significant apoptotic potency. This could be attributed to the typical characteristic of acetyl groups in increasing the lipophilicity of drugs (compounds), thus improving their absorption. Once in the cells the acetyl groups can also be easily hydrolyzed to give a free hydroxyl groups necessary for hydrogen bonding which may be responsible for bioavalability.[373] The same reason may account for the high activity exhibited by the other acetylated derivatives other than compound **5.13**, which had a hydroxyl group in the *p*-menthane skeleton in comparison to Camptothecin.

The acetyl group has indeed been found to be essential for the anticancer activities of some known compounds. For instance, Colin *et al.*,[374] reported improved antiproliferative activities of several resverotrol derivatives containing acetyl groups. In addition, previous studies by Fragopoulou *et al.*,[375] demonstrated that acetylation of standard sphingolipids resulted in the formation of compounds with significantly enhanced biological activity.

From the results, it may also be inferred that the carbonyl group plays a major role towards increasing the apoptotic potential as indicated by compounds **5.11** and **5.12**, which exhibited the highest activities against Jurkat T cells amongst the synthesized compounds. The high activity exhibited by the limonene derivative **5.14** especially against CHO cells may imply that the cyclohexenyl skeleton played a protagonistic role towards inducing apoptosis.

5.5   Conclusion

The apoptosis inducing potential exhibited by the compounds (**5.11-5.16**) investigated in this chapter indicated that most of them posses some level of activity against the cell lines tested, with some of the acetylated *C*-glycosides showing higher activity than that of Camptothecin, which was used as the standard drug. The TiCl$_4$ catalyzed condensation reaction was found to be a convenient regioselective approach to unsaturated *C*-glycosides since most of the products were obtained *via* attack on the less hindered terminal olefin group for all the precursors used. However, a very low yield

was obtained for compound **1.83** which was attributed to the presence of a hydroxyl group as discussed in the relevant section.

5.6   Experimental Procedures

5.6.1   General Experimental Procedures

These were the same as described in Chapter Two.

5.6.2   General Procedure for the Condensation of *p*-Menthanes with Triacetyl-D-glucal (5.10)

A round-bottomed flask equipped with a magnetic stirrer and sealed with a rubber septum was filled successively with triacetyl-D-glucal (**5.10**) dissolved in CH_2Cl_2 and the corresponding *p*-menthane monoterpenes, and the reaction cooled to 0 $^{\circ}$C. $TiCl_4$ was added *via* a metallic syringe and the reaction was monitored by TLC. When the starting material had disappeared the reaction mixture was poured into a mixture of diethyl ether and saturated aqueous Na_2HPO_4 (1:1 v/v). The aqueous layer was extracted twice with diethyl ether (20 ml each) then dried ($MgSO_4$). The solvent was removed under reduced pressure followed by flash chromatography of the residue on silica gel.

Di-(4,6-C-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol-8-chloro-p-menthan-2-one   (**5.11**). This was obtained as a yellow oil by treating dihydrocarvone (**2.1**, 3.9 g, 27 mM) with triacetyl-D-glucal (8.7 g, 40 mM) in the presence of $TiCl_4$ (7.5 g, 40 mM) for 3 min; yield, 0.98 g (90.7 %); ^{1}H NMR (600 MHz, CDCl$_3$), δ 5.82 (2H, m, H-2' and H-3'), 5.11 (1H, m, H-4'), 4.61 (1H, m, H-1'), 4.48 (1H, m, H-1'), 4.21 (1H, m, H-6'), 4.12 (1H, m,

H-6'), 3.87 (1H, m, H-5'), 2.60 (1H, dddd, $J_{H1, H7}$ = 3.6, 3.6, $J_{H1, H6}$ = 2.4, 2.4, Hz, H-1), 2.34-2.13 (1H, m, H-4), 2.08 (3H, bs, MeCO), 2.06 (3H, s, MeCO), 1.93-1.97 (4H, m, H-5 and H-6), 1.66 (3H, s, H-10), 1.61 (2H, d, J = 11.4 Hz, H-9) and 1.03 (3H, d, J = 6.0 Hz, H-7); ^{13}C NMR (150 MHz, CDCl$_3$), δ 211.9 (C-2), 170.7 (MeCO), 170.3 (MeCO), 75.8 (C-8), 69.8 and 69.4 (C-1'), 69.1 and 69.0 (C-4'), 64.6 (C-5'), 62.6 (C-6'), 51.0 (C-9), 47.9 (C-1), 44.9 and 44.7 (C-4), 43.5 (C-3), 33.7 (C-10), 28.3 (C-6), 26.8 (C-5) and 14.2 (C-7); MS, *m/z* (% rel. int.) 401 ([M]$^+$, 75), 365 (30), 341 (98), 307 (3), 305 (25) and 213 (5).

9-[Di-(4,6-C-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol]-8-chloro-p-menthen-2-one

(5.12). Carvone (**1.85**, 1.44 g, 9.6 mM) upon stirring for 3 min with triacetyl-D-glucal (5.22 g, 19.2 mM) and TiCl$_4$ (3.62 g, 19.2 mM) afforded compound **5.12** as a yellow syrup; yield, 2.38 g (62.3 %); R$_f$, 0.58 (hexane:EtOAc 2:3 v/v); IR, ν$_{max}$ (CHCl$_3$) cm^{-1} 3460, 2936, 2334, 1743 and 1232; ^{1}H NMR (600 MHz, CDCl$_3$), δ 6.75 (1H, d, J = 3.8 Hz, H-6), 5.79 (2H, m, H-2' and H-3'), 5.09 (1H, t, $J_{H4', H3' \& H6'}$ = 6.6 Hz, H-4'), 4.63 (1H, d, J = 8.4 Hz, H-1'), 4.46 (1H, d, J = 8.6 Hz, H-1'), 4.17 (2H, m, H-6'), 3.89 (1H, m, H-5'), 2.67-1.94 (5H, m, H-3, H-4 and H-5), 2.08 (2MeCO, s), 2.04 (s, 3H, H-7), 1.78 (3H, bs, H-10) and 1.66 (2H, d, J = 3.8 Hz, H-9); ^{13}C NMR (150 MHz, CDCl$_3$), δ 199.0 (C-2), 170.6 (MeCO) 170.3 (MeCO), 144.4 and 144.2 (C-6), 135.3 and 135.2 (C-1), 132.8 and 132.6 (C-2'), 124.3 and 123.8 (C-3'), 75.1 and 74.6 (C-8), 69.9 and 69.4 (C-1'), 68.9 (C-5'), 64.9 and 64.6 (C-4'), 62.6 (C-6'), 46.8 (C-4), 44.6 and 43.9 (C-9), 39.9 (C-3), 28.4 (C-10), 27.7 (C-5), 21.7 and 21.0 (MeCO) and 15.5 (C-7); MS, *m/z* (% rel. int.) 315 ([M+1], 30), 279 (100), 243 (90) and 135 (30).

9-[Di-(4,6-C-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol]-8-chloro-p-menthen-2-ol (**5.13**).

p-Mentha-1,8-diene-2-ol (**1.84**, 1.42 g, 9 mM), treated with triacetyl-D-glucal (**5.10**, 5.22 gm, 19.2 mM) and TiCl$_4$ (3.56 gm, 19 mM) for 10 min afforded compound **5.13** as a white syrup; yield, 251 mg, (7 %); Rf, 0.46 (hexane:EtOAc 3:2 v/v); ^{1}H NMR (600 MHz, CDCl$_3$), δ 5.88 (1H, m, H-3'), 5.79 (1H, m, H-2'), 5.55 (1H, m, H-6), 5.10 (1H, m, H-4'), 5.02 (1H, m, H-4'), 4.68 (1H, m, H-1'), 4.58 (1H, m, H-1'), 4.26 (1H, m, H-6'), 4.19 (1H, m, H-6'), 3.86 (1H, dd, $J_{H2, H3}$ = 4.8, 4.8 Hz, H-2), 2.39-1.67 (5H, m, H-3, H-4 and H-5), 1.80 (3H, bs, H-7), 1.64 (2H, bs, H-9) and 1.58 (3H, s, H-10); ^{13}C NMR (150 MHz, CDCl$_3$), δ 170.8 (MeCO), 170.3 (MeCO), 135.0 and 134.2 (C-1), 133.9 and 133.7 (C-2'), 124.6 and 124.2 (C-3'), 123.9 and 123.1 (C-6), 76.7 and 76.2 (C-8), 69.5 and 69.2 (C-5'), 68.7 (C-2), 68.2 (C-1'), 64.7 and 64.6 (C-4'), 62.4 and 61.9 (C-6'), 46.0 and 43.7 (C-9), 40.2 and 39.9 (C-4), 37.1 and 36.7 (C-3), 33.4 and 33.3 (C-10), 27.8 and 27.0 (C-5), 21.1 (MeCO), and 20.8 and 20.7 (C-7); MS, *m/z* (% rel. int.) 400 ([M]$^+$, 3), 383 (48), 347 (50), 287 (30) and 227 (30).

9-[Di-(4,6-C-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol]-8-chloro-p-menthe-1-ne (**5.14**).

Treatment of *p*-mentha-1,8-diene (**1.83**, 1.26 g, 9.3 mM), with triacetyl-D-glucal (**5.10**, 5.22 g, 19.2 mM) and TiCl$_4$ (3.56 g, 19 mM) for 10 min yielded **5.14** as a yellow syrup; yield, (117 mg, 32 %); R$_f$, 0.64, (hexane:EtOAc 7:3 v/v); ^{1}H NMR (600 MHz, CDCl$_3$), δ 5.65 (1H, m, H-2'), 5.58 (1H, m, H-3'), 5.53 (1H, m, H-2), 5.16 (1H, dddd, $J_{H4', H3}$ = 1.8, 1.8 Hz, $J_{H4', H5'}$ = 1.2 Hz and $J_{H4', H6'}$ = 1.8 Hz, H-4'), 4.26 (1H, m, H-6'), 4.13 (1H, m, H-6'), 3.90 (1H, m, H-1'), 3.79 (1H, dd, $J_{H5', H4'}$ = 3.0, 2.4 Hz, H-5'), 3.74 (1H, dd, $J_{H5', H4'}$ = 3.0, 3.0 Hz, H-5'), 2.43 (1H, m, H-6), 2.25 (1H, m, 6), 2.33-1.71 (5H, m, H-3, H-

4 and H-5), 2.08 (6H, s, 2MeCO), 1.72 (3H, m, H-7), 1.68 (3H, m, H-10) and 1.59 (2H,

m, H-9); ^{13}C NMR (150 MHz, CDCl$_3$), δ 170.9 (MeCO), 170.5 (MeCO), 138.6 (C-1),

125.7 (C-3'), 123.0 (C-2'), 77.5 (C-8), 69.0 (C-1'), 68.5 (C-4'), 66.3 (C-5'), 64.2 (C-6'),

45.2 (C-4), 40.2 (C-9), 35.9 (C-6), 31.4 (C-10), 24.8 (C-3) and 22.8 (C-7), 21.1 (MeCO)

and 20.9 (C-5); MS, *m/z* (% rel. int.) 412 ([412]$^+$, 5), 353 (5), 319 (8), 303 (100), 243

(100), 225 (38).

5.6.3 General procedure for the deacetylation of the acetylated C-glycosides

Into a round-bottomed three necked flask equipped with a magnetic stirrer and sealed

with a rubber septum dry MeOH (10 ml) was added followed by NaOMe and the

mixture stirred to dissolution. The acetylated C-glycosides dissolved in dry MeOH were

added *via* a syringe and the reaction mixture was stirred for 30 min at room temperature

under nitrogen.

*9-(2,3-Dideoxy-α,β-**D**-hex-2-enitol)-8-chloro-p-menthan-2-one* (**5.15**). Compound **5.11**

(0.9 g, 23 mM) treated with NaOMe (0.27 g, 5.1 mM) and stirred for 30 min under

nitrogen in dry MeOH afforded **5.15** as white crystals; yield, 0.61 g (86 %); R$_f$, 0.52

(EtOAc); m.p. 62-65 $^\circ$C; ^{1}H NMR (600 MHz, CD$_3$OD), δ 5.80 (1H, m, H-3'), 5.75 (1H,

m, H-2'), 4.57 (1H, m, H-4'), 4.01 (1H, m, H-6'), 3.75 (1H, m, H-6'), 3.68 (1H, m, H-1'),

3.37 (1H, m, H-5'), 2.59 (1H, m, H-1), 2.45 (1H, m, H-3), 2.38-1.73 (5H, m, H-4, H-5

and H-6), 2.16 (1H, m, H-3), 1.68 (3H, s, H-10), 1.65 (3H, s, H-10), 1.00 (3H, d *J* = 3.0

Hz, H-7) and 0.99 (3H, d *J* = 3.0 Hz, H-7); ^{13}C NMR (150 MHz, CD$_3$OD), δ 215.0 and

214.8 (C-2), 131.7 (C-2'), 130.4 (C-'), 77.5 (C-5'), 75.0 (C-1'), 73.2 (C-8), 71.4 (C-4'), 63.6 and 62.5 (C-6'), 52.2 (C-9), 49.6 (C-1), 45.7 (C-4), 44.3 (C-3), 35.2 (C-10), 28.3 (C-6), 28.0 (C-5) and 14.5 (C-7); MS, *m/z* (% rel. int.) 281 ([M+1], 35), 263 (85), 245 (100), 151 (10), 129 (5).

9-(2,3-Dideoxy-α,β-D-hex-2-enitol)-8-chloro-p-menthen-2-one **(5.16).** Treatment of compound **5.12** (1.85 g, 4.6 mM) with NaOMe (0.25 g, 4.6 mM) while stirring for 30 min under nitrogen in MeOH afforded **5.16** as white crystals; yield, 1.225 g (85 %); R_f, 0.48 (EtOAc); m.p. 69-71 $^{\circ}$C; IR, ν_{max} (CHCl$_3$) cm^{-1} 3255, 2923, 2490, 1644 and 721; ^{1}H NMR (600 MHz, CD$_3$OD), δ 6.88 (1H, d, *J* = 6.0 Hz, H-6), 5.81 (1H, tt, $J_{H3', H2 \& H'}$ = 1.8 and 1.8, Hz, H-3'), 5.75 (1H, dddd, $J_{H2', H1'}$ = 1.8, 2.4 Hz, $J_{H2', H3'}$ = 2.4 and 1.8 Hz, H-2'), 4.56 (1H, tt, $J_{H1', H2'}$ = 2.4, 2.4 Hz, H-1'), 3.94 (1H, dd, $J_{H4', H3'}$ = 2.4 Hz, $J_{H4', H5'}$ = 2.4 Hz, H-4'), 3.77 (1H, dd, $J_{H6', H5'}$ = 3.0, 2.4 Hz, H-6'), 3.65 (1H, m, H-6'), 3.39 (1H, dddd, $J_{H5', H4'}$ = 3.0, 2.4, 2.4, and 3.0 Hz, H-5'), 2.67 (1H, m, H-3), 2.54 (m, 1H, H-3), 2.43 (1H, m, H-4), 2.28 (2H, q, *J* = 9.0 Hz, H-5), 1.92 (3H, d, *J* = 1.8 Hz, H-7), 1.89 (3H, d, *J* = 1.8 Hz, H-7), 1.75 (2H, t, *J* = 1.2 Hz, H-9) and 1.71 (3H, s, H-10); ^{13}C NMR (150 MHz, CD$_3$OD), δ 201.6 (C-2), 147.3 (C-6), 135.9 (C-1), 131.9 (C-2'), 130.4 (C-3'), 77.3 (C-8), 75.2 (C-5'), 71.2 (C-1'), 63.6 (C-4'), 62.8 (C-6'), 47.6 (C-4), 44.2 (C-9), 40.7 (C-3), 29.0 (C-10), 27.8 (C-5) and 15.5 (C-7).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

This study was aimed at carrying out chemical modifications of menthane derivatives so as to test the resulting products for mosquito repellence and their apoptotic induction potential. The syntheses of the compounds were carried out largely through functional group interconversions and the resulting derivatives were evaluated for mosquito repellence activity and apoptotic induction using standard bioassay protocols.

The mosquito repellence bioassay was first carried out for the precursor compounds that included (*R*)-(-)-carvone, (+)-dihydrocarvone, (-)-dihydrocarveol, (*R*)-(+)-limonene, (-)-carveol (**1.84**), α-terpinene, γ-terpinene, α-terpeneol, and *trans*-*p*-menth-6-ene-2,8-diol. These compounds exhibited varying degrees of repellence activity, with all of them recording low activity in comparison to *p*-menthane-3,8-diol (**1.79**) that was used as the standard repellent. Indeed, the mosquito repellence of the standard compound **1.79** was about four times as active as compound **1.84**, which was the most active precursor. Despite their relatively good level of activity the compounds have not been very useful in combating the mosquito menace due to their transient protective nature when applied as mosquito repellents. It was for this reason that structural modifications of the monoterpenoids was pursued as part of investigations reported in this Thesis, aimed at obtaining potentially more active and longer lasting repellent compounds. Thus, through epoxidation of the precursor monoterpenoids via a bicarbonate activated peroxidation

process, halohydrin formation and via the use of *m*-CPBA, twelve *p*-menthane epoxide derivatives were obtained.

Interestingly, all the epoxides obtained in this study exhibited a higher degree of mosquito repellence as compared to their precursors. This suggested that the presence of an epoxide moiety in the *p*-menthane skeleton might have played a protagonistic role towards their effectiveness as repellents. Indeed, some of the tested epoxides showed greater repellence than the known repellent monoterpenes, *p*-menthane-3,8-diol (**1.79**). For instance, the highest level of improvement was recorded for diepoxylimonene (**2.15**) that was found to be approximately 19 times more effective than its precursor, limonene and about three times as active as compound **1.79**. Another notable improvement in mosquito repellence activity was observed for 8,9-epoxy-*p*-menthan-2-one formed from dihydrocarvone that was ninefolds as active as its precursor, carvone and twice more active as the monoterpene **1.79**. The hydroxylated derivatives obtained in this study also indicated varying levels of mosquito repellence activity, with the most active compound being *p*-menthane-2,8-diol a dihydroxylated derivative of dihydrocarveol obtained upon hydroxylation *via* oxymercuration demercuration reaction. This compound was twice as active as the monoterpene **1.79**, and more than twelve times active than its precursor.

The mosquito repellence bioassay results clearly indicated that the changes in the functional groups on the precursors, which involved the introduction of epoxide and hydroxyl groups, had a major effect towards their repellence potential, with most of the compounds synthesized showing a marked increase in activity. However, it was noted

that amongst the hydroxylated compounds, the presence of more than two hydroxyl groups led to a diminished level of repellent activity and in some cases exhibited attractive tendency, as was observed for *cis-p*-menthane-1,2,8-triol and *p*-Menthane-1,2,3,8-tetrol.

Since the study was based on a dose response method, it would be worthwhile to undertake a time response study for the most active compounds obtained in this study, in order to asses their effectiveness as a function of time. Such a study would reveal their viability as potentially applicable mosquito repellents. Carrying out formulation experiments to identify viable means of making the highly active yet very volatile compounds longer lasting upon application would be a valuable endeavor. In addition, evaluation of their toxicity would be important in order to ascertain the safety levels for humans use. It would also be interesting to evaluate their repellence potential against other insects, such as tick and fleas that are easily brought into contact with humans by domestic pets and animals.

In addition to the mosquito repellence assays, apoptosis induction potential bioassays were conducted for the precursors, epoxides and the hydroxylated compounds against Chinese Hamster Ovary (CHO) and Jurkat T cell lines and their activity compared with that of Camptothecin, a known anticancer drug that was used as the standard drug. In general, the precursor compounds exhibited higher apoptotic induction potential than Camptothecin. This did not come as a surprise since some of the compounds have been tested and found to possess anticancer activity. Indeed, limonene, which showed very

high apoptotic potential, has been subjected to clinical trials as an anticancer agent. The epoxide derivatives exhibited varying degrees of apoptosis induction potential while showing some level of selective activity against the two cell lines. Similarly, the hydroxylation products obtained in this study also exhibited apoptosis induction potential that also varied depending on the substitution pattern and the number of hydroxyl groups present. For instance the lowest activity against Jurkat T cells was observed for *cis-p*-menthane-1,2,8-triol and *p*-Menthane-1,2,3,8-tetrol.

Since *β*-amino alcohol and *β*-hydroxysufide units have been known to be responsible for anticancer activity in a number of compounds, several of the epoxide containing compounds were subjected to nucleophilic oxirane ring opening reactions using, benzyl amine, aniline, piperidine and benzyl mercaptan so as to furnish their respective amino alcohols and hydroxysulfides. To obtain the targeted compounds, the reactions were carried out in a solvent system that contained 30 % MeCN in water and catalyzed by Et$_3$N for the amino alcohols, while for the benzyl mercaptans the reactions were conducted in water alone. The yields were varying, with the lowest yields being recorded for the amino alcohols, which was mainly attributed to the low nucleophilicity of the nucleophiles especially aniline and to some extent benzylamine. Steric hindrance was another factor that might have led to the low yield. On the other hand the hydroxybenzylmercaptans were obtained in relatively good to excellent yields.

These *β*-amino alcohols when assayed against CHO and Jurkat T cell lines exhibited apoptosis induction potential at the level that varied from low to very good, with some

of the derivatives showing higher activity than the standard drug Camptothecin. Amongst the seven amino alcohols assayed, 2-piperidinyl-4,5-epoxy-*p*-menthane-1-ol exhibited the highest apoptotic activity, implying the presence of the piperidinyl group and an epoxide moiety might have had a positive apoptosis induction influence. However, the compound did not show selective activity against the two cell lines.

Eight hydroxybenzylmercaptans obtained in this study also indicated varying degrees of bioactivity as apoptosis inducing agents against CHO and Jurkat T cell lines. The most active amongst the mercaptans was 6,9-dimercaptobenzyl-*p*-menthane-1,2,8-triol. The presence of two mercaptobenzyl groups in the latter compound may have been responsible for the increased activity of the compound, thereby outweighing the negative effect that was initially observed for the highly hydroxylated compounds *cis-p*-menthane-1,2,8-triol and *p*-Menthane-1,2,3,8-tetrol.

Since many clinically important chemotherapeutics are derived from glycosylated natural products and that the presence of a sugar moiety has been known in a number of anticancer agents, it was considered worthwhile to synthesize glycal bearing compounds and to test the latter for their potential as apoptosis inducing agents against CHO and Jurkat T calls. Thus, four 2,3-unsaturated *C*-glycals were prepared *via* a known method that utilized $TiCl_4$ as the Lewis catalyst. The compounds were di-(4,6-C-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol-8-chloro-p-menthan-2-one (**5.11**), 9-[di-(4,6-C-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol]-8-chloro-p-menthen-2-one (**5.12**), 9-[di-(4,6-C-acetyl)-2,3-dideoxy-α,β-D-hex-2-enitol]-8-chloro-p-menthen-2-ol (**5.13**) and 9-[di-(4,6-C-acetyl)-2,3-dideoxy-

α,β-D-hex-2-enitol]-8-chloro-p-menthe-1-ne (**5.14**). Compounds **5.11** and **5.12** were deacetylated and, as for their precursors also subjected to apoptosis assay. The TiCl$_4$ catalyzed condensation reaction was found to be regioselective since the unsaturated *C*-glycoside were obtained *via* attack on the less hindered terminal olefin group for all the precursor compounds used.

In general high apoptotic induction potential was observed for all the acetylated compounds **5.11**-**5.14**, with the highest activity being exhibited by compound **5.12** against Jurkat T cells and **5.14** against CHO cells. Also noted for these compounds was a relatively good level of selective activity against the two cell line. Interestingly, the two deacetylated compounds **5.15** and **5.16** exhibited the least activity amongst this group of compounds, which was attributed to the reduced lipophilicity in comparison to their acetylated precursors. However, the trend of activity was in agreement with the previous observations, where the ester carbonyl group was found to enhance bioactivity as compared to compounds without such groups.

Another general observation that was made from this study in relation to the activity of the compounds studied against the two cell lines was the presence of an α,β-unsaturated carbonyl system and epoxide groups. Most of the compounds that had such groups exhibited relatively high apoptosis induction potential. As explained in the relevant sections in this Thesis the two functional groups have indeed been established in other studies to increase the bioactivity level in compounds in which they are present.

The findings and observations accrued from this study prompt for a need to carry out further studies that will augment scientifically the leads that have been established. Thus, the following are being recommended.

To gauge their spectrum of apoptotic induction potential and their selectivity profile, it would be valuable to test the compounds obtained in this study against other cell lines. It would also be important to identify the mode of apoptotic activity of the tested compounds, which may be achieved by conducting different assays that target specific pathways, such as Caspase activity bioassay etc.

In respect to repellent properties, since most of the compounds evaluated in this study had more than one functional group it would be of great value to ascertain whether the individual chemical moieties were functioning alone to impart the observed activity, or the observed activity was the result of synergy. Thus, synthesis of *p*-menthane derivatives having only one functional group would be of significance to this end.

Furthermore, since most of the compounds obtained in this study were stereoisomeric mixtures that interestingly gave a good lead to pursue. For further research it would be important to pursue stereoselective reactions that would afford pure stereoisomers that would indicate which amongst the possible stereoisomers is more potent and which compound is less toxic.

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