A PHYTOCHEMICAL INVESTIGATION OF MEMBERS OF THE HYACINTHACEAE FAMILY AND BIOLOGICAL SCREENING OF HOMOISOFLAVANONES AND STRUCTURALLY RELATED COMPOUNDS

BY

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Soli Deo Gloria

PREFACE

The phytochemical investigation described in this thesis was carried out in the School of Pure and Applied Chemistry, University of KwaZulu-Natal, Durban and in the Chemistry Department, University of KwaZulu-Natal, Pietermaritzburg. The biological assays were carried out in the Research Centre for Plant Growth and Development, University of KwaZulu-Natal, Pietermaritzburg and the quantitative structure-activity relationship studies were performed in the Pharmaceutical Chemistry Department, Potchefstroom Campus, North-West University, South Africa. This study was supervised by Professor D.A. Mulholland and Professor S.E. Drewes.

The study represents original work by the author and has not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

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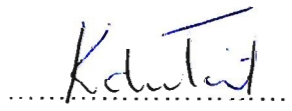

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I hereby certify that the above statement is correct.

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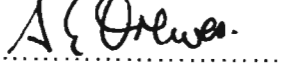
Professor D.A. Mulholland
(Supervisor)



Professor S.E. Drewes
(Co-supervisor)

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List of Abbreviations

AIA - anti-inflammatory activity

ACD - Advanced Chemistry Development

AM1 - Austin Method 1

AMA - antimicrobial activity

AMP - adenosine monophosphate

ax - axial

BC - bacteriostatic concentration

br d - broad doublet

br s - broad singlet

CE - Coulombic interaction energy

CoA - coenzyme A

COSY - correlated nuclear magnetic resonance spectroscopy

COX - cyclooxygenase

D - density

d - doublet

dd - doublet of doublets

ddd - double doublets

DEPT – distortionless enhancement by polarization transfer

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

DP - dipole moment

EDTA – ethylenediaminetetra-acetate

EF - heat of formation

eq - equatorial

ES - aqueous phase energy

FDA - United States Food and Drug Administration

FSA - Flora of southern Africa

HMBC - heteronuclear multiple bond coherence

HMQC - heteronuclear multiple quantum coherence

HSQC - heteronuclear single quantum coherence

Hz -hertz

IC_{50} - concentration needed to inhibit 50% of the enzyme

INT - *p*-iodonitrotetrazolium violet

IR - infra red

J - coupling constant

m - multiplet

m/z - mass-to-charge ratio

MH Broth - Mueller-Hinton Broth

mM - millimole

MM^+ - molecular mechanics force field

MMFF94 - Merck Molecular Force Field

MR - molar refractivity

NIE - total nonbonded interaction energy

NMR - nuclear magnetic resonance

NOESY - nuclear Overhauser enhancement spectroscopy

NSAID - nonsteroidal anti-inflammatory drugs

ORTEP - Oak Ridge Thermal Ellipsoid Program

P - parachor

POL - polarizability

ppm - parts per million

q - quartet

QSAR - quantitative structure-activity relationships

RDA - *retro*-Diels-Alder

RI - refractive index

s - singlet

SA - surface area

SE - strain energy

ST - surface tension

SV - volume of a space-filling model

t - triplet

TE - vibrational entropy

TLC - thin layer chromatography

UV - ultra-violet

VE - Van der Waals interaction energy

VEL - vibrational enthalpy

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5.2. An example of the microplate method. Bacterial growth can be seen as dark pink wells. Wells in which inhibition of bacterial growth occurred are a yellowish colour and a light pink colour indicates bacteriostatic activity. Compound numbers are indicated at the bottom of the plate as well as the controls, neomycin (N), ethanol (E), DMSO (D), Bacteria (B1) and Broth (B2)

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Abstract

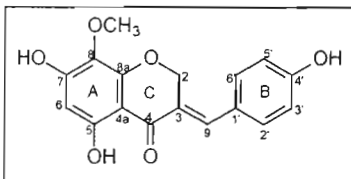
The Hyacinthaceae family is richly represented in southern Africa. Of the five subfamilies, three are found in southern Africa. These are the Urgineoideae (URG), Ornithogaloideae (ORN) and the Hyacinthoideae (HYA). The overview of Pfosser and Speta (1999), revealed chemotaxonomic trends at a subfamily level for the Hyacinthaceae family of the Flora of southern Africa region. Homoisoflavanones were found to define the Hyacinthoideae subfamily whilst the Ornithogaloideae subfamily and the Urgineoideae subfamily are defined by steroidal compounds namely, cholestane glycosides and bufadienolide glycosides respectively.

Representatives of all three subfamilies were investigated phytochemically. From *Eucomis comosa* (HYA), five homoisoflavanones were isolated. *Ornithogalum tenuifolium* (ORN) contained a spirostanol saponin of which the crystals were amenable to X-ray analysis. Evidence of a novel stereoisomer was obtained. Extraction of the bulbs of *Galtonia princeps* (ORN) led to the isolation of two cholestane glycosides, one known and one novel, and a homoisoflavanone. Two novel bufadienolides were isolated from *Urginea lydenburgensis* (URG). Structures were elucidated on the basis of spectroscopic data and chemical evidences.

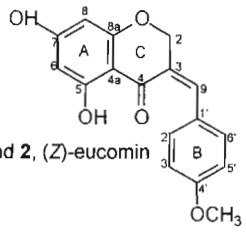
Homoisoflavanones and related compounds were then screened for antibacterial and anti-inflammatory activity. Several compounds showed antibacterial activity against *Staphylococcus aureus*, a gram-positive bacteria. Inhibition of the inflammatory process in microsomal cells was first evaluated, followed by screening of specific inhibition of cyclooxygenase enzymes. These are membrane-associated enzymes occurring in different isoforms. High levels of anti-inflammatory activity were detected especially in microsomal cells. This biological information made it possible to rationalize the ethnomedicinal use of some of the plants from which the compounds were isolated.

Biological screening was followed by a computer-based quantitative structure-activity relationship (QSAR) study. This study produced five equations with significant prediction value of anti-inflammatory and antibacterial activity for homoisoflavanones and related compounds. The derived models also provided valuable parameter guidelines of those properties influencing the anti-inflammatory and antimicrobial activity of the studied compounds.

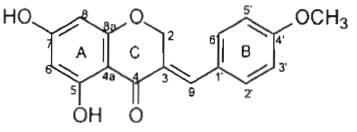
Structures of compounds isolated from the Hyacinthaceae



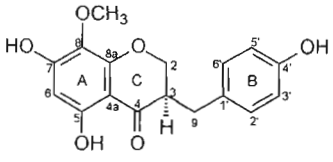
compound **1**, punctatin



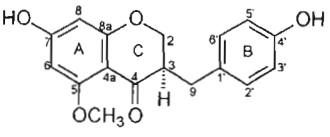
compound **2**, (*Z*)-eucomin



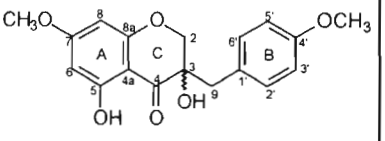
compound **3**, (*E*)-eucomin



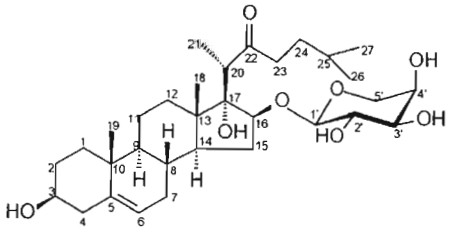
compound **4**, 3,9-dihydropunctatin.



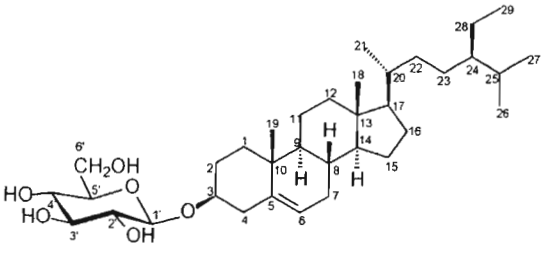
compound **5**, 5-methoxy-7-hydroxy-3-(4'-hydroxybenzyl)-4-chromanone



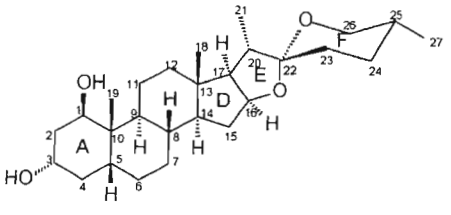
compound **6**, 7-*O*-methyleucomol



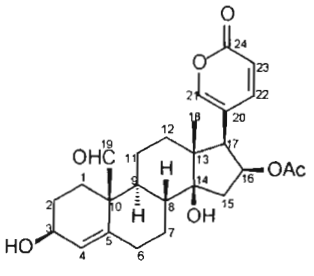
Compound **7**, 3β,17α-dihydroxycholest-5-en-22-one 16β-*O*-α-*L*-arabinopyranoside



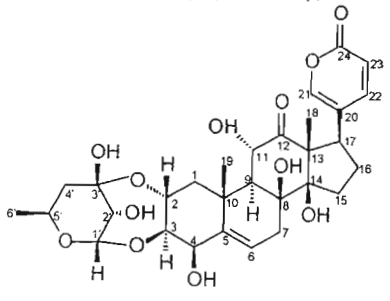
compound **9**, sitosterol 3-*O*-β-*D*-glucopyranoside



compound **10**, 25*R*,5β-spirostane-1β,3α-diol.



compound **11**, 16β-acetoxyberscillogenin.

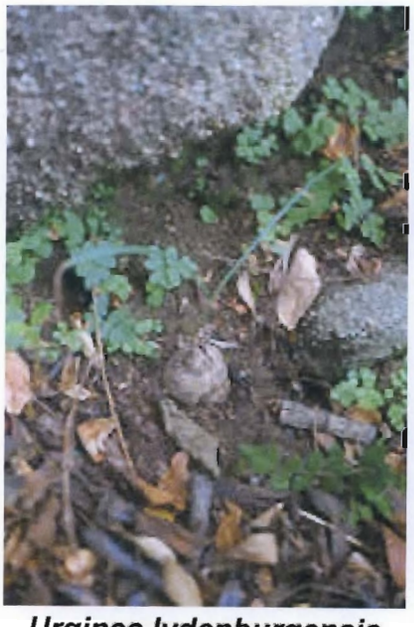


compound **12**, lydenburgenin

Photographs of plants from the Hyacinthaceae family

Galtonia princeps
(photographed by Dr N. Crouch)

Ornithogalum tenuifolium
(photographed by Dr N. Crouch)



Urginea lydenburgensis
(photographed by Dr N. Crouch)

Eucomis comosa
(photographed by Dr N. Crouch)

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CHAPTER 1

INTRODUCTION

1.1. BACKGROUND

The Lilliaceae family *sensu lato*, was one of the largest families of flowering plants wordwide. It formed a heterogeneous group mainly consisting of herbs with a variety of underground storage organs like corms, bulbs, tubers and tuberous roots [1]. Dalgren and Clifford [2] proposed a restructuring of the Lilliaceae family in order to form more homogenous families arranged according to relationships found in comparative studies.

One of the families formed was the Hyacinthaceae family. It consists of geophytes with bulbs (sometimes covered with a membranous tunic) as underground parts and basal leaves. Inflorescence is a simple raceme and perianth segments are free or united at the base. The fruit is a capsule [1].

The Hyacinthaceae family consists of approximately sixty-seven genera and nine hundred species across the world [3]. The two main areas in which they are most abundant, are:

- the area south of the Kunene, Okavango and Limpopo rivers including Namibia, Botswana, Swaziland, South Africa and Lesotho (Flora of Southern Africa (*FSA* area) [4],
- the area stretching from the Mediterranean to South-West Asia [5].

Hyacinthaceae representatives are also found from North-West Europe through to central and eastern Asia and in the Americas [3].

1.2. SOUTHERN AFRICAN HYACINTHACEAE

1.2.1. Introduction

The southern African Hyacinthaceae are highly endemic. This family seems to have originated in southern Africa according to DNA analyses [6]. Of the five subfamilies of the Hyacinthaceae, three occur in southern Africa. These are the Urgineoideae, Ornithogaloideae and the Hyacinthoideae [3].

Members of this family are most usually found in open, sunny habitats [3]. They are best adapted to a fluctuating moist-arid climate. During arid periods they wither down to bulbs [5]. In drought years these plants draw on their own reserves and are sometimes the only green sprouts in the veld [7]. Many genera provide widely cultivated, mainly spring-flowering, ornamentals [5].

1.2.2. Toxicology

Interest in the chemistry of the South African Hyacinthaceae was stimulated by reports of stock poisoning during the 1900's, when South Africa's economic prosperity depended mostly on agriculture and gold [8]. Under normal circumstances farm animals and game do not eat large amounts, if any, of poisonous plants. However, certain factors like drought or veld fires cause shortages of grazing, and animals then eat poisonous plants. Sometimes hungry animals, being transported to new areas, also eat those plants they have not yet learnt to avoid [9].

A variety of toxicity tests were conducted by Steyn [9] at Onderstepoort Veterinary Research Institute in Pretoria. Steyn defined poisonous plants as plants which, when consumed in such quantities as will be taken by animal or man over short or prolonged periods, exert harmful effects on the system, or cause death by virtue of toxic substance(s) normally contained in that plant. Fresh bulbs and leaves of hyacinthaceous plants were given to rabbits and

repeated drenching experiments on sheep were performed. Steyn [10] also found that toxicity in plants varied with soil and cultivation, climatic conditions, nature and intensity of light, season, stage of development and parts of plant used e.g. leaves, and state of plants e.g. fresh or dry.

'Secondary metabolites' in plants are often defence mechanisms to deter plant-eaters or to kill them. Most of these secondary metabolites are bioactive and their toxicity depends on concentration. In the 15th century, Paracelsus declared that "only dose distinguishes a poison from a remedy" [11].

1.2.3. Ethnobotany

Hyacinthaceous plants have been employed in Xhosa and Zulu remedies ("imithi") for many years. The remedies are used for purposes ranging from the treatment of rheumatic fever, hangovers and syphilis to bewitchment of neighbours and for good fortune [12]. Indigenous uses of plants indicate their possible biological activities. A specific therapeutic area can thus be targeted by the ethnobotanical approach. However, the existing knowledge of medicinal uses of plants can be lost because of climate change, urban expansion, destruction of habitats of plant species and the breakdown of traditional society structures through which this knowledge was transmitted [11]. The high demand for popular ethnomedicinal or "*muthi*" plants may cause them to be exploited to extinction [8].

1.3. A REVIEW OF THE PHYTOCHEMISTRY AND BIOLOGICAL ACTIVITY OF COMPOUNDS ISOLATED FROM THE HYACINTHACEAE

Chemotaxonomic trends are evident at a sub-family level for the southern African Hyacinthaceae family. The Hyacinthoideae subfamily is defined by homoisoflavanones whilst the Ornithogaloideae subfamily and the Urgineoideae subfamily are defined by steroidal compounds, namely cholestane glycosides and bufadienolide glycosides respectively [12].

1.3.1. Homoisoflavanones

Homoisoflavanones belong to a small homogeneous group of naturally occurring oxygen heterocycles. The first homoisoflavanones to be isolated were eucomin and eucomol [13]. Since then a large number of these compounds have been isolated from several genera within the Hyacinthaceae family including *Eucomis*, *Merwilla*, *Ledebouria*, *Veltheimia* and *Drimiopsis* [12]. Isolation of homoisoflavanones from the extra-*FSA* Hyacinthaceae, *Chionodoxa luciliae* [14] and *Muscari comosum* [15] has also been reported. The homoisoflavanones consist of a sixteen carbon skeleton which includes a chromanone, chromone or chromane system with a benzyl or benzylidene group at position 3 [16]. Three basic structural types of homoisoflavanones can be identified, namely, 3-benzyl-4-chromanone, 3-benzyl-3-hydroxy-4-chromanone and 3-benzylidene-4-chromanone types.

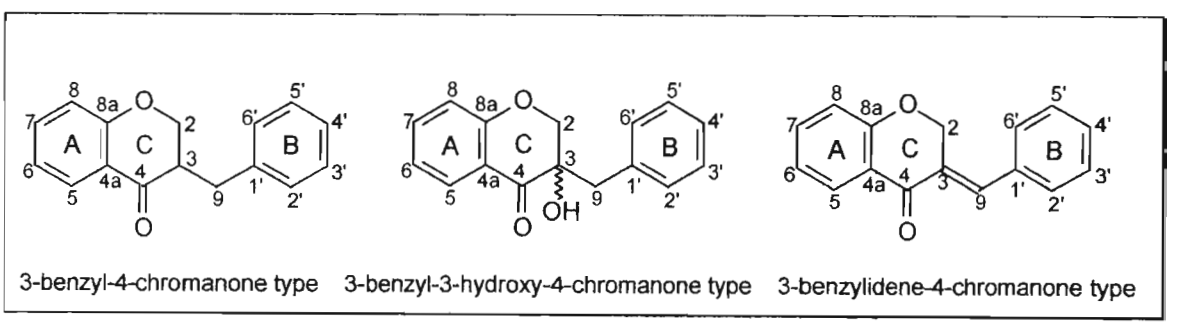
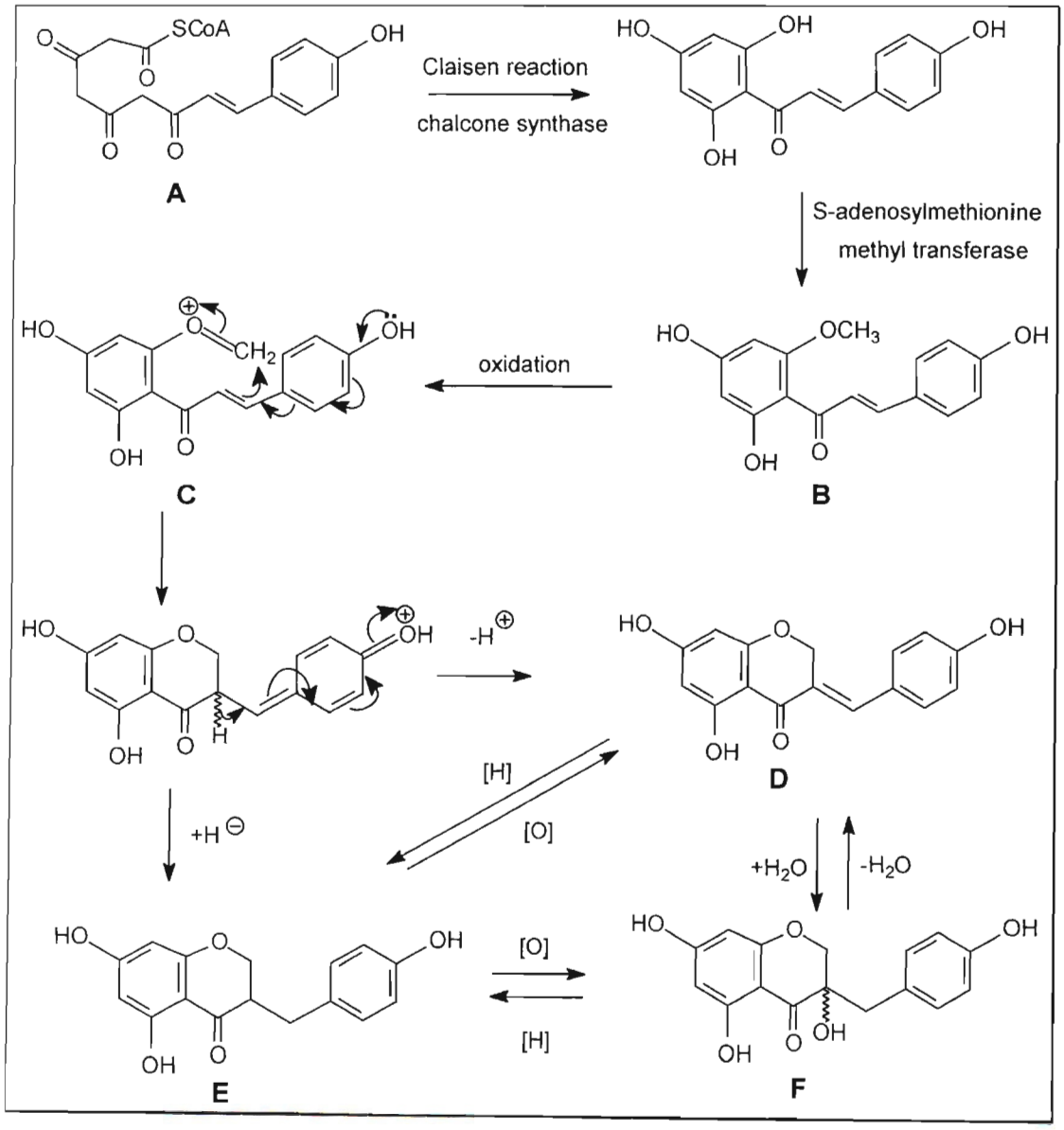


Figure 1.1. The three basic structural types of homoisoflavanones

The 3-benzylidene-4-chromanones can undergo a variety of chemical transformations of which two will be mentioned here. Firstly, interconversion between (*Z*)- and (*E*)-isomers can take place [17]. Secondly, isomerization through migration of the exocyclic double bond into the pyrone ring causes the formation of 3-benzylchromones. The second transformation requires drastic conditions and is irreversible [17].

Dewick [18,19] proposed a biosynthetic pathway for homoisoflavanones. The chalcone formation phase [19], starts from a 4-hydroxycinnamoyl-CoA unit. Three molecules of malonyl-CoA are used for chain extension forming a polyketide (**A**). This is followed by a Claisen-like reaction and cyclizations occur. The chalcone synthase enzyme is responsible for the formation of chalcones (**B**). Dewick [18], found that L-methionine is responsible for the methylation of the hydroxyl group at C-2 of the chalcones to form 2'-methoxychalcones which are the biosynthetic precursors of homoisoflavanones.



Scheme 1.1. Modified proposed biosynthesis of homoisoflavanones [18, 19].

Oxidation of the 2'-methoxychalcone (**B**) forms an intermediate (**C**) which, with cyclization, would produce either a 3-benzilidene-4-chromanone (**D**) or a 3-benzyl-4-chromanone (**E**) by loss of a proton or addition of a hydride ion respectively. 3-Hydroxy-derivatives (**F**) may be produced by hydration of (**D**) or hydroxylation of (**E**) [18].

Identification of the different types of homoisoflavanones can be made by the defining resonance of C-3 in the ^{13}C-NMR spectra. This resonance will appear as a oxygenated, aliphatic, quaternary resonance for the 3-benzyl-3-hydroxy-4-chromanone type and as a quaternary carbon resonance in the alkene region for the 3-benzylidene-4-chromanone type [20]. The carbonyl resonance for the 3-benzylidene-4-chromanone type is typically observed at approximately δ182 due to the conjugative effect of the 3,9-double bond, whilst the carbonyl resonances for the 3-benzyl-4-chromanone and 3-benzyl-3-hydroxy-4-chromanone types are usually observed between δ193 and δ195 [17]. The oxygenation pattern for ring A can be confirmed by shifts in the main UV bands after addition of anhydrous sodium acetate and anhydrous aluminium chloride. These bathochromic shifts are characteristic of hydroxyl groups on carbons 7 and 5 respectively [21].

In addition to the three basic structural types of homoisoflavanones, the unusual scillascillin type has been isolated [15]. These homoisoflavanones possess a unique 3-*spiro*-cyclobutane system.

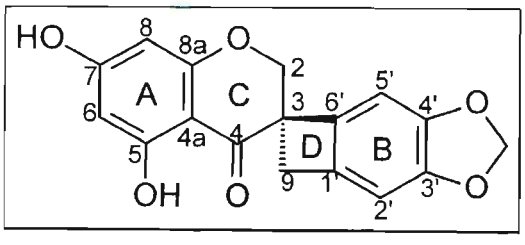


Figure 1.2. The structure of scillascillin [15]

Few reports on the biological activity of homoisoflavanones have been found. However, according to previous studies, homoisoflavanones have anti-inflammatory, antibacterial, antihistaminic, antimutagenic and angioprotective properties as well as their being potent phosphodiesterase inhibitors [20, 22, 23].

1.3.2. Steroidal compounds

Cholesterol has the fundamental steroidal structure. Modification of this structure gives rise to a variety of biologically active compounds like steroidal saponins, cardioactive glycosides, bile acids and mammalian sex hormones. Their biological activities range from anti-inflammatory activity and regulation of mineral metabolism, to anabolic activity.

Steroid nomenclature is based on the parent hydrocarbons like estrane, androstane, cholestane and stigmastane [19].

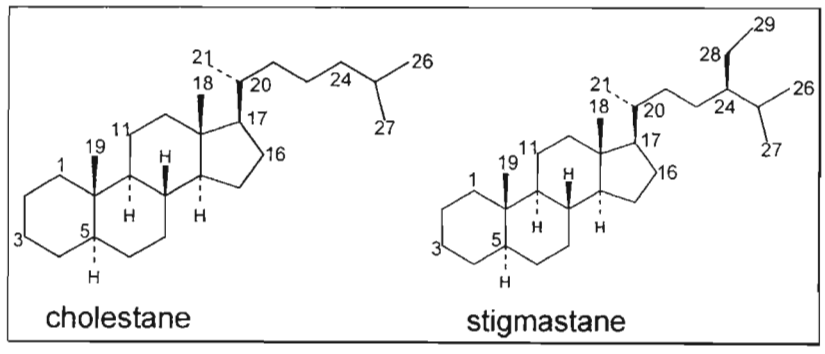
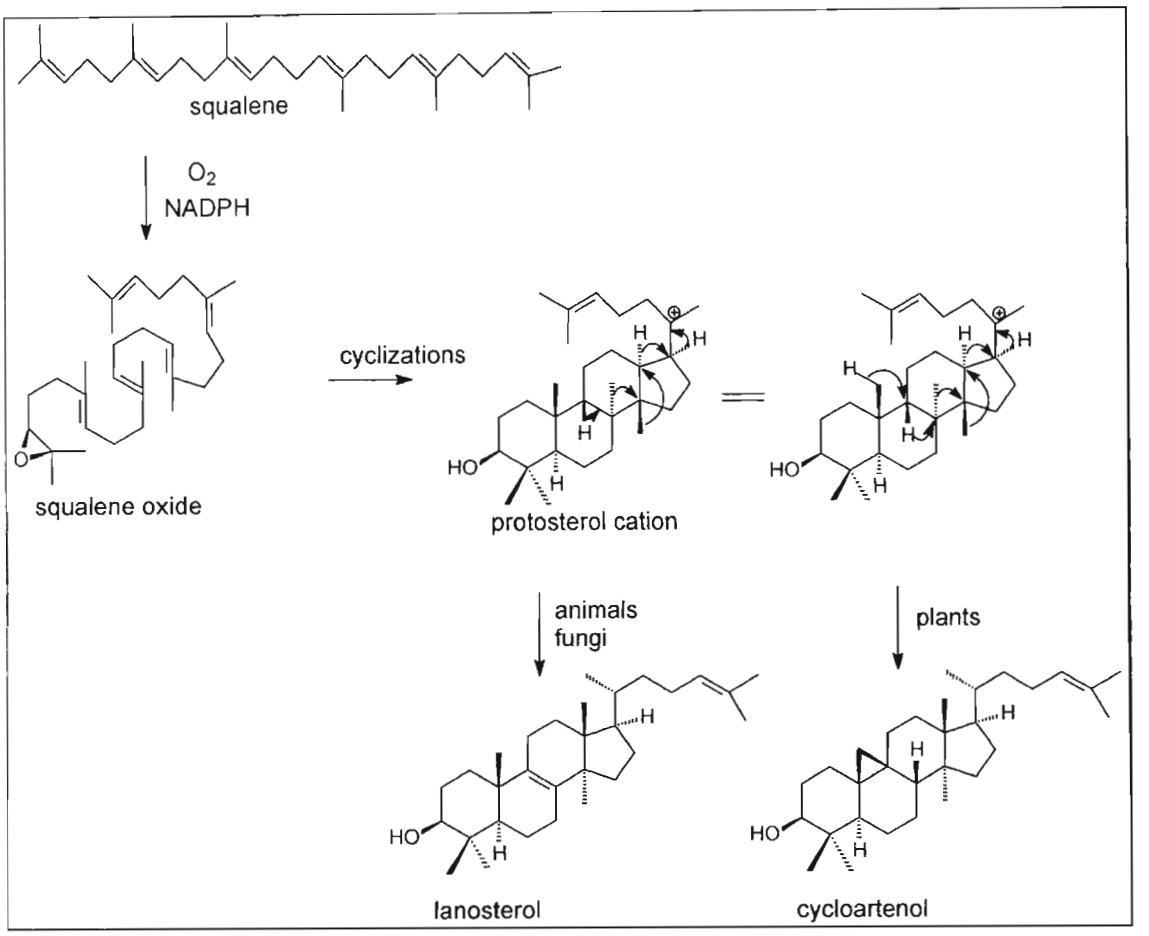


Figure 1.3. Two parent hydrocarbons important for this study [19]

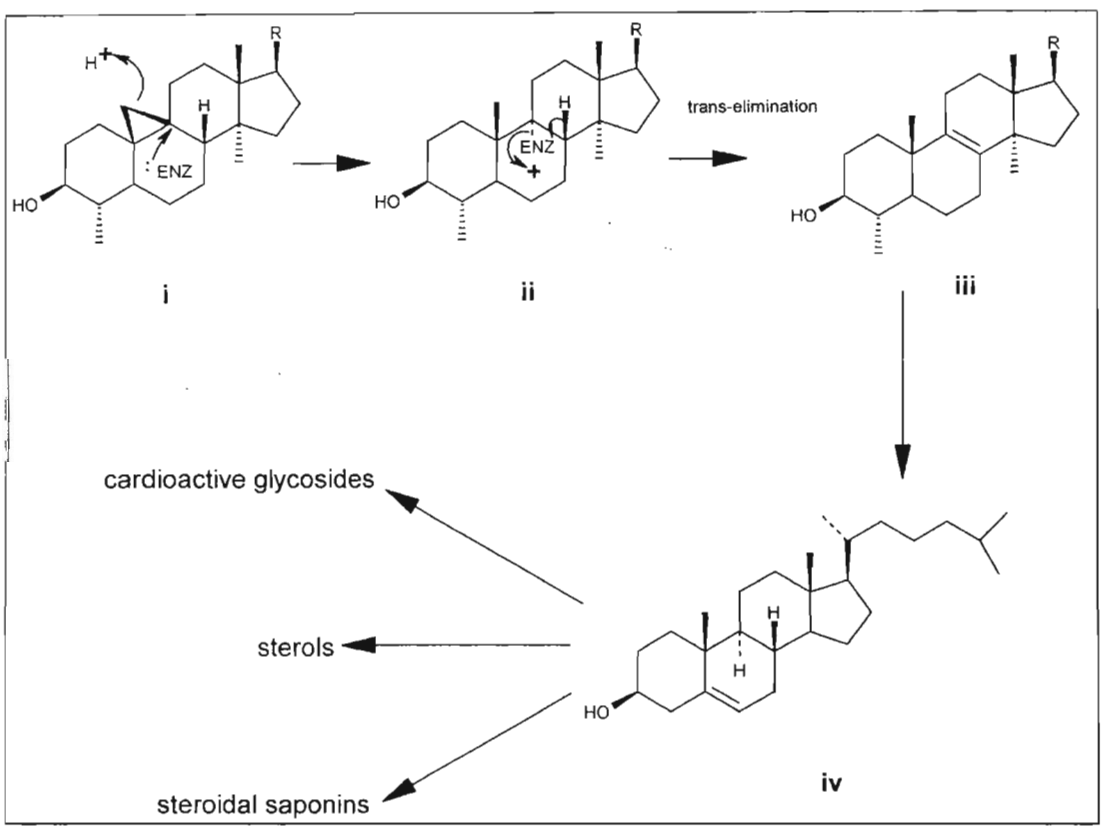
The hydrocarbon squalene which was originally isolated from the liver oil of a shark species (*Squalus* sp.) is a biosynthetic precursor of triterpenes and steroids [19]. Cyclization of squalene takes place *via* the squalene-2,3-oxide intermediate. Carbocation mediated cyclizations then take place in a step-wise sequence and this is followed by Wagner-Meerwein migrations of methyls and hydrides (Scheme 1.2) [19].



Scheme 1.2. The biosynthesis of lanosterol and cycloartenol from squalene [19]

In animals, lanosterol, a triterpenoid alcohol, is formed. However, photosynthetic organisms like plants do not contain lanosterol but cycloartenol. In cycloartenol the H-9 proton is not lost, but migrates to C-8. A number of plant sterols maintain the cyclopropane feature at C-10, but the majority contain a methyl group at C-10. The cyclopropane ring-opening process is specific to 4α-monomethyl sterols and in plants the 4α-methyl group is cleaved off *via* a decarboxylation mechanism and the 4β-methyl group takes up the α-orientation. The substrate (**i**) thus has both 4α- and 14α-methyls. It has been suggested that the cyclopropane ring opening is initiated by an attack on C-9 by the nucleophilic group of a specific enzyme incorporating a proton from water [19]. A *trans* elimination then generates the 8,9-double bond to form a 4α-monomethyl lanosterol derivative

(**iii**). Cholesterol (**iv**) is formed *via* cycloartenol and cholesterol modifications take place to form a wide variety of compounds [19].



Scheme 1.3. Modified conversion of cycloartenol to steroidal compounds [19].

The stereochemistry of steroids influences the biological activity of these compounds significantly. In natural steroids the A/B ring can be *trans* or *cis* fused and unsaturation at C-4 or C-5 is possible. All natural steroids have a *trans* B/C ring fusion and the C/D ring fusion is also usually *trans*, except in cardioactive glycosides where the C/D ring fusion is usually *cis* [19]. Spirocyclic nortriterpenes, cholestane glycosides, steroidal saponins and their derivatives have been isolated from members of the *FSA* Hyacinthaceae family.

1.3.2.1. Spirocyclic nortriterpenes

Spirocyclic nortriterpenoids have a basic lanosterol skeleton and variation is due to differences in oxygenation in the aglycones and the combinations of sugars attached. These compounds have been extensively isolated from various *Eucomis species* [24], but also from *Veltheimia viridifolia* [25] and *Ledebouria cooperi* [26]. Recently two new spirocyclic norterpenes, natalensis A and natalensis B were isolated from *Merwilla natalensis* (previously *Scilla natalensis*) [27].

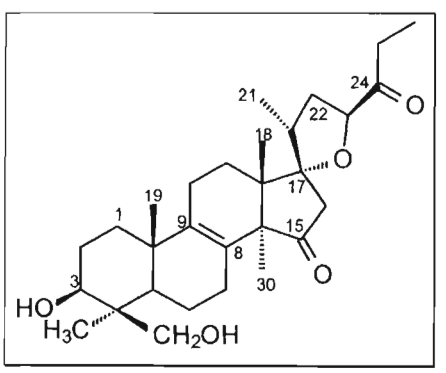


Figure 1.4. The spirocyclic nortriterpene, eucosterol/ (23*S*)-17α,23-epoxy-3β,29-dihydroxy-27-nor-lanost-8-ene-15,24-dione, isolated from different *Eucomis* species [24].

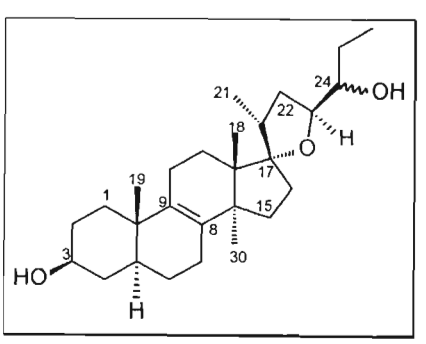
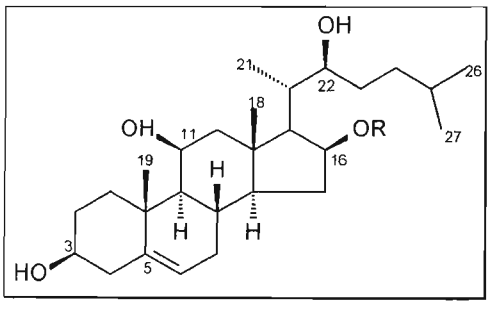


Figure 1.5. The spirocyclic nortriterpene, natalensis A, isolated from *Merwilla natalensis* [27].

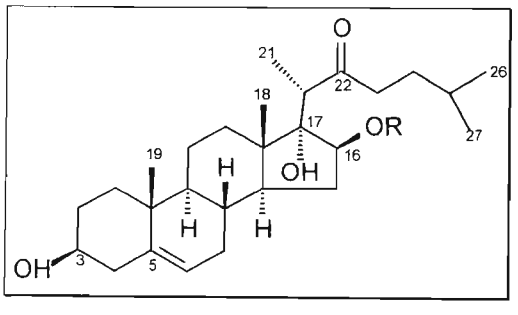
1.3.2.2. Cholestane glycosides

The cholestane glycosides consist of a basic cholestane triterpenoid aglycone skeleton in which the degree of oxygenation varies considerably and 5,6-double bonds as well as 24,25-double bonds are common. Glycosides have been observed to occur at C-1, C-3 and C-16. These compounds have been isolated extensively from the *FSA* Hyacinthaceae *Ornithogalum species*, *O. thyrsoides* [28] and *O. saundersiae* [29, 30].



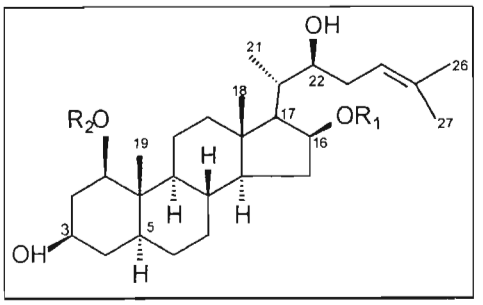
I R= α-*L*-rha
II R= (3'-*O*-acetyl)-α-*L*-rha
III R= (2'-*O*-acetyl)-α-*L*-rha

Figure 1.6. The cholestane glycosides, (22*S*), 3β, 11β, 22β-trihydroxycholest-5-ene 16β-*O*-α-*L*-rhamnopyranoside and its two acetyl derivatives isolated from *O. saundersiae* [29].



IV R=β-*D*-xyl-(1→3)-(2'-*O*-acetyl)-α-*L*-arab
V R=β-*D*-xyl-(1→3)-α-*L*-arab
VI R=2"-*O*-4-methoxybenzoyl-β-*D*-xyl-(1→3)-(2'-*O*-acetyl)-α-*L*-arab
VII R=2"-*O*-3, 4-dimethoxybenzoyl-β-*D*-xyl-(1→3)-(2'-*O*-acetyl)-α-*L*-arab

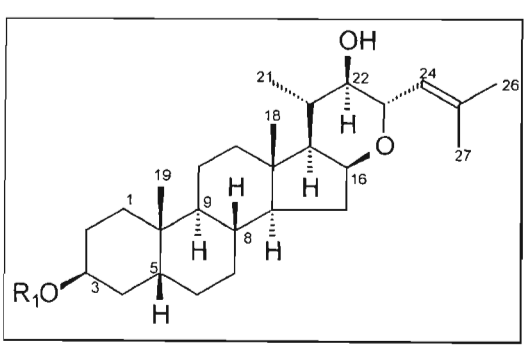
Figure 1.7. Acylated cholestane glycosides isolated from *O. saundersiae* [30].



VIII R_1 = β-*D*-glc, R_2 = α-*L*-rha
VIX R_1 = 6'-*O*-acetyl-β-*D*-glc, R_2 = α-*L*-rha

Figure 1.8. Cholestane bisdesmosides from *O. thyrsoides* [28].

The biological activity of compounds isolated from *O. saundersiae* [29,30] and *O. thyrsoides* [28] was detected using cyclic AMP phosphodiesterase as a screening tool. A correlation between pharmacological activity *in vivo* and inhibition of phosphodiesterase *in vitro* has been reported. The pharmacological effects do not necessarily arise from alteration of cyclic AMP metabolism – compounds with a variety of pharmacological activities can be detected [31]. Compounds **II**, **VI**, **VII** and **VIX** have considerable AMP phosphodiesterase inhibitory activity. It seems as if the acetyl group linked to the rhamnose C-3' of compound **II** and the benzoyl derivatives attached to the sugar moiety in compounds **VI** and **VII** contribute to the activity. The other compounds were inactive. In 1995 another cholestane glycoside was isolated from *O. saundersiae* by Kuroda *et al.*, [32]. This compound showed potential as a new immunosuppressive agent.



XX R_1 = α-*L*-rha-(1→2)-β-*D*-glc-(1→2)-β-*D*-glc

Figure 1.9. Structure of 5β-16β, 23-epoxy-22β-hydroxy-18-oxocholest-24-ene 3β-*O*-α-*L*-rhamnopyranosyl-(1→2)-β-*D*-glucopyranosyl-(1→2)-β-*D*-glucopyranoside [32].

Apart from these *Ornithogalum* species, cholestane glycosides have also been isolated from other species. A novel cholestane glycoside, galtonioside A, was recently isolated from *Galtonia candicans* [33].

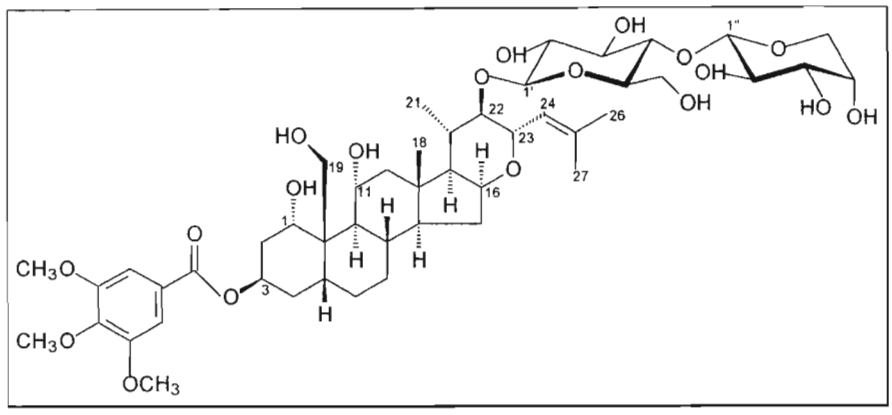


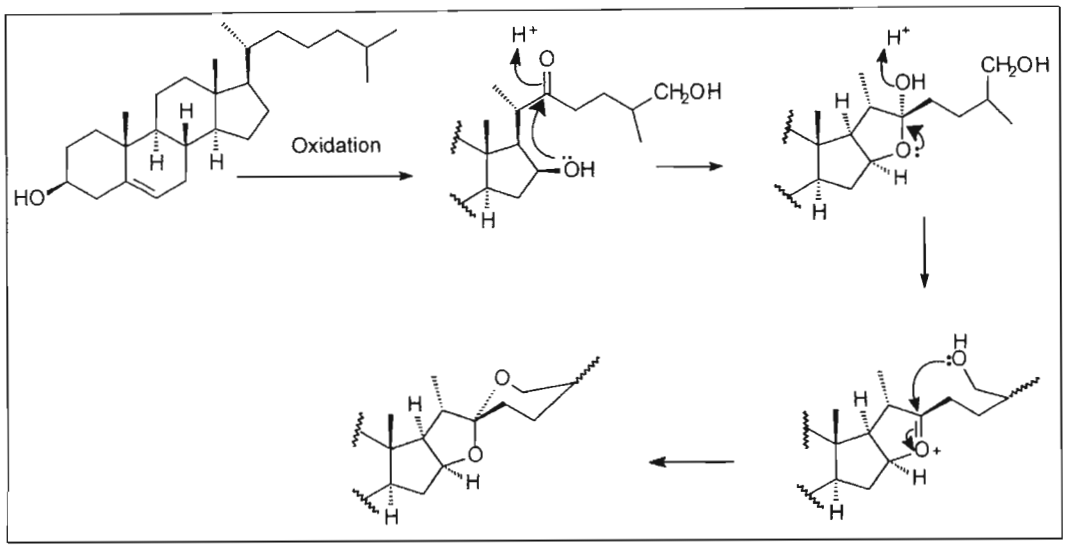
Figure 1.10. The structure of galtonioside A, isolated from *Galtonia candicans* [33].

Galtonioside A showed differential cytotoxicity in cell line assays. The 3,4,5-trimethoxy-benzoyl group attached to the aglycone plays an important role in this activity. The pattern of differential cytotoxicity of this compound did not correlate with that shown by other known cytotoxic compounds and indicated a possible new mode of action [33].

1.3.2.3. Steroidal saponins

The steroidal saponins are plant glycosides which have the property of forming a soapy lather when shaken in water. The steroidal saponins are found in many plants like *Allium chinese* [34], *Cordyline stricta* [35], as well as in *Urginea sanguinea* which forms part of the *FSA* Hyacinthaceae family [36]. Although the cardiac glucosides also posses this property, they are classified separately because of their biological activity [37]. Steroidal sapogenins are C_{27} sterols in which the cholesterol side chain has been modified to a spiroketal. A series of oxygenation reactions take place in which C-16 and a side-chain terminal methyl

group are hydroxylated and C-22 is oxidized to a ketone. A hemiketal is then formed and converted to a spiroketal [19].



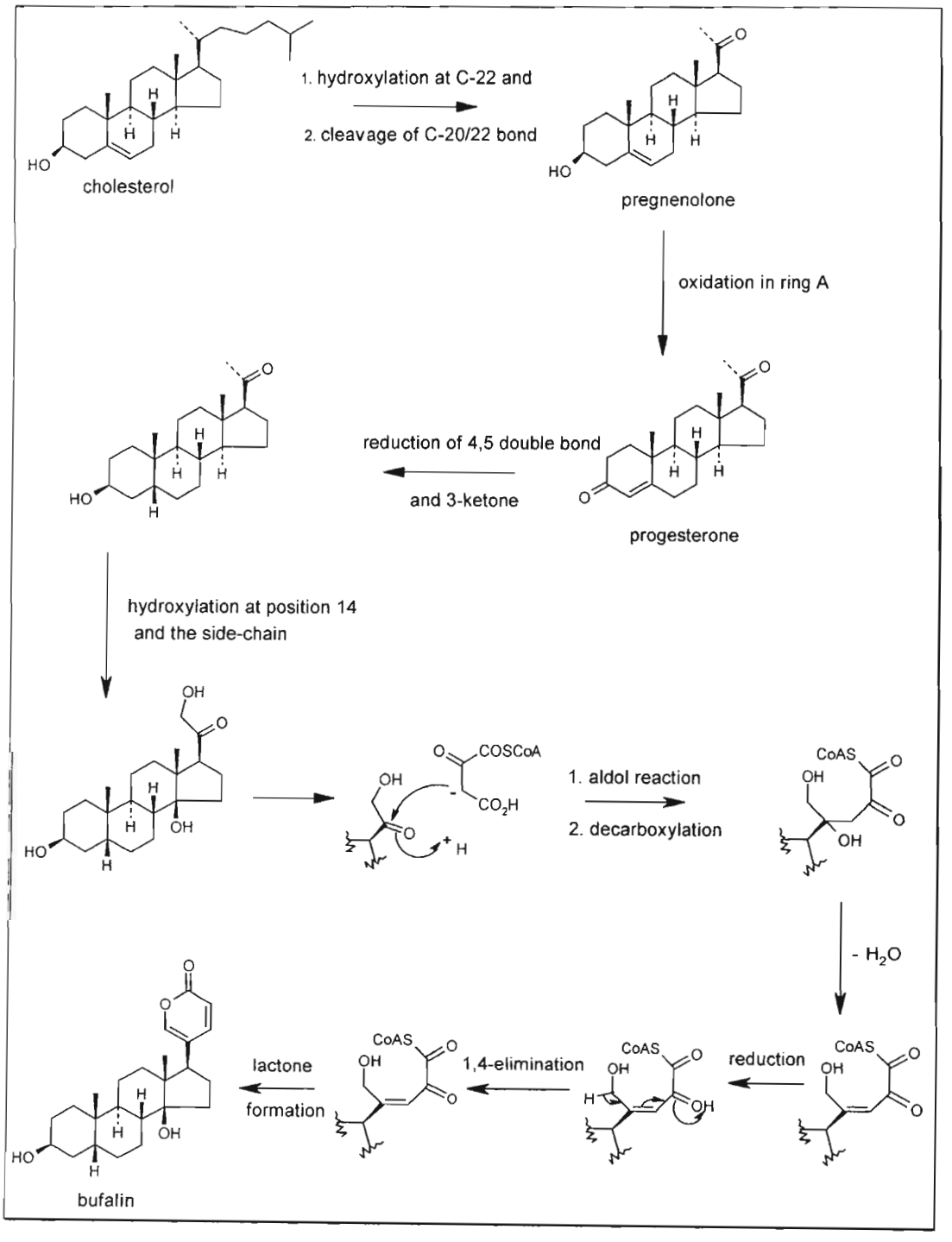
Scheme 1.4. The proposed biosynthesis of spiroketal compounds [19].

Saponins, in general, are powerful emulsifiers, and are toxic, haemolytic and able to form complexes with cholesterol. Thus, they decrease blood cholesterol levels, lipid deposition in the aorta and liver arterial tension. Saponins are also generally good antifungal and antibacterial agents [37].

1.3.3. Bufadienolides

The subfamily Urgineoideae of the Hyacinthaceae is phytochemically characterized by the presence of bufadienolides. Several *Urginea* species have been investigated: *U. sanguinea* [36], *U. maritima* [38], *U. rubella* [39], *U. altissima* [40], *U. burkei* [41] and *U. physodes* [42]. Bufadienolides have been found to be the major chemical constituents of this genus. Knowledge of the cardiotoxic activity of *Urginea* species dates back to as early as 1554 BC. *U. maritima* (the sea onion) was mentioned in the Ebers Papyrus of the Middle Empire of Egypt as a cure for dropsy [3,19]. Bufadienolides are known for their digitalis-like cardiac activity and have been used in Europe as both a heart

medicine and a rat poison [43]. Steyn *et al.* declared in 1949 [44], that *U. lydenburgensis* presumably has a digitalis action.



Scheme 1.5. Proposed biosynthesis of bufalin [19]

Bufalin was originally isolated from the skin of a toad (Bufa *spp.*), and this class of compounds has taken the general name bufadienolides. The basic bufadienolide structure arises biosynthetically from the metabolism of cholesterol. These compounds are structurally related to the cardenolides and share the digitalis-like cardiac activity of the cardenolides [19] (see Figure 1.11).

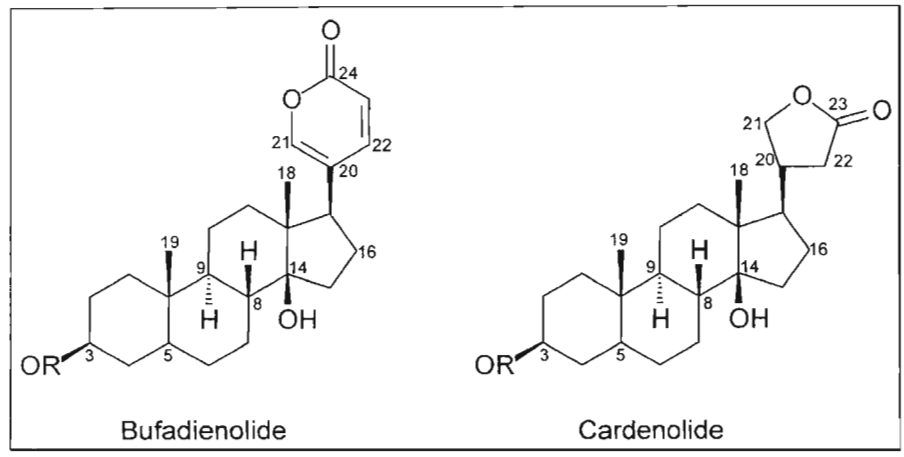
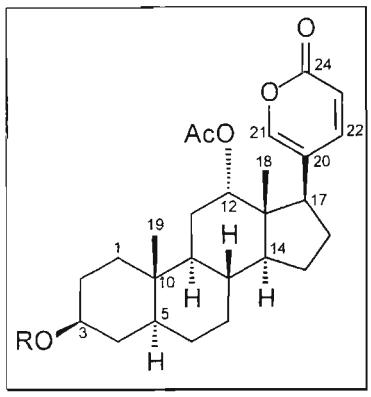


Figure 1.11. Comparison of the structures of a bufadienolide and a cardenolide

Bufalin can undergo other reactions like 5β-hydroxylation and C-19 oxidation to form different bufadienolides. A six membered α,β-unsaturated lactone ring at C-17β is characteristic of the bufadienolides. Tertiary methyl groups are present at C-10β and C-13β although an aldehyde group can sometimes replace the group at C-10. A 4,5-double bond is also a common feature. The stereochemistry of bufadienolides is very important for their activity. The compounds usually have *cis*-fused C/D rings as well as a β-hydroxy group at C-14 [19]. The 14-deoxybufadienolides however, were found to have *trans*-fused C/D rings [42]. They were also found to be non-toxic and this confirmed that the 14β-hydroxy group is essential for biological activity [42]. A sugar (if present) can usually be found at the 3β-position [19].



X R = β-*D*-xyl-(1→4)-*O*-α-*L*-rha
XI R = β-*D*-xyl-(1→3)-*O*-(4'-O-acetyl- α-*L*-rha)

Figure 1.12. The structures of physodine C (**X**) and physodine D (**XI**) which were the first examples of 14-deoxybufadienolides [42]

The isolation of physodine C and D suggests that an alternative biosynthetic pathway exists for these bufadienolides. Physodine C and D could be derived from cholic acid derivatives [42].

The novel bufadienolide compound with a trisaccharide moiety, urginin, was recently isolated from *U. altissima* by Pohl *et al.* [40].

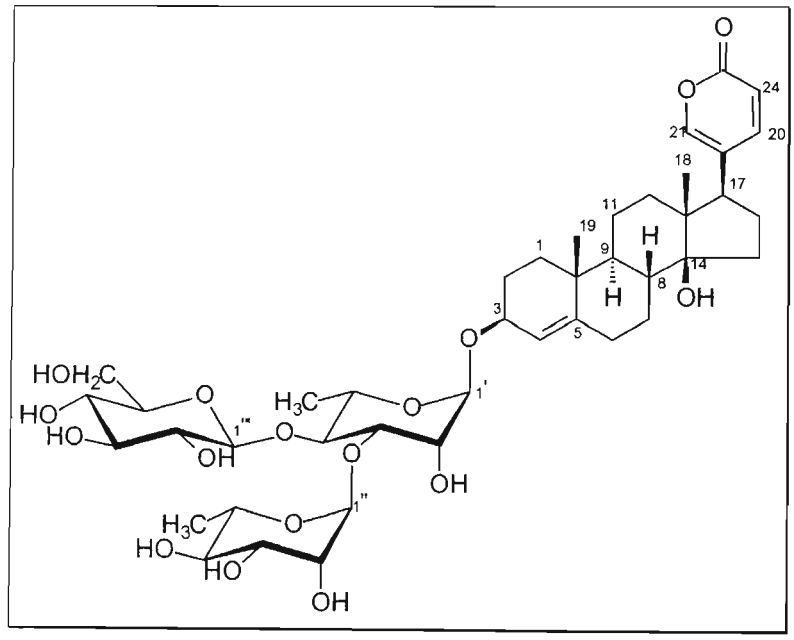


Figure 1.13. Structure of urginin, isolated from *U. altissima* [40].

Different types and numbers of sugars are often linked to C-3 and although the biological activity resides in the aglycone, the sugars influence the water solubility and thus bioavailability of the bufadienolides [19].

Although bufadienolides show typical digitalis-like activity, the therapeutic usefulness of these compounds is unfortunately impaired by side effects like severe gastric irritation [19,45].

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CHAPTER 2

EXTRACTIVES FROM THE HYACINTHACEAE

2.1. INTRODUCTION

The phytochemical investigation of plants is important as nature is an abundant source of novel chemotypes. However, the aim of this study was to bridge the gap between chemistry and biology through interdisciplinary collaboration.

Four members of the Hyacinthaceae family were investigated phytochemically. These were *Eucomis comosa* (Houtt.) Wehrh, *Ornithogalum tenuifolium* Delaroche, *Urginea lydenburgensis* R.A. Dyer and *Galtonia princeps* (Bak.) Decne. The biological investigation focused on the homoisoflavanones isolated from *E. comosa*, as well as compounds from other plant sources (see Chapter 4).

Plant material was collected and identified by Dr. N. Crouch and voucher speciments were lodged at the Natal Herbarium.

2.2. EXTRACTIVES FROM *EUCOMIS COMOSA* (HOUTT.) WEHRH.

comosa = hairy tufts, shaggy [1]

E. comosa forms part of the Hyacinthoideae subfamily [2] and is also known as *E. punctata* and *E. pallidiflora*. Common names of this plant are: slender pineapple flower, krulkoppie (Afrikaans) [3], ubuhlungu-becanti (Xhosa, Zulu) [4].

Plants are distributed inland in the Northern Cape, Transkei and Eastern Cape [5], in swamps and on grassy hillsides [6]. Leaves are firm and bright

green with purple streaks underneath. Small cream flowers with purple ovaries are borne on stout cylindrical stems [6].

Eucomis species have been suspected of causing human poisoning [7]. They also cause poisoning in sheep [7] with symptoms of abdominal pain, diarrhoea and renal failure [4]. It seems that the bulbs contain a dangerous haemolytic poison [8]. However extracts of these species are used in small amounts by Zulu traditional healers without them poisoning their patients. *E. comosa* is used by Xhosa people for rheumatism and also for teething infants. Frequent application produces a rash [7]. Taylor and Van Staden [9] screened extracts of *Eucomis* species for anti-inflammatory activity employing COX-1 and -2 assays. COX enzymes are important role players in the inflammatory process. High levels of COX-1 and -2 inhibitory activity were associated with several *Eucomis* species including *E. comosa*. This study seemed to validate the ethnomedicinal use of *Eucomis* species for conditions associated with inflammation. Watt and Breyer-Brandwijk [7] reported that *E. comosa* contains saponins, that neither herb nor bulb has any cardiac glycosidal effects and that extracts of the herb gave negative bactericidal results.

A specimen of *E. comosa* was purchased from the Warwick Triangle herbal market in Durban and a specimen grown to flowering (*Crouch 940*, NH).

2.2.1. Structural elucidation of compounds from *E. comosa*

Five homoisoflavanones, compounds **1-5**, were isolated from the ethyl acetate extract of *Eucomis comosa*. The remainder of the compounds were identified as fatty acids and common sterols.

2.2.1.1. Structural elucidation of compound **1**, punctatin

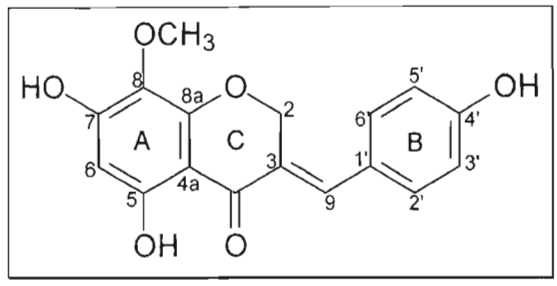
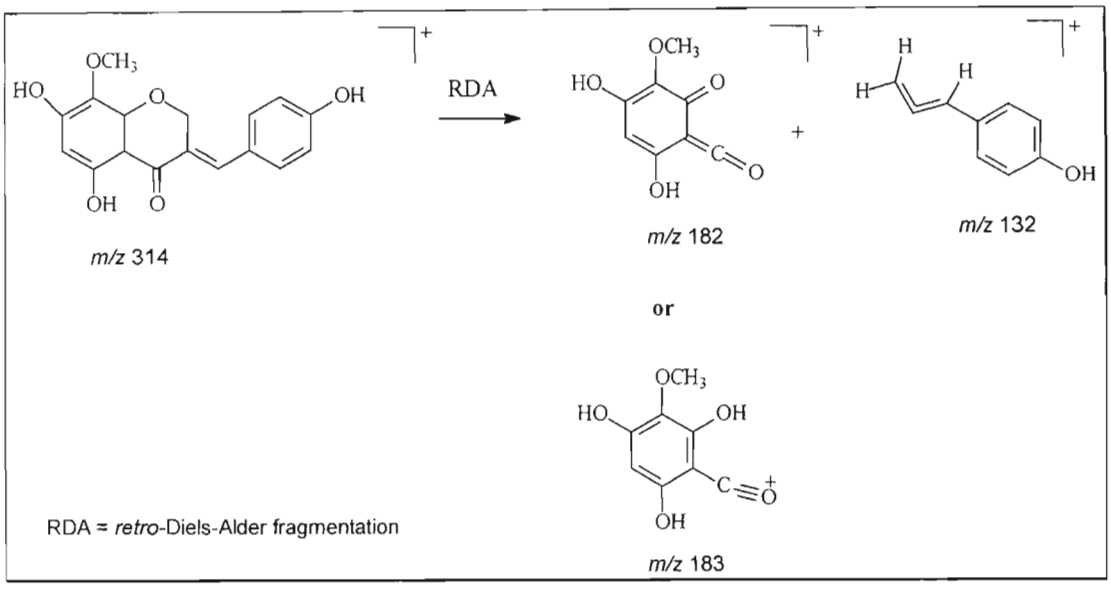


Figure 2.1. The structure of compound **1**, punctatin

The parent ion peak at *m/z* 314 in the mass spectrum was the base peak. An α,β-unsaturated carbonyl group carbon resonance at δ185.2 in the ^{13}C-NMR spectrum, as well as a quaternary carbon resonance at δ130.2 and the resonance at δ137.4 (CH) ascribed to C-3 and C-9, indicated that compound **1** was a homoisoflavanone of the 3-benzylidene-4-chromanone type [16]. The substitution pattern of ring B was revealed by the appearance of two aromatic doublets, integrating to two protons each, at δ6.90 (*J*=8.5Hz) and δ7.20 (*J*=8.7Hz) which are typical of a *para*-disubstituted benzene ring. These resonances were assigned to the chemically equivalent proton pairs H-2', H-6' and H-3', H-5'. The peak at *m/z* 132 in the mass spectrum corresponded to the fragment indicating a hydroxylated ring B.

A singlet resonance at δ6.13 in the ^{1}H-NMR spectrum integrating for one proton suggested ring A was penta-substituted. The peaks at *m/z* 182 and 183 in the mass spectrum, due to the *retro*-Diels-Alder cleavage, indicated a dihydroxy, monomethoxy substituted ring A (Scheme 2.1).



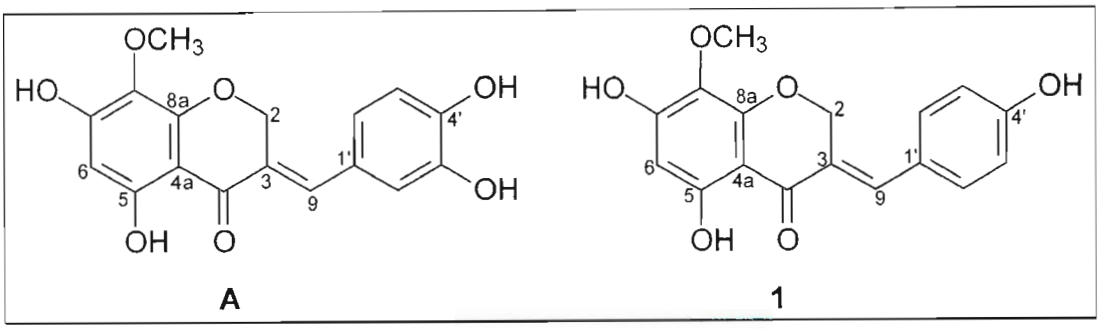
Scheme 2.1. The mass spectrometric fragmentation pattern of compound **1** [11].

Bathochromic shifts on addition of both $AlCl_3$ (+32nm) and NaOAc (+35nm) suggested that two hydroxyl groups were attached to ring A at the 5 and 7 positions [12]. A singlet resonance at δ12.56 in the ^1H NMR spectrum was assigned to the proton of the hydroxyl group at position 5 as this is a signal typically seen when a hydrogen bond is formed with the carbonyl group at position 4. The NOESY correlation between the 5-OH proton and the singlet resonance at δ6.13 indicated that C-6 was unsubstituted. The singlet at δ3.82 in the ^1H NMR spectrum integrated to three protons and was assigned to an aromatic methoxy group. The HMBC data confirmed the position of the aromatic methoxy group in ring A. The C-8 carbon resonance at δ127.2 showed a HMBC correlation to the methoxy group proton resonance at δ3.82 which, in turn, showed a NOESY correlation to the 2H-2 resonance at δ5.36. Thus the methoxy group was placed at C-8.

The ^{13}C-NMR data of compound **1** (in CD_3OD) was compared to the literature data of the known compound 5,7-dihydroxy-8-methoxy-3-(3',4'-dihydroxybenzylidene)-chroman-4-one (compound **A**) [10], which differs from

compound **1** in having an extra hydroxyl group at C-3'. No other suitable data was available for comparison. Compound **1** correlated well with compound **A**.

Table 2.1. Comparison of ^{13}C NMR data of compound **1** and compound **A** [10].



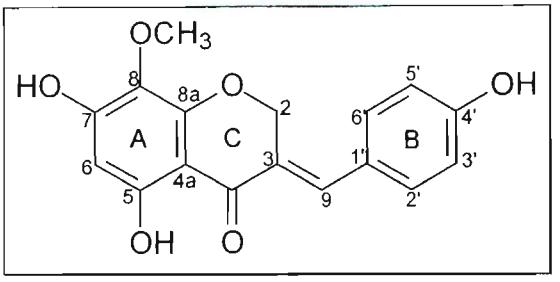
	Compound 1 *	Compound A *[10]
2	68.8 (CH_2)	69.0 (CH_2)
3	129.8 (C)	128.0 (C)
4	186.2 (C)	188.9 (C)
4a	103.2 (C)	103.9 (C)
5	**160.7 (C)**	**161.3 (C)**
6	**97.7 (CH)**	**97.3 (CH)**
7	**161.8 (C)**	**161.6 (C)**
8	**130.1 (C)**	**129.8 (C)**
8a	**153.8 (C)**	**154.9 (C)**
9	138.1 (CH)	138.8 (CH)
1'	132.3 (C)	127.4 (C)
2'	133.5 (CH)	118.4 (CH)
3'	116.8 (CH)	149.0 (CH)
4'	154.7 (C)	146.7 (C)
5'	116.8 (CH)	116.7 (CH)
6'	133.5 (CH)	124.8 (CH)
8-OCH_3	61.4 (CH_3)	61.6 (CH_3)

*NMR data obtained in CD_3OD, 100MHz

The (*E*)-configuration of the 3,9-double bond was determined by the downfield chemical shift of the H-9 proton at δ7.79. The (*Z*)-configuration places the C-9 proton away from the anisotropic region of the carbonyl group and causes this proton to resonate at a higher field [11]. The NOESY spectrum showed no correlations between the 2H-2 and the H-9 proton resonances as would be expected for the (*Z*)-isomer. Only correlations between the H-9 and the H-2'/H-6 resonances were found.

The IR data obtained for compound **1** was consistent with the proposed structure. Peaks were observed at 3395 cm^{-1} (OH stretching), 2928 cm^{-1} (aliphatic stretching), 1638 cm^{-1} (carbonyl stretching), 1512 cm^{-1} (aromatic C=C stretching) (see Chapter 3, p115). It was concluded that compound compound **1** was the known compound (*E*)-punctatin, isolated previously from *Eucomis comosa* (Houtt.) Wehrh [12].

Table 2.2. NMR data for compound **1**, punctatin



	^{1}H NMR data for compound 1*	^{13}C NMR data for compound 1**	HMBC correlations C→H	NOESY correlations
2	5.36 *d* (1.7)	67.6 (CH_2)	9	8-OCH_3, 2', 6'
3	-	130.2 (C)	-	-
4	-	185.2 (C)	2, 9	-
4a	-	103.1 (C)	6	-
5	-	157.7 (C)	6	-
6	6.13 *s*	96.0 (CH)	-	-
7	-	160.8 (C)	6	-
8	-	127.2 (C)	8-OMe, 2, 6	-
8a	-	152.1 (C)	2	-
9	7.79 br *t* (1.7)	137.4 (CH)	2, 2'/6'	2', 6'
1'	-	126.9 (C)	3'/5', 9	-
2'	7.20 *d* (8.7)	132.2 (CH)	9, 6'	2, 9, 3'
3'	6.90 *d* (8.5)	115.8 (CH)	5'	2'
4'	-	157.1 (C)	2', 6', 3', 5'	-
5'	6.90 *d* (8.5)	115.8 (CH)	3'	6'
6'	7.20 *d* (8.7)	132.2 (CH)	2', 9	2, 9, 5'
8-OCH_3	3.82 *s*	61.5 (CH_3)		2
5-OH	12.56 *s*	-	-	6

*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$, 100MHz

2.2.1.2. Structural elucidation of compound **2**, (*Z*)-eucomin.

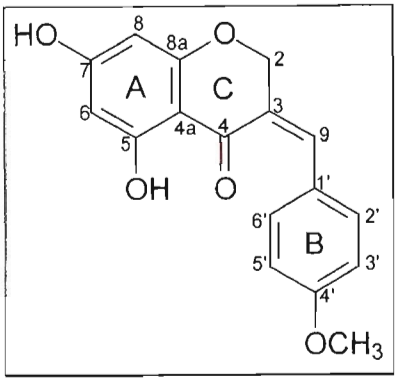
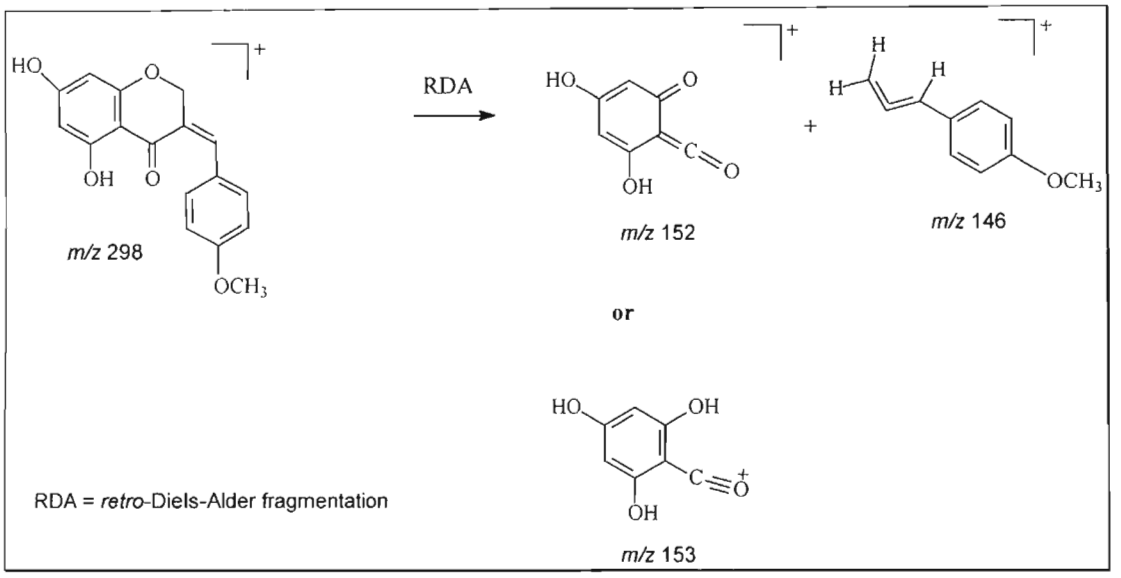


Figure 2.2. The structure of compound **2**, (*Z*)-eucomin

Compounds **2** and **3** were isolated as a mixture from *Eucomis comosa* and were separated from each other by solvent extraction first using chloroform and then chloroform / methanol 9:1 (see Chapter 3, p116). However, small amounts of impurities from compound **3** were still evident in the spectrum of compound **2**.

Analysis of the ^{13}C-NMR spectrum indicated a 3-benzylidene-4-chromanone system with the carbonyl carbon resonance at δ187.1 and the C-3 and C-9 resonances at δ125.5 and δ140.8 respectively (see compound **1**). A singlet integrating to three protons, was observed at δ3.84 indicating the presence of an aromatic methoxy group. Two aromatic doublets integrating for two protons each at δ7.78 (*J*=8.7Hz) and δ6.87 (*J*=8.7Hz) in the ^{1}H NMR spectrum, indicated a *para*-disubstituted B ring. The C-4' resonance showed a correlation to the methoxy group protons resonance at δ3.84 in the HMBC spectrum which, in turn, showed a correlation to H-3' and H-5' in the NOESY spectrum, and thus the methoxy substituent was placed at C-4'. The presence of a peak at at *m/z* 146 in the mass spectrum of compound **2** due to *retro*-Diels Alder fragmentation confirmed the substitution on ring B (Scheme 2.2).

The proposed structure was confirmed by the observed molecular ion peak (base peak) at *m/z* 298. Hydroxyl groups were placed at C-5 and C-7 on biosynthetic grounds. The fragmentation peaks at *m/z* 152 and 153, due to *retro*-Diels-Alder cleavage confirmed that two hydroxy groups were present on ring A. As a result of an effective hydrogen transfer from C-2 to ring A, the peak at *m/z* 153 was of much higher intensity [11].



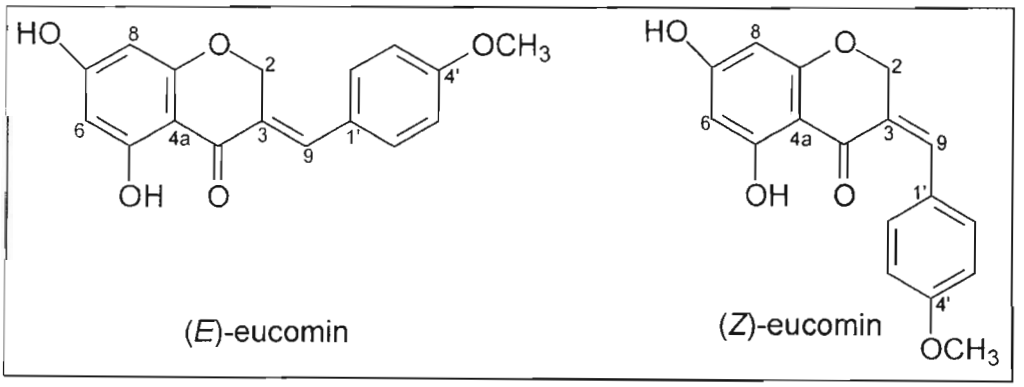
Scheme 2.2. The mass spectrometric fragmentation pattern of compound **2** [11]

Bathochromic shifts with $AlCl_3$ (+37nm) and NaOAc (+21nm) also confirmed the presence of the hydroxyl groups at the 5 and 7 positions. The upfield shift (< 6.00ppm) of the H-6 and H-8 resonances in the 'H-NMR spectrum at δ5.97 and δ5.90 are typical of these protons in the presence of a hydroxyl group at C-7 [13].

The C-9 proton resonance in the ^{1}H-NMR spectrum occurred more upfield at δ6.84 in comparison to that of compound **1** because the (Z)-configuration places the C-9 proton away from the anisotropic region of the carbonyl group and causes this proton to resonate at higher field [11]. Correlations between the 2H-

2 and H-9 protons in the NOESY spectrum indicated that this compound was the (*Z*)-isomer since the model of the (*Z*)-isomer revealed a close proximity between the protons. The geometry of the (*E*)-isomer will make NOESY correlations between 2H-2 and H-9 protons impossible. In Table 2.3, ^{1}H NMR data of compound **2** is compared to that of (*E*)-eucomin and (*Z*)-eucomin [14].

Table 2.3. ^{1}H NMR data of compound **2**, (*E*)-eucomin and (*Z*)-eucomin [14].



	Compound 2 *	(*E*)-eucomin [15] **	(*Z*)-eucomin [14] **
2H-2	4.88 *s*	5.31 d (1.7)	4.91 s
H-6	5.97*s*[2]	6.00 d (2.3)	5.99 d (2.3)
H-8	5.90*s*[2]	5.90 d (2.3)	5.93 d (2.3)
H-9	6.84 [1]	7.80 t (1.7)	6.87 s
H-2'/H-6'	7.78*d* (8.7)	7.27 d (8.8)	7.81 d (8.8)
H-3'/H-5'	6.87*d* (8.7)[1]	6.96 d (8.8)	6.90 d (8.8)
4'-OCH_3	3.84 *s*	3.87 s	3.85 s
5-OH	12.75 *s*	12.77 s	12.75 s

*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$, 90MHz

[1]overlapping resonances

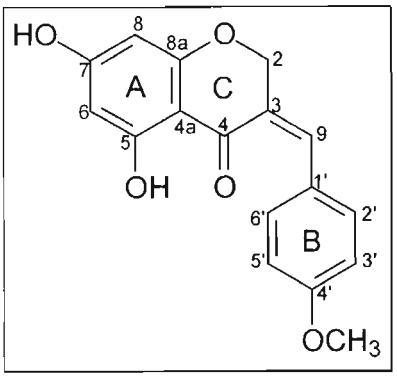
[2]resonances not well resolved

The data of (*Z*)-eucomin correlates well with those of compound **2**. All the analytical data and tabled data agreed with the proposed structure.

The IR data obtained for compound **2** was consistent with the proposed structure. Peaks were observed at 3133 cm^{-1} (OH stretching), 2925 cm^{-1}

(aliphatic stretching), 1637 cm^{-1} (carbonyl stretching), 1509 cm^{-1} (aromatic C=C stretching). Compound **2** is the (*Z*)-isomer of the known compound, eucomin isolated previously from *Eucomis bicolor* (Bak.)[15].

Table 2.4. NMR data for compound **2**, (*Z*)-eucomin



	^{1}H NMR data for compound 2 *	**^{13}C NMR data for compound 2****	**HMBC correlations C→H**	**NOESY correlations**
2	4.88 *s*	74.9 (CH_2)	9	9
3	-	125.5 (C)	2, 9	-
4	-	187.1 (C)	2, 9	-
4a	-	104.6 (C)	-	-
5	-	164.2 (C)	5-OH	-
6	5.97 *s*[2]	96.7 (CH)	5-OH	-
7	-	165.4 (C)	-	-
8	5.90 *s*[2]	94.9 (CH)	-	-
8a	-	162.8 (C)	2	-
9	6.84 [1]	140.8 (CH)	2, 2', 6'	2
1'	-	126.6 (C)	-	-
2'	7.78 *d* (8.7)	132.9 (CH)	9, 6'	3'
3'	6.87 *d* (8.7)[1]	113.4 (CH)	5'	2', 4'-OCH_3
4'	-	161.0 (C)	4-OCH_3, 3' ,5', 2',6'	-
5'	6.87 *d* (8.7)[1]	113.4 (CH)	-	6', 4'-OCH_3
6'	7.78 *d* (8.7)	132.9 (CH)	9, 2'	5'
4'-OCH_3	3.84 *s*	55.3 (CH_3)	-	3', 5'
5-OH	12.75 *s*	-	4a, 5, 6	-

*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$, 100MHz

[1]overlapping resonances

[2]resonances not well resolved

2.2.1.3. Structural elucidation of compound **3**, (*E*)-eucomin.

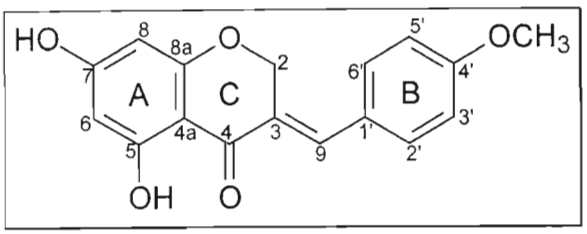


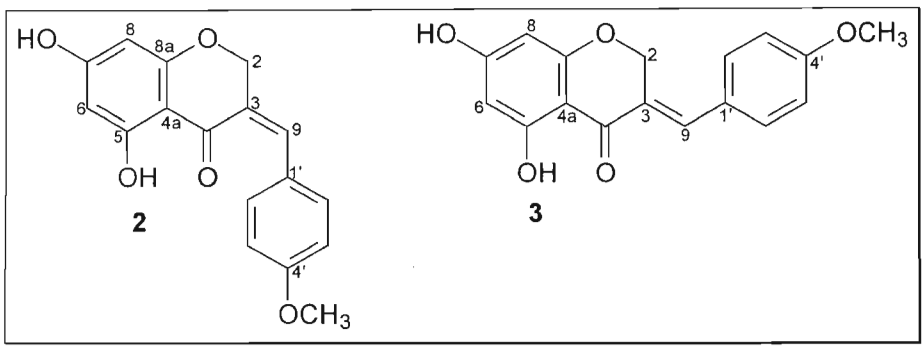
Figure 2.3. The structure of compound **3**, (*E*)-eucomin

The molecular ion peak of compound **3** was observed at *m/z* 298. The ^{1}H NMR spectrum for compound **3** closely resembled that of compound **2**, which suggested that these compounds were very similar. As before, two aromatic doublets integrating for two protons each at δ7.22 (*J*=8.7Hz) and δ6.93 (*J*=8.8Hz) in the ^{1}H NMR spectrum of compound **3**, indicated a *para*-disubstituted B ring. A singlet at δ3.82 integrating to three protons indicated the presence of an aromatic methoxy group. Correlations between the methoxy group protons at δ3.82 and H-3'/H-5' in the NOESY spectrum confirmed that the methoxy group was present at C-4' in ring B. The H-6 and H-8 resonances appeared as two *meta* coupled doublets at δ5.94 (*J*=2.2Hz) and δ5.85 (*J*=2.2Hz) upfield of 6.00 ppm and suggested the presence of a hydroxyl group at C-7 [13]. Since only one methoxy group proton resonance was observed, it was assumed that another hydroxyl group was present on C-5. The highly deshielded proton resonance at δ12.72 could be assigned to this hydroxyl proton. More convincing evidence for the presence of the hydroxyl group at C-5, is the infra-red absorption of the C=O group at 1638 cm^{-1}. This low frequency is indicative of a hydrogen bond being formed with the oxygen of the carbonyl group.

The C-3 and C-9 resonances at δ127.8 and δ136.9 in the ^{13}C NMR of compound **3** indicated a 3-benzylidene-4-chromanone system. The corresponding resonance of the H-9 proton in the ^{1}H NMR occurred more downfield at δ7.67 in

relation to the H-9 proton of compound **2** which appeared at δ6.84 [14]. No NOESY correlations were observed between the H-9 proton and the 2H-2 protons of compound **3** opposed to the correlations found in the NMR data of compound **2**. This observation gave proof that compound **3** was the (*E*)-isomer of compound **2**.

Table 2.5. Comparison of the ^{1}H NMR spectral data of compounds **2** and **3** and (*E*)-eucomin [14]



	Compound 2 *	Compound 3**	(*E*)-eucomin [14]***
2H-2	4.88 *s*	5.18 *d* (1.7)	5.31 *d* (1.7)
H-6	5.97*s*[2]	5.94 *d* (2.2)	6.00 *d* (2.3)
H-8	5.90 *s*[2]	5.85 *d* (2.2)	5.90 *d* (2.3)
H-9	6.84 [1]	7.67 (complex multiplet)	7.80 *t* (1.7)
H-2'/H-6'	7.78 *d* (8.7)	7.22 *d* (8.7)	7.27 *d* (8.8)
H-3'/H-5'	6.87*d* (8.7)[1]	6.93 *d* (8.8)	6.96 *d* (8.8)
4'-OCH_3	3.84 *s*	3.82 *s*	3.87 *s*
5-OH	12.75 *s*	12.72 *s*	12.77 *s*

*NMR data obtained in $CDCl_3$, 400MHz

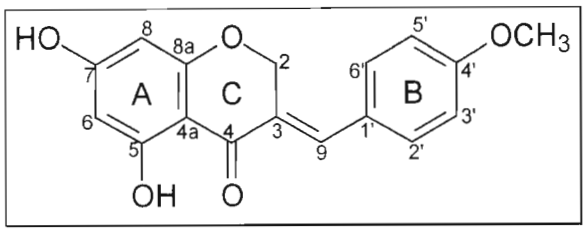
**NMR data obtained in $CDCl_3$/ CD_3OD, 400MHz

***NMR data obtained in $CDCl_3$, 90MHz

[1]overlapping resonances

[2] resonances not well resolved

Table 2.6. NMR data for compound **3**, (*E*)-eucomin



	^{1}H NMR data for compound 3 *	^{13}C NMR data for compound 3**	HMBC correlations C→H	NOESY correlations
2	5.18 *d* (1.7)	67.4 (CH_2)	2', 6'	-
3	-	127.8 (C)	2	-
4	-	185.2 (C)	2	-
4a	-	102.7 (C)	-	-
5	-	164.8 (C)	-	-
6	5.94 *d* (2.2)	96.7 (CH)	-	-
7	-	166.9 (C)	-	-
8	5.85 *d* (2.2)	95.4 (CH)	-	-
8a	-	162.5 (C)	2	-
9	7.67 (complex multiplet)	136.9 (CH)	2', 6'	2', 6'
1'	-	127.0(C)	3', 5'	-
2'	7.22 *d* (8.7)	132.1 (CH)	2, 9, 3'	2, 9, 3'
3'	6.93 *d* (8.8)	114.4 (CH)	4'-OCH_3, 2'	4'-OCH_3, 2'
4'	-	160.9(C)	4'-OCH_3, 2', 6'	-
5'	6.93 *d* (8.8)	114.4 (CH)	4'-OCH_3, 6'	4'-OCH_3, 6'
6'	7.22 *d* (8.7)	132.1 (CH)	2, 9, 5'	2, 9, 5'
4'-OCH_3	3.82 *s*	48.4(CH_3)	-	3', 5'
5-OH	12.72 *s*	-	-	-

*NMR data obtained in $CDCl_3$/ CD_3OD, 400MHz

**NMR data obtained in $CDCl_3$ / CD_3OD 100MHz

The IR data obtained for compound **3** was consistent with the proposed structure. Peaks were observed at 3134 cm^{-1} (OH stretching), 2928 cm^{-1} (aliphatic stretching), 1638 cm^{-1} (carbonyl stretching), 1514 cm^{-1} (aromatic C=C stretching) (see Chapter 3, p117). Compound **3**, (*E*)-eucomin is a known compound and has been isolated before from *Eucomis bicolor* [14].

2.2.1.4. Structural elucidation of compound **4**, 3,9-dihydropunctatin.

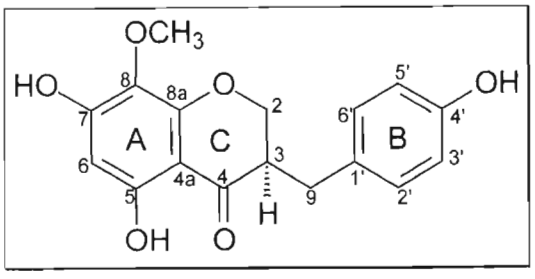
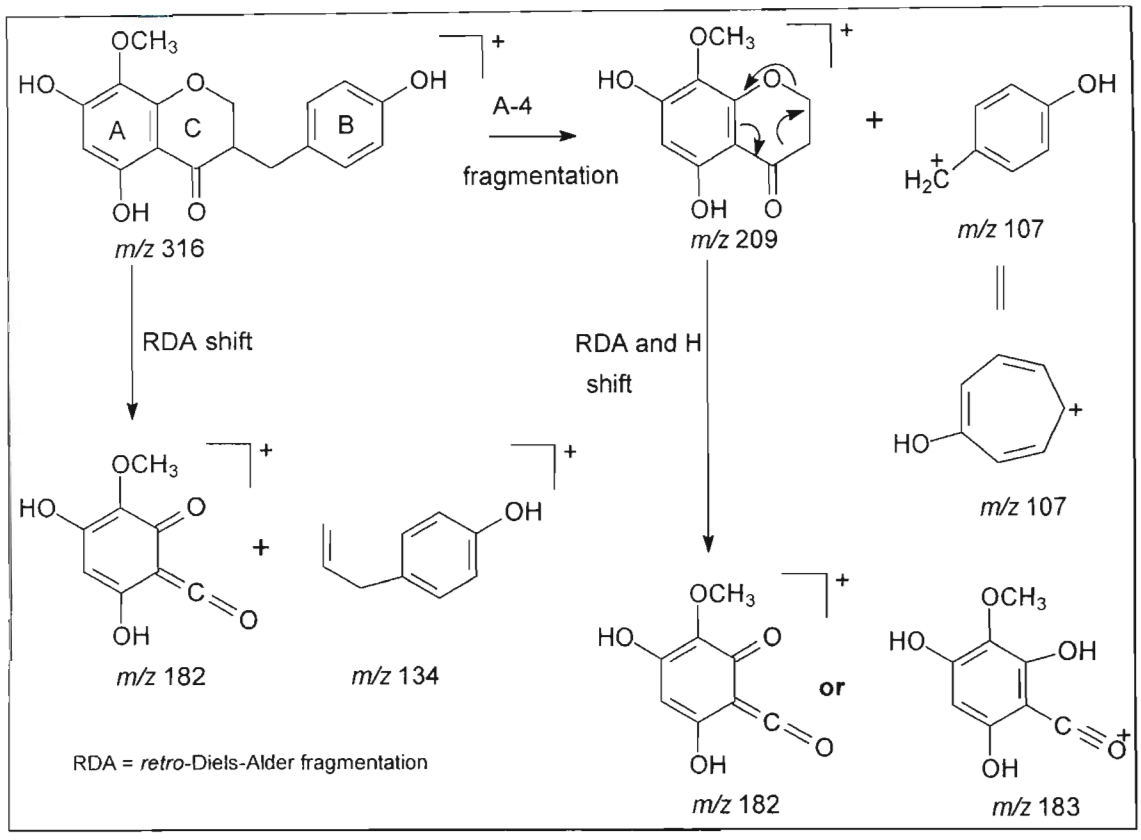


Figure 2.4. The structure of compound **4**, 3,9-dihydropunctatin.

The molecular ion peak of compound **4** was found to occur at *m/z* 316, and the ^{1}H NMR data of compound **4** indicated that it was a homoisoflavanone of the 3-benzyl-4-chromanone type. Two pairs of double doublets at δ4.34, δ4.18 and δ3.17, δ2.63 ascribed to the two H-2 and two H-9 protons, together with the multiplet at δ2.81 ascribed to the H-3 protons formed two typical ABX systems. The absolute stereochemistry of H-3 has been determined by Professor D. Ferreira at the University of Mississippi, USA for a series of homoisoflavanones of this type using circular dichroism. In all cases the absolute stereochemistry at C-3 was shown to be *R*, implying that H-3 must be α [17]. It is assumed that the stereochemistry would be the same for these compounds.

The protons of one aromatic methoxy group were observed as a singlet at δ3.83 in the ^{1}H NMR spectrum, and the presence of one aromatic proton singlet at δ6.11 indicated that ring A was *penta*-substituted. The fragmentation ion peak at *m/z* 209 in the mass spectrum, is typical of the A-4 fragmentation pattern common for saturated homoisoflavanones of the 3-benzyl-4-chromanone type, and corresponded to the dihydroxymethoxychromanone fragment ion.



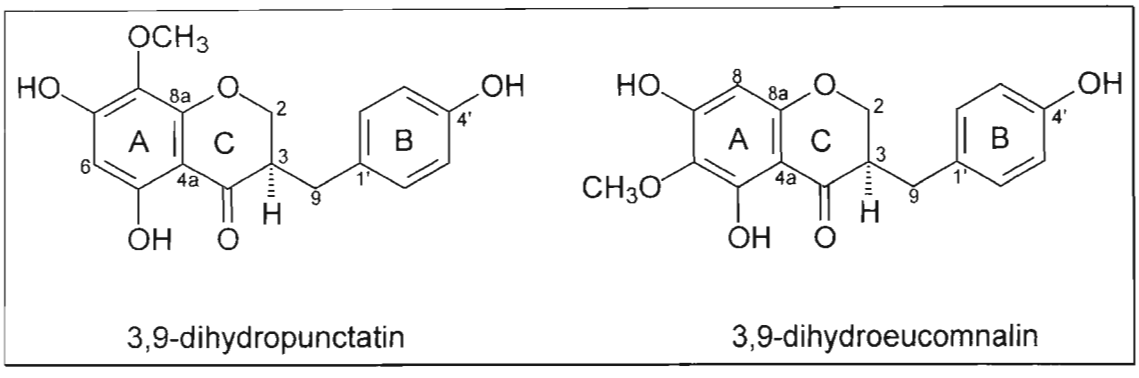
Scheme 2.3. Fragmentation pattern of the 3-benzyl-4-chromanone type applied to compound **4** [11].

The aromatic AA'BB' system in the ^{1}H NMR spectrum of compound **4** indicated that ring B was a *para*-disubstituted benzene ring. An intense peak at *m/z* 107 in the mass spectrum of compound **4** also due to A-4 fragmentation, corresponded to a hydroxybenzyl / hydroxytropylium ion and a hydroxy group was thus placed at position 4'.

The ^{1}H NMR spectrum revealed a low field resonance at δ11.95 due to the C-5 hydroxyl group proton which forms a hydrogen bond with the oxygen of the carbonyl group at position 4. This resonance, assigned to the 5-OH proton, showed a NOESY correlation with the aromatic singlet at δ6.11 ascribed to H-6, which gave further support for the structure. Positive bathochromic shifts with $AlCl_3$ (+27nm) and NaOAc (+39nm) confirmed the existence of hydroxyl groups

at position 5 and 7. The HMBC spectrum showed correlations between the C-8 resonance and the singlet at δ3.83 integrating to three protons and the methoxy group was placed at position 8. Finally the NMR data of compound **4** (in CD_3OD) was compared to the literature NMR data of 3,9-dihydroeucomnalin [13] and 3,9-dihydropunctatin [18].

Table 2.7. ^{13}C NMR data of compound **4,** 3,9-dihydroeucomnalin [13] and 3,9-dihydropunctatin [18].



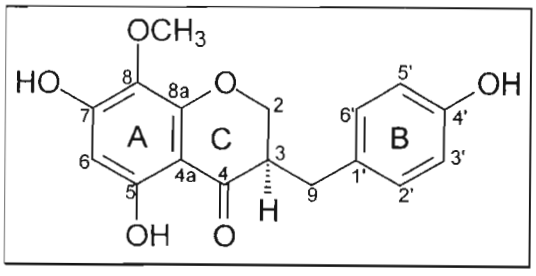
	Compound 4 *	**3,9-dihydroeucomnalin [13]****	**3,9-dihydropunctatin [18]****
2	70.5	70.5	70.5
3	48.0	49.1	48.0
4	199.4	199.9	199.3
4a	102.8	102.7	102.8
5	161.0	156.7	161.0
6	**97.0**	**130.4**	**97.2**
7	161.2	161.1	161.6
8	**129.6**	**95.9**	**130.1**
8a	155.6	160.0	157.1
9	33.0	32.9	33.1
1'	130.0	130.0	129.8
2'	131.1	131.0	131.1
3'	116.3	116.3	116.4
4'	157.2	157.1	155.6
5'	116.3	116.3	116.4
6'	131.1	131.0	131.1
6-OCH_3	-	60.9	-
8-OCH_3	61.4	-	61.5

*NMR data obtained in CD_3OD, 100MHz

**NMR data obtained in CD_3OD, 67.88MHz

The NMR data of compound **4** was in agreement with the proposed structure which was 3,9-dihydropunctatin. The IR data obtained for compound **4** was consistent with the proposed structure. Peaks were observed at 3384 cm^{-1} (OH stretching), 2930 cm^{-1} (aliphatic stretching), 1638 cm^{-1} (carbonyl stretching), 1514 cm^{-1} (aromatic C=C stretching) (see Chapter 3, p117). This compound was isolated from *Eucomis comosa* (Houtt.) Wehrh. before [12].

Table 2.8. NMR data for compound **4**, 3,9-dihydropunctatin.



	^{1}H NMR data for compound 4 *	^{13}C NMR data for compound 4**	HMBC Correlations C→H	NOESY correlations
2α	4.34 *dd* (4.2, 11.3)	69.4 (CH_2)	9α, 9β	2β, 3
2β	4.18 *dd* (7.2, 11.3)			2α, 9β, 2', 6'
3	2.81 *m*	46.7 (CH)	9β	2α, 9α, 2', 6'
4	-	197.7 (C)	2α,2β	-
4a	-	102.4 (C)	6	-
5	-	157.7 (C)	6	-
6	6.11 *s*	95.8 (CH)	-	-
7	-	160.2 (C)	6	-
8	-	129.0 (C)	8-OCH_3, 6	-
8a	-	152.9 (C)	2β	-
9α	3.17 *dd* (4.2, 13.8)	31.9 (CH_2)	2',6'	9β, 3, 2', 6'
9β	2.63 *dd* (10.2,13.5)			9α, 2', 6'
1'	-	129.7 (C)	3', 5', 2', 6'	-
2'	7.07 *d* (8.5)	130.2 (CH)	3, 9α, 9β	3, 9α, 9β, 3'
3'	6.78 *d* (8.5)	115.5 (CH)	-	2', 6'
4'	-	154.4 (C)	3',5',2',6'	-
5'	6.78 *d* (8.5)	115.5 (CH)	-	2', 6'
6'	7.07 *d* (8.5)	130.2 (CH)	3, 9α, 9β	3, 9α, 9β, 5'
8-OCH_3	3.83 *s*	61.5 (CH_3)	-	-
5-OH	11.95 *s*	-	4a, 6, 7	6

*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$, 100MHz

2.2.1.5. Structural elucidation of compound **5**, 5-methoxy-7-hydroxy-3-(4'-hydroxybenzyl)-4-chromanone

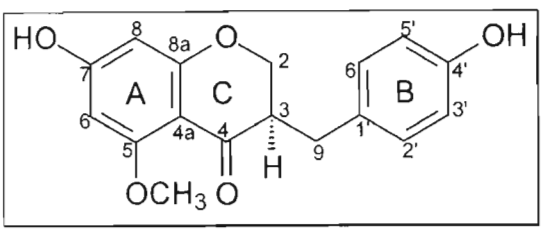
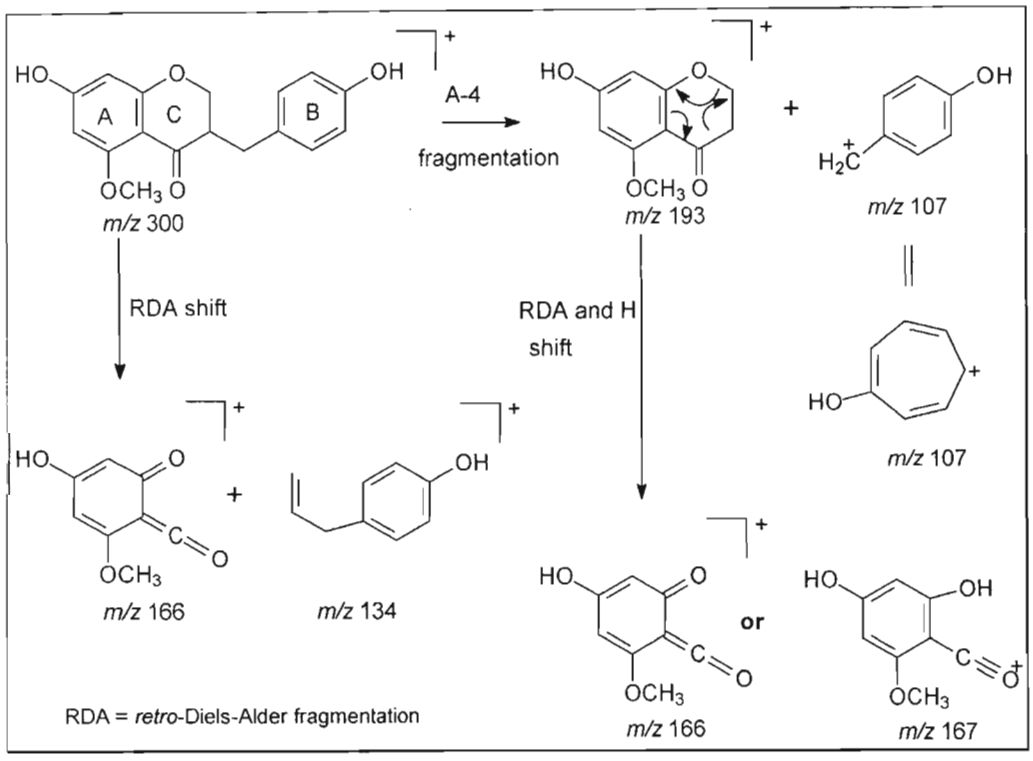


Figure 2.5. The structure of compound **5**, 5-methoxy-7-hydroxy-3-(4'-hydroxybenzyl)-4-chromanone

The ^{1}H NMR data indicated that this compound was a homoisoflavanone of the 3-benzyl-4-chromanone type. Two sets of double doublets at δ4.23, δ4.08 and δ3.03, δ2.63 in conjunction with the multiplet at δ2.72 form two ABX systems consisting of the two H-2, two H-9 and the H-3 protons. Again, the absolute stereochemistry is given here on the basis of the work done by Professor D. Ferreira [17]. The ^{1}H-NMR spectrum of compound **5** showed two *meta*-coupled proton doublets at δ6.11 (*J*=2.2) and δ5.99 (*J*=2.2) indicative of a 5,7-disubstituted ring A. The chemical shift of the C-4 carbonyl resonance in the ^{13}C NMR spectrum of homoisoflavanones can be used as a diagnostic feature to determine whether a hydroxyl or methoxy group is present at C-5. A hydroxyl group at C-5 results in a C-4 resonance at approximately δ198 ppm and a methoxy group at C-5 results in a C-4 resonance at approximately δ194 ppm [11]. The C-4 resonance for compound **5** was observed at δ194.2 and this suggested that a C-5 methoxy group was present [16]. A NOESY correlation between H-6 and the methoxy group proton resonance, gave further evidence of a C-5 methoxy group. Only one methoxy group proton resonance at δ3.84, could be found in the ^{1}H-NMR spectrum and position 7 must therefore have a hydroxyl group attached on biosynthetic grounds. The presence of low intensity peaks at *m/z* 193, 166 and 167 in the mass spectrum, corresponding to A4, RDA and H-

shift fragments confirmed the presence of a monohydroxy, monomethoxy substituted ring A.



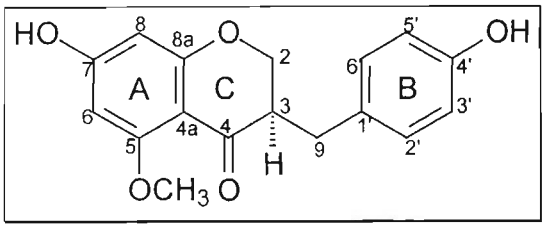
Scheme 2.4. Fragmentation pattern of the 3-benzyl-4-chromanone type applied to compound **5** [11].

The AA'BB' system in the ^{1}H-NMR spectrum consisting of two aromatic doublets integrating to two protons each at δ6.76 (*J*=8.4) and δ7.07 (*J*= 8.4) indicated that ring B was *para*-disubtituted. An intense peak at *m/z* 107 in the mass spectrum corresponded to the hydroxybenzyl / hydroxytropylium ion. The parent ion peak at *m/z* 300 was consistent with the proposed structure.

The IR data obtained for compound **5** was consistent with the proposed structure. Peaks were observed at 3186 cm^{-1} (OH stretching), 2927 cm^{-1} (aliphatic stretching), 1655 cm^{-1} (carbonyl stretching), 1516 cm^{-1} (aromatic C=C stretching) (see Chapter 3, p119). It was concluded that compound **5** is the

known compound 5-methoxy-7-hydroxy-3-(4'-hydroxybenzyl)-4-chromanone isolated previously from *Eucomis comosa* (Houtt.) Wehrh [12].

Table 2.9. NMR data for compound **5**, 5-methoxy-7-hydroxy-3-(4'-hydroxybenzyl)-4-chromanone



	^{1}H NMR data for compound 5 *	**^{13}C NMR data for compound 5****	**HMBC correlations C→H**	**NOESY correlations**
2α	4.23 *dd* (3.5,11.3)	69.6 (CH_2)	9α	3, 2β
2β	4.08 *dd* (5.9,11.3)		9α	2α
3	2.72[b] *m*	49.9 (CH)[a]	2β, 9α, 9β	2α
4	-	194.2 (C)	2β	-
4a	-	105.0 (C)	6,8	-
5	-	164.4 (C)	5-OCH_3, 6	-
6	6.11 *d* (2.2)	96.8 (CH)	-	5-OCH_3
7	-	167.0 (C)	6	-
8	5.99 *d* (2.2)	94.3 (CH)	-	-
8a	-	166.3 (C)	2β	-
9α	3.03 *dd* (9.1,10.3)	33.4 (CH_2)	2α, 2', 6'	9β, 3
9β	2.63[b] *dd* (10.3,c)		2α, 2', 6'	9α, 2', 6'
1'	-	130.6 (C)	-	-
2'	7.07 *d* (8.4)	131.1 (CH)	9α, 9β, 3'	9β, 3'
3'	6.76 *d* (8.4)	116.3 (CH)	-	2'
4'	-	156.8 (C)	3', 5', 2', 6'	-
5'	6.76 *d* (8.6)	116.3 (CH)	-	6'
6'	7.07 *d* (8.4)	131.1 (CH)	9α, 9β, 5'	9β, 5'
5-OCH_3	3.84 *s*	56.2 (CH_3)	-	6

*NMR data obtained in CD_3OD, 400MHz

**NMR data obtained in CD_3OD 100MHz

[a] hidden under solvent peaks

[b] resonances are obscured by each other

[c] unable to determine *J*-value

2.3. EXTRACTIVES FROM *GALTONIA PRINCEPS* (BAK.) DECNE.

princeps = first [1]

G. princeps belongs to the Ornithogaloideae subfamily [2] and is commonly known as the Berg Lily or Berglelie (Afrikaans) [3]. The plant was first found on the slopes of Mount-Aux-Sources in the Drakensberg Mountains, at seven to eight thousand feet by Flanagan [19]. Usually these plants can be found in marshy areas, amongst rocks and along the coast [6]. They have erect green leaves and pale green flowers are produced between November and February [6]. No documentation on the toxicity or ethnobotany of *G. princeps* could be found.

The collection was made in Richmond, Kwa-Zulu Natal (*Crouch 847*, NH).

2.3.1. Structural elucidation of compounds from *G. princeps.*

A stigmastene glycoside (compound **9**), an acylated cholestane glycoside (compound **7**) and a homoisoflavanone (compound **6)** were isolated from the methanol extract of *Galtonia princeps*. Compound **7** was acetylated and compound **8** was formed.

2.3.1.1. Structural elucidation of compound **6**, 7-*O*-methyleucomol.

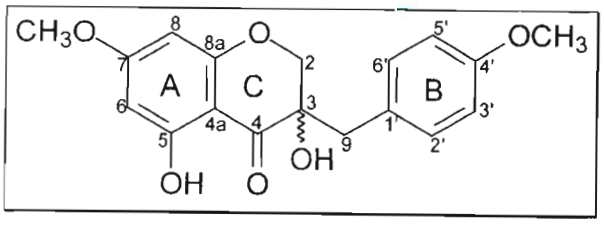
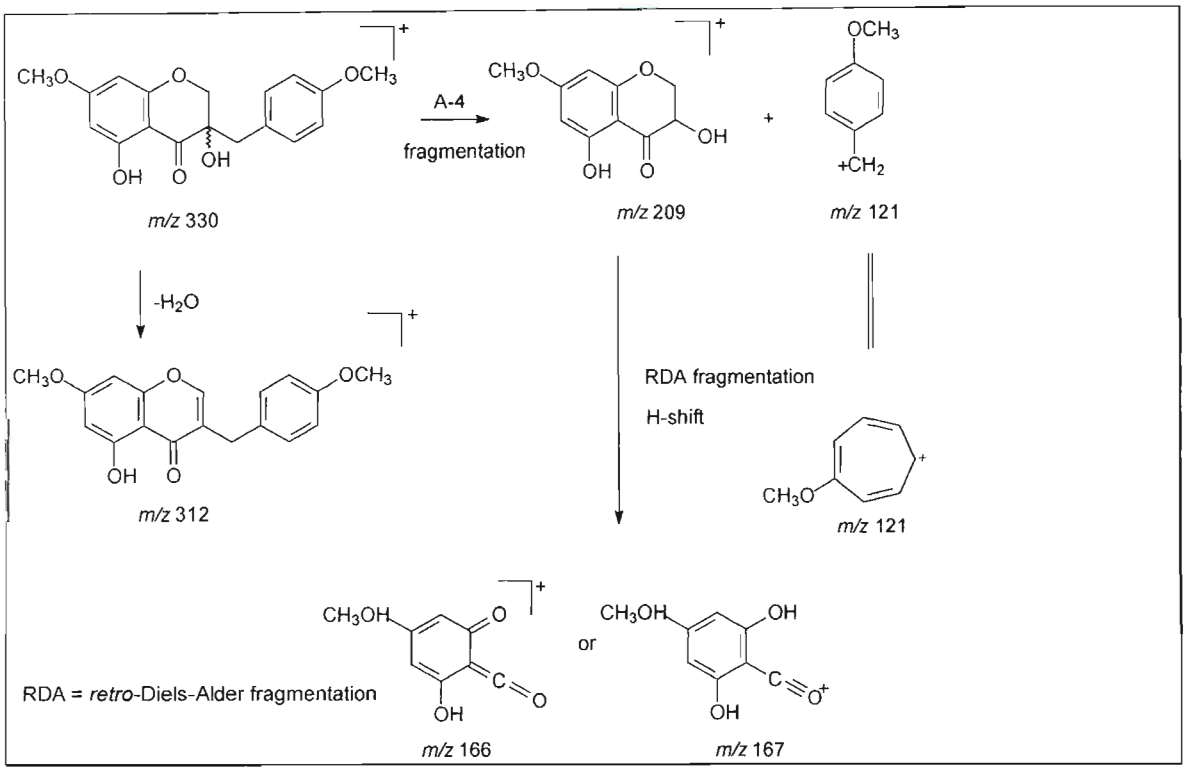


Figure 2.6. The structure of compound **6**, 7-*O*-methyleucomol

The ^{1}H NMR data revealed a pair of doublets at δ4.20 (*J*=11.0) and δ4.05 (*J*=11.0) ascribable to the two non-equivalent H-2 protons. The resonance, integrating to two protons at δ2.95 is typical of the two non-equivalent H-9 protons. If the chemical shift difference between resonances of coupling protons is small, which is the case with the 2H-9 resonances, the inner resonances become larger whilst the outer resonances become smaller to form an AB system [20]. The 2H-2, H-3, 2H-9 coupled system of the 3-benzyl-4-chromanone type of homoisoflavanones did not occur in this spectrum. The C-3 resonance, was found to occur as a fully substituted resonance at δ72.2, indicating that a tertiary hydroxyl group was present at C-3. The absolute stereochemistry of 3-OH determined by Professor D. Ferreira at the University of Mississippi, USA, for homoisoflavanones of this type indicated that these compounds occur as racemic mixtures. The $[\alpha]_D$ value of -35° observed for compound **6** in this study would therefore indicate that the compound is probably not a equal mixture of the two epimers. The pair of doublets at δ7.12 (*J*=8.7) and δ6.85 (*J*=8.7) integrated to two protons each and were assigned to H-3',5' and H-2',6' respectively. These are the typical resonances of the aromatic protons for a *para*-disubstituted B ring.

The ^{1}H-NMR spectrum revealed proton resonances for protons of two methoxy groups and one hydroxyl group at δ3.79 (*s*), δ3.84 (*s*) and δ11.24 (*s*) respectively. The remaining aromatic doublets at δ6.12 (*J*=2.2) and δ6.05 (*J*=2.2) were ascribed to the H-6 and H-8 protons and indicated that ring A was *tetra*-substituted. A downfield shift (>6.00 ppm) of the resonances of H-6 and H-8 is also an indication of a methoxy group at C-7 [13]. The H-6 and H-8 proton resonances showed correlations in the NOESY spectrum to the methoxy proton resonance at δ3.84. The doublet at δ6.85 ascribed to H-3'/5' showed correlations to the other methoxy protons resonance at δ3.79 in the NOESY spectrum. This resulted in the placement of the two methoxy groups at C-7 and C-4'. A proton resonance at δ11.24 was due to the proton of the hydroxyl group at C-5. The

downfield shift indicated hydrogen bonding between the oxygen of the C-4 carbonyl group and the proton of the hydroxyl group at C-5.

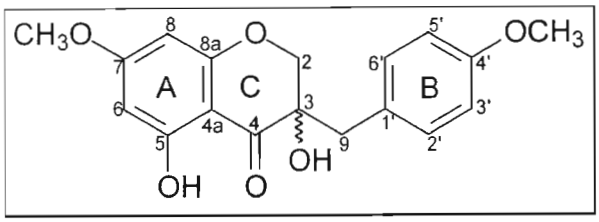


Scheme 2.5. Fragmentation pattern of the 3-benzyl-4-chromanone type applied to compound **6** [11].

The mass spectrum revealed a parent ion peak (which could not be clearly seen) at *m/z* 330 which was consistent with the proposed structure. The fragmentation ion peak at *m/z* 209 in the mass spectrum corresponded to the 3,5-dihydroxy-7-methoxy-4-chromanone fragment and gave further evidence to the suggested substitution in ring A. The base peak observed at *m/z* 121, due to the methoxybenzyl / methoxytropylium ion, made it possible to confirm the substitution on ring B. Loss of water produced the peak at *m/z* 312. The IR data obtained for compound **6** was also consistent with the proposed structure. Peaks were observed at 3142 cm^{-1} (OH stretching), 2927 cm^{-1} (aliphatic stretching), 1640 cm^{-1} (carbonyl stretching) and 1512 cm^{-1} (aromatic C=C stretching) (see

Chapter 3, p 120). These observations led to the conclusion that compound **6** is the known compound 7-*O*-methyleucomol isolated previously from *Eucomis bicolor* (Bak.) [14].

Table 2.10. Comparison of the ^{1}H NMR spectral data of compounds **6** and 7-*O* methyleucomol [14]



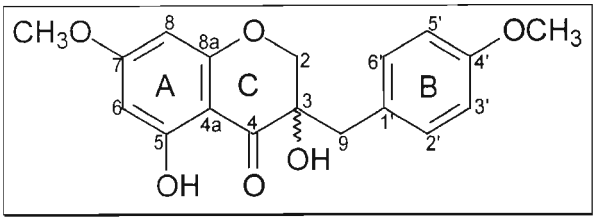
	Compound 6 *	**7-O-methyleucomol** [14]**
2a	4.20 *d* (11.0)	4.21 d (11.2)
2b	4.05 *d* (11.0)	4.06 d (11.2)
H-6	6.12 *d* (2.2)	6.12 d (2.3)
H-8	6.05 *d* (2.2)	6.05 d (2.3)
H-9a	2.95 *d* (13.0)	2.95
H-9b	2.95 *d* (13.0)	2.95
H-2'/H-6'	7.12 *d* (8.7)	7.12 d (8.8)
H-3'/H-5'	6.85 *d* (8.7)	6.85 d (8.8)
7-OCH_3	3.84 *s*	3.85 s
4'-OCH_3	3.79 *s*	3.8 s

*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$, 90MHz

The overview of Speta [2], revealed chemotaxonomic trends at a subfamily level for the Hyacinthaceae family of the *FSA* region and homoisoflavanones were found to define the Hyacinthoideae subfamily. This homoisoflavanone was, however, isolated from the Ornithogaloideae subfamily. The same compound was isolated from two species of this subfamily, *Galtonia princeps* and *Ornithogalum longibracteatum* [21].

Table 2.11. NMR data for compound **6**, 7-*O*-methyleucomol.



	^{1}H NMR data for compound 6 *	^{13}C NMR data for compound 6**	HMBC correlations C→H	NOESY correlations
2a	4.20 *d* (11.0)	71.8 (CH_2)	-	2b, 9', 2', 6'
2b	4.05 *d* (11.0)			2a
3	-	72.2 (C)	9a, 9b	-
4	-	198.2 (C)	2a, 2b, 9a, 9b	-
4a	-	100.5 (C)	5-OH, 6, 8	-
5	-	164.0 (C)	6, 5-OH	-
6	6.12 *d* (2.2)	95.4 (CH)	5-OH, 8	7-OCH_3, 5-OH
7	-	168.6 (C)	7-OCH_3, 6, 8	-
8	6.05 *d* (2.2)	94.5 (CH)	6	7-OCH_3
8a	-	162.7 (C)	2a, 8	-
9a	2.95 *d* (13.0)	40.7 (CH_2)	2a, 2b, 2', 6'	2', 6', 2a
9b	2.95 *d* (13.0)			
1'	-	126.0 (C)	3', 5', 9a, 9b	-
2'	7.12 *d* (8.7)	131.5 (CH)	6', 9a, 9b	3', 2b, 9'
3'	6.85 *d* (8.7)	113.7 (CH)	5'	2', 4'-OCH_3
4'	-	158.8 (C)	4-OCH_3,3',5',2',6'	-
5'	6.85 *d* (8.7)	113.7(CH)	3'	6', 4'-OCH_3
6'	7.12 *d* (8.7)	131.5 (CH)	9a, 9b, 2'	5', 2a, 9'
7-OCH_3	3.84 *s*	55.8 (CH_3)	-	6, 8
4'-OCH_3	3.79 *s*	55.2 (CH_3)	-	3', 5'
5-OH	11.24 *s*	-	-	6

*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$ 100MHz

2.3.1.2. Structural elucidation of compound **7**,3β,17α-dihydroxycholest-5-en-22-one 16β-*O*-α-*L*-arabinopyranoside.

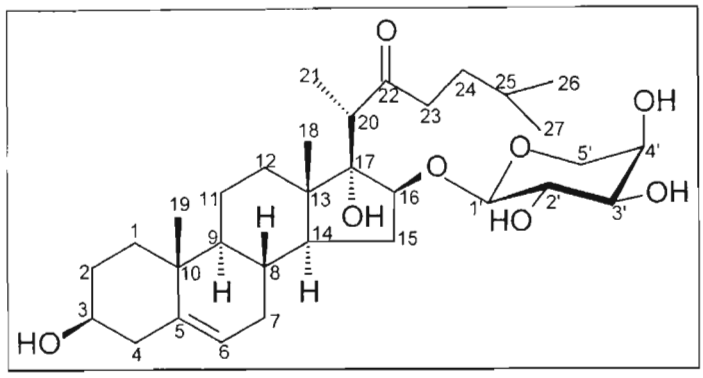


Figure 2.7. The structure of compound **7**, 3β,17α-dihydroxycholest-5-en-22-one 16β-*O*-α-*L*-arabinopyranoside.

Compound **7** was obtained as an amorphous powder from the methanol extract of *Galtonia princeps*. Although the parent molecular ion peak was not observed in the positive-ion FAB mass spectrum, a fragmentation ion peak at *m/z* 416 corresponding to the aglycone part of the molecule was observed. ^{1}H, ^{13}C, HSQC, HMQC, COSY, TOCSY and NOESY spectra were employed to determine the structure of compound **7** and to assign all the ^{1}H and ^{13}C NMR resonances (Table 2.12 and 2.13). The steroidal nature of the aglycone was indicated by the two angular methyl resonances at δ0.72 (3H-18, *s*) and δ0.86 (3H-19, *s*) in the ^{1}H NMR spectrum. Three methyl group proton doublets occurred at δ1.08, δ0.75 and δ0.72 and were assigned to 3H-21, 3H-26 and 3H-27. The 3H-21 resonance was seen to be coupled to the H-20 resonance (*q*, δ3.00) in the COSY spectrum and both the 3H-26 and 3H-27 resonances were seen to be coupled to the H-25 resonance at δ1.39. This made the assignment of C-20 (δ44.8, CH) and C-25 (δ26.5, CH) possible. The H-20 resonance occurred as a quartet with no further coupling and indicated fully substituted carbon atoms at C-17 and C-20. The resonance at δ82.6 (C) showed correlations to the 3H-18 and 3H-21 resonances in the HMQC spectrum and was assigned to C-17 and a

hydroxyl group was placed at this position. (This correlation confirmed the correct assignment of the 3H-18 and 3H-19 resonances). A carbonyl resonance at δ220.6 was placed at C-22 on the basis of a correlation to the 3H-21 resonance in the HMQC spectrum. The peak at 1642 cm^{-1} in the IR spectrum confirmed the presence of the carbonyl group (see Chapter 3, p120).

The methine carbon resonance at δ47.0, ascribed to C-14, showed correlations to the 3H-18 resonance in the HMQC spectrum. The corresponding H-14 resonance at δ1.49 was seen to be coupled to the two H-15 resonances at δ1.29 and δ2.10 in the TOCSY and COSY spectra. The two H-15 resonances were further coupled to a resonance at δ3.77 assigned to H-16. The corresponding C-16 resonance at δ88.3 was seen to correlate to the anomeric proton at δ102.6 in the HMQC spectrum, indicating the presence of a monosaccharide at C-16. The H-16 resonance showed correlations to the H-15α resonance which, in turn, showed correlations to the H-14α resonance in the NOESY spectrum. A literature search showed that, in similar compounds groups were attached at C-16β [24].

A trisubstituted alkene double bond was indicated by the resonances at δ140.5 (C) and δ120.2 (CH) in the ^{13}C NMR spectrum. A secondary hydroxyl group was present at C-3β as is usually seen in these compounds. The H-3 resonance at δ3.35 was seen to be coupled to the resonance at δ2.10 ascribed to the superimposed H-4 resonances. The H-4 resonances, in turn, were seen to be long range coupled to the H-6 resonance at δ5.15, indicating a 5,6-double bond. The C-5 resonance at δ140.5 showed a correlation to the 3H-19 resonance in the HMQC spectrum.

The anomeric carbon resonance was displayed at δ102.6 in the ^{13}C-NMR spectrum of compound **7**, and the other resonances arising from the sugar moiety occurred at δ70.6 (CH), δ71.8 (CH), δ66.6 (CH), and δ64.4 (CH_2). Overlapping peaks made it difficult to define the nature of the sugar and

compound **7** was acetylated to give compound **8**. Since a tetra-acetate was found, acetylation took place at the hydroxyl group at C-3 and at the three hydroxyl groups which were associated with the monosaccharide. The hydroxyl group at C-17 was not acetylated as it is tertiary.

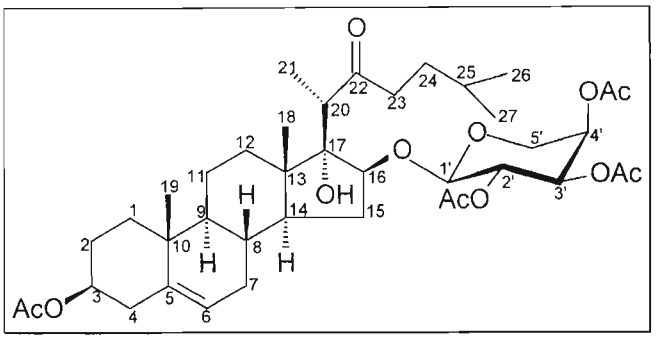
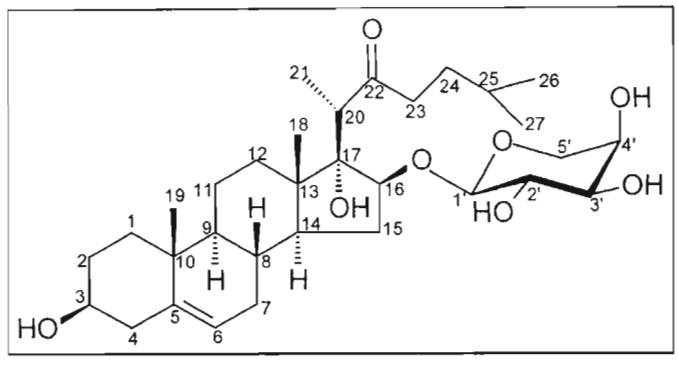


Figure 2.8. The structure of compound **8**, the acetylation product of compound **7**.

The ^{1}H-NMR spectrum of compound **8** showed better resolution of the proton resonances of the sugar moiety. Using the anomeric proton resonance at δ4.33 as a starting point, the COSY spectrum was analysed. Correlations between H-1'/H-2', H-2'/H-3', H-3'/H-4', H-4'/2H-5' could be identified. This sugar was either arabinopyranoside or xylopyranoside, depending on the stereochemistry. In the NOESY spectrum of compound **8** correlations between H-4' and H-3' were observed. Since these correlations would have been impossible in a xylose molecule the sugar molecule was assigned to be arabinopyranoside. In pyranosides, the six-membered ring usually adopts a fixed conformation and protons are classified as axial or equatorial. Therefore coupling patterns are characteristic of the stereochemistry of the pyranosides [22,23]. The ^{1}H NMR spectrum of compound **7** showed the anomeric proton's resonance as a doublet at δ3.85 with a coupling constant of 6.4 Hz. According to Agrawal [23], a coupling constant of *J*~7 Hz is assignable to the anomeric proton of α-*L*-arabinopyranose because the *J*-value (following the Karplus relation) suggests a *trans-diaxial* relationship of the protons at C-1' and C-2' of pyranose residues [22,23]. Compound **7** was thus assigned as 3β,17α-dihydroxycholest-5-en-22-one 16β-*O*-

α-*L*-arabinopyranoside, a new compound. This compound is closely related to the acylated cholestane glycosides (3β,17α-dihydroxycholest-5-en-22-one compounds) from *Ornithogalum saundersiae* isolated by Kubo *et al.*, [24], it is a 16β-*O*-α-*L*-arabinopyranoside derivative.

Table 2.12. NMR data for the aglycone of compound **7**.



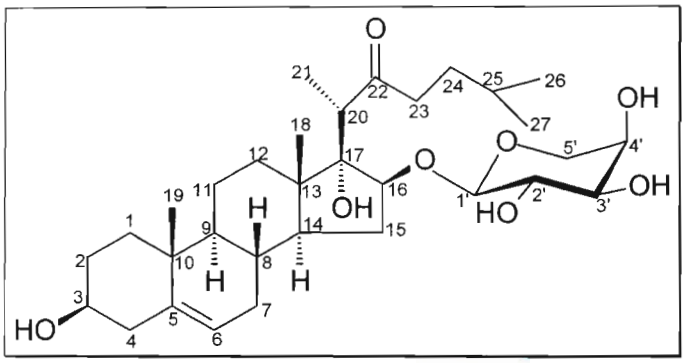
	^{1}H NMR data for compound 7 *	**^{13}C NMR data for compound 7****	**HMQC correlations C→H**	**NOESY correlations**	**COSY correlations**
1	0.95α	36.1 (CH_2)	6, 19	3	2α, 2β
	1.72β			19	2α, 2β
2	1.65 α	31.1 (CH_2)	-	3	1α,1β, 3
	1.33 β			-	1α,1β, 3
3	3.35 [1]	70.1 (CH)	4	1α	2α, 2β, 4
4	2.10[3]	40.7 (CH_2)	6	-	3, 6
5	-	140.5 (C)	19, 4	-	-
6	5.15 *br d* (4.1)	120.2 (CH)	4	4	4, 7α, 7β
7	1.43 α[3]	30.7 (CH_2)	6	9	6, 8
	1.81 β[3]			8	6, 8
8	1.32[3]	30.6 (CH)	-	7β, 18	7α, 7β, 9
9	0.82[3]	48.4 (CH)	19	14	8, 11
10	-	36.8 (C)	-	-	-
11	1.42[3]	19.4 (CH_2)	-	-	-
12	1.28[3]	30.8 (CH_2)	18	-	-
13	-	[2]	-	-	-
14	1.49[3]	47.0 (CH)	18	9	15α, 15β
15	2.10 α[3]	33.3 (CH_2)	-	14, 16	14, 16
	1.29 β[3]			18	14, 16
16	3.77 *dd* (5.4, 8.6)	88.3 (CH)	15β, 1'	15α	15α, 15β
17	-	82.6 (C)	15α, 16, 18, 21	-	-
18	0.72 *s*	12.3 (CH_3)	-	20	-
19	0.86 *s*	18.1 (CH_3)	-	1β, 4, 8	-
20	3.00 *q* (7.3)	44.8 (CH)	18, 21	18	21
21	1.08 *d* (7.3)	10.6 (CH_3)	20	20	20

Table 2.12 continued

22	-	220.6 (C)	20, 21, 23	-	-
23	2.55 *t*-like	38.1 (CH_2)	-	25, 26	24
24	1.35[3]	30.0 (CH_2)	23	-	-
25	1.39[3]	26.5 (CH)	23, 26, 27	-	26, 27
26	0.75 *d* (6.4)[4]	21.4 (CH_3)[4]	25	-	25
27	0.72 *d* (6.4)[4]	20.9 (CH_3)[4]	25	-	25

*NMR data obtained in $CDCl_3$ / CD_3OD, 400MHz
**NMR data obtained in $CDCl_3$ / CD_3OD, 100MHz
[1]Resonance overlapping with sugar resonances
[2]Resonance not observed
[3]Overlapping resonances
[4]Interchangeable resonances

Table 2.13. NMR data for the glycoside moiety of compound **7**.



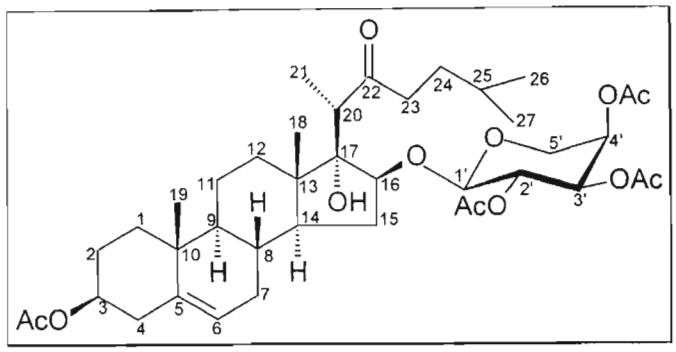
	^{1}H NMR data for glycoside moiety of compound 7 *	**^{13}C NMR data for glycoside moiety of compound 7****	**HMQC correlations C→H**	**COSY correlations**
1'	3.85 *d* (6.4)	102.6 (CH)	16, 5'	2'
2'	3.35[1]	70.6(CH)	-	-
3'	3.35[1]	71.8 (CH)	5'	-
4'	3.68 *br s*	66.6 (CH)	5'	3', 5'
5'	3.35[1] 3.73 *dd* (12.3,3.2)	64.4 (CH_2)	1'	4'

*NMR data obtained in $CDCl_3$/CD_3OD, 400MHz

**NMR data obtained in $CDCl_3$ / CD_3OD, 100MHz

[1] Overlapping resonances

Table 2.14. NMR data for the aglycone of compound **8**.



	^{1}H NMR data for compound 8 *	^{13}C NMR data for compound 8**	HMQC correlations C→H	NOESY correlations	COSY correlations
1	1.13α[1]	38.8 (CH_2)	19	-	2α, 2β
	1.87β[1]			2β	2α, 2β
2	1.85α[1]	33.7(CH_2)	-	-	1α,1β, 3
	1.53β[1]			-	1α,1β
3	4.60 *m*	75.8 (CH)	-	-	2α, 4a
4	2.37a[1]	41.2 (CH_2)	-	-	3
	2.53b[1]			6	4a
5	-	141.6 (C)	19	-	-
6	5.36 *br d* (5.5)	124.3 (CH)	-	4b	4a, 7a, 7b
7	1.93a[1]	34.2 (CH_2)	-	-	6, 8
	1.61b[1]			-	6, 8
8	1.52[1]	33.8 (CH)	-	18	7a, 9
9	0.98[1]	51.3 (CH)	19	-	8, 11
10	-	38.5 (C)	-	-	-
11	2.05[1]	22.4 (CH_2)	-	-	9
12	1.21[1]	29.4 (CH_2)	13, 14	-	-
13	-	48.0 (C)	-	-	-
14	1.65[1]	49.8 (CH)	18	-	15α
15	2.25α[1]	40.0 (CH_2)	-	-	14, 16
	1.32β[1]			18	16
16	3.90 *dd* (5.5, 8.2)	91.1(CH)	1'	1'	15α, 15β
17	-	87.2 (C)	18, 21	-	-
18	0.86 *s*	15.3 (CH_3)	-	20	-
19	1.01 *s*	21.2 (CH_3)	-	1β, 8	-
20	3.00 *q* (7.3)	47.8 (CH)	18, 21	18	21
21	1.20 *d* (7.3)	13.5 (CH_3)	-	-	20
22	-	220.5 (C)	21	-	-
23	2.25[1]	35.7 (CH_2)	-	-	24
24	1.73[1]	31.6 (CH_2)	26/27	-	23, 25
25	1.35[1]	29.7 (CH)	26/27	-	24, 26, 27
26	0.89 *d* (6.4)[2]	24.1(CH_3)[2]	-	-	25
27	0.90 *d* (6.4)[2]	24.5 (CH_3)[2]	-	-	25

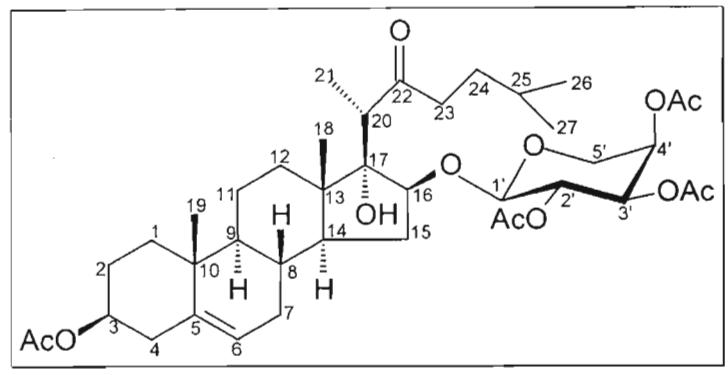
*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$, 100MHz

[1]Overlapping resonances

[2]Interchangeable resonances

Table 2.15. NMR data for the glycoside moiety of compound **8**.



	^{1}H NMR data for compound 8 *	**^{13}C NMR data for compound 8****	**HMQC correlations C→H**	**NOESY correlations**	**COSY correlations**
1'	4.33[1]	101.5(CH)	5b	16	2'
2'	4.90 *dd* (7.3, 5.0)	71.6 (CH)	-	-	1', 3'
3'	5.03 *dd* (7.3, 3.2)	71.2 (CH)	-	4'	2', 4'
4'	5.19 *m*	68.7 (CH)	-	3'	5'a, 5'b
5'	3.58 *dd* (12.3,2.7)a 3.95 *dd* (12.3,5.5)b	63.3(CH_2)	1'	5'b	4', 5'a
				5'a	4', 5b

*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$, 100MHz

[1]Resonance obscured by other resonances

2.3.1.3. Structural elucidation of compound **9**, sitosterol 3-*O*-β-*D*-glucopyranoside.

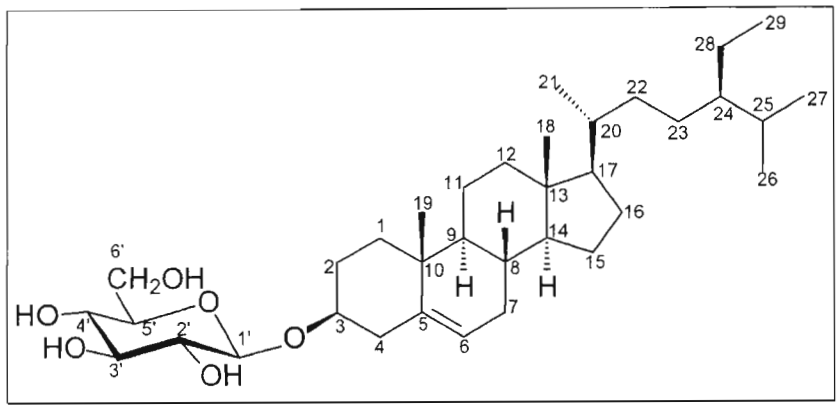


Figure 2.9. The structure of compound **9**, sitosterol 3-*O*-β-*D*-glucopyranoside.

Compound **9** was isolated from the methanol extract of *Galtonia princeps* as an amorphous powder (see Chapter 3, p121). This powder was insoluble in normal solvents and a mixture of methanol and chloroform was generally used as solvent system. Two tertiary methyl singlets were observed at δ0.56 (3H-18) and δ0.89 (3H-19) and three secondary methyl resonances at δ0.80 (3H-21, *d*, *J*=6.4), δ0.71 (3H-26/27, *d*, *J*=6.8) and δ0.72 (3H-26/27, *d*, *J*=6.8) and a primary methyl group at δ0.71 (3H-29, *t*, *J*=6.4) in the ^{1}H NMR spectrum. These are typical resonances associated with sitosterol. One anomeric carbon resonance was observed at δ100.9 in the ^{13}C NMR spectrum and indicated the presence of a monosaccharide.

The C-3 resonance showed correlations to the anomeric proton doublet at δ4.39 in the HMQC spectrum and the sugar moiety was placed at C-3. All the glycoside resonances were assigned making use of HSQC, HMQC and COSY spectra as well as literature values [22,23]. The NMR data indicated that the sugar could be a glucopyranoside or a galactopyranoside. These two sugars differ only in the stereochemistry of the H-4' proton. However the limited chemical shift range and the presence of homonuclear ^{1}H-^{1}H spin-coupling in sugars often

lead to non-first order spectra. The resonance of importance (H-4') in this case overlaps with the H-3' proton resonance. A NOESY correlation between the H-2' resonance and the H-3'/H-4' resonance gave evidence that the sugar is a glucopyranoside. A NOESY correlation would not be possible between the H-2' resonance and the H-3' resonance in gluco- or galactopyranoside. Thus, this NOESY correlation can only exist between the resonances of H-2' and H-4' of glucose.

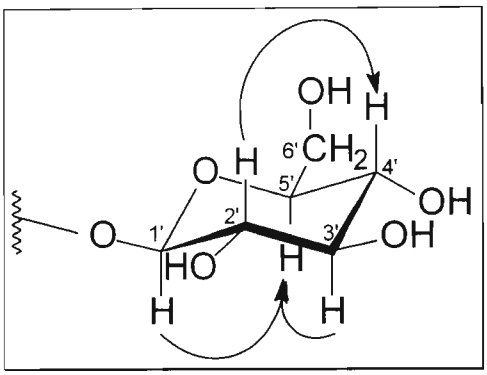


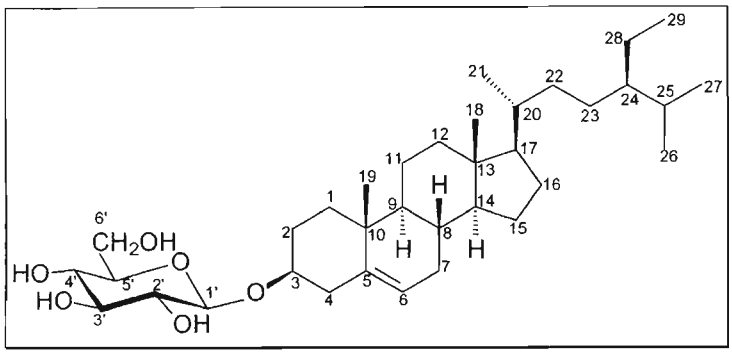
Figure 2.10. The NOESY correlations giving evidence of a β-*D*-glucopyranoside

A molecular ion peak was observed at *m/z* 577 $[M+H]^+$ which corresponded to the $C_{35}H_{60}O_6$ structure of compound **9** suggested by NMR data. The fragmentation peak at *m/z* 414 corresponded to the loss of a glucose molecule.

Finally the ^{13}C NMR data of compound **9** was compared to the literature ^{13}C NMR data. A literature search showed that the ^{13}C NMR data was generally run in pyridine. Compound **9** was dissolved in chloroform / methanol (80:10) mixture because it was easier to recover the compound from this mixture, for biological tests, than from pyridine. However, the chemical shift values of compound **9** was, in all cases, in conformity with the reported values for sitosterol 3-*O*-β-*D*-glucopyranoside. The melting point and $[\alpha]_D$ values of compound **9** were also identical to those reported for sitosterol 3-*O*-β-*D*-glucopyranoside [25,26]. The literature data of the aglycone of sitosterol 3-*O*-β-*D*-tri-*O*-acetylxylopyranose

which was run in a suitable solvent system was compared to the aglycone of compound **9** in Table 2.16 [27].

Table 2.16. ^{13}C NMR data of the aglycone of compound **9** and the aglycone of sitosterol 3-*O*-β-*D*-tri-*O*-acetylxylopyranose [27]

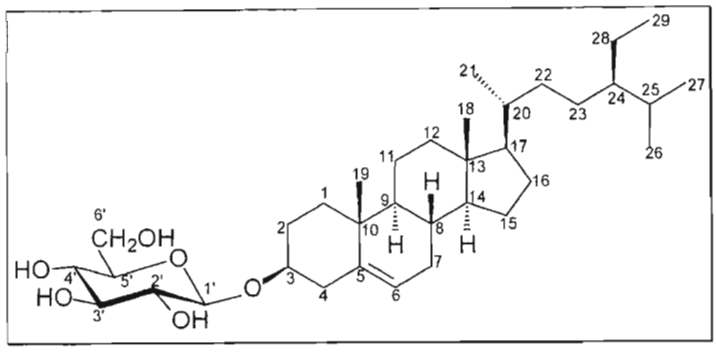


	Compound 9 *	aglycone of sitosterol 3-*O*-β-*D*-tri-*O*-acetylxylopyranose [27]**
1	37.0 (CH_2)	37.0 (CH_2)
2	29.4 (CH_2)	29.5 (CH_2)
3	78.9 (CH)	79.9 (CH)
4	38.5 (CH_2)	38.7 (CH_2)
5	140.3 (C)	140.2 (C)
6	121.9 (CH)	121.9 (CH)
7	31.7 (CH_2)	31.7 (CH_2)
8	31.7 (CH)	31.7 (CH)
9	50.0 (CH)	50.0 (CH)
10	36.5 (C)	36.6 (C)
11	20.8 (CH_2)	21.0 (CH_2)
12	39.5 (CH_2)	39.6 (CH_2)
13	42.5 (C)	42.2 (C)
14	55.8 (CH)	56.6 (CH)
15	24.1(CH_2)	24.2 (CH_2)
16	28.0 (CH_2)	28.1 (CH_2)
17	56.5 (CH)	55.9 (CH)
18	11.6 (CH_3)	11.7 (CH_3)
19	19.0 (CH_3)	19.2 (CH_3)
20	35.9 (CH)	36.0 (CH)
21	18.5 (CH_3)	18.6 (CH_3)
22	33.7 (CH_2)	34.0 (CH_2)
23	25.8 (CH_2)	26.0 (CH_2)
24	45.6 (CH)	45.7 (CH)
25	28.9 (CH)	29.2 (CH)
26	19.5 (CH_3)	19.6 (CH_3)
27	18.7 (CH_3)	18.9 (CH_3)
28	22.8 (CH_2)	23.0 (CH_2)
29	11.7 (CH_3)	11.8 (CH_3)

*NMR data obtained in $CDCl_3/CD_3OD$, 100MHz

**NMR data obtained in $CDCl_3$, 20.15MHz

Table 2.17. NMR data for the aglycone of compound **9**.



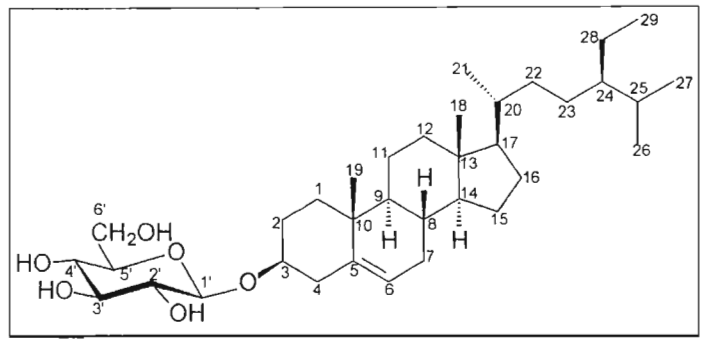
	^{1}H NMR data for compound 9 *	^{13}C NMR data for compound 9**	HMQC correlations C→H	NOESY correlations
1	0.91[1] 1.22[1]	37.0 (CH_2)	9	-
2	1.42β[1] 1.86α[1]	29.4 (CH_2)	-	19 -
3	3.45 *m*	78.9 (CH)	1', 4α	4α
4	2.28α[1] 2.14β[1]	38.5 (CH_2)	-	- 6
5	-	140.3 (C)	19	-
6	5.25 *br s*	121.9 (CH)	4α	-
7	1.38[1] 1.88[1]	31.7 (CH_2)	-	-
8	1.36[1]	31.7 (CH)	-	18, 19
9	0.81[1]	50.0 (CH)	19	-
10	-	36.5 (C)	-	-
11	0.72[1] 1.41[1]	20.8 (CH_2)	-	-
12	1.07[1] 1.90[1]	39.5 (CH_2)	18	-
13	-	42.5 (C)	18	-
14	1.05[1]	55.8 (CH)	9, 18	9, 17
15	1.04[1]	24.1(CH_2)	-	-
16	1.79[1]	28.0 (CH_2)	-	-
17	0.90[1]	56.5 (CH)	18	21
18	0.56 *s*	11.6 (CH_3)	-	8, 20
19	0.89 *s*	19.0 (CH_3)	9	8
20	1.26[1]	35.9 (CH)	21	18
21	0.80 d (6.4)	18.5 (CH_3)	-	
22	1.18[1] 1.73[1]	33.7 (CH_2)	21	-
23	1.12[1] 1.18[1]	25.8 (CH_2)	-	-
24	0.80[1]	45.6 (CH)	26, 27, 28, 29	-
25	1.51[1]	28.9 (CH)	26, 27	-
26	0.71 *d* (6.8)	19.5 (CH_3)	27	-
27	0.72 *d* (6.8)	18.7 (CH_3)	26	-
28	0.73[1]	22.8 (CH_2)	26, 27	-
29	0.71 *t* (6.4)	11.7 (CH_3)	-	-

*NMR data obtained in $CDCl_3$/CD_3OD, 400MHz

**NMR data obtained in $CDCl_3$ / CD_3OD, 100MHz

[1]Overlapping resonances

Table 2.18. NMR data for the sugar moiety of compound **9**



	^{1}H NMR data for compound 9 *	**^{13}C NMR data for compound 9****	**HMQC correlations C→H**	**NOESY correlations**
1'	4.30 *d* (7.8)	100.9 (CH)	3, 2'	5'
2'	3.20	73.3 (CH)	-	4'
3'	3.39[1]	76.2 (CH)	2', 4'	5'
4'	3.39[1]	70.0 (CH)	3'	2'
5'	3.31 m	75.5 (CH)	6' b	1'
6'	3.72a *dd* (3.2,11.9)	61.6 (CH_2)	4'	-
	3.65b *dd* (4.5,11.9)			

*NMR data obtained in $CDCl_3/CD_3OD$, 400MHz

**NMR data obtained in $CDCl_3$ / CD_3OD, 100MHz

[1]Resonances overlapping

Compound **9** is the common compound, sitosterol 3-*O*-β-*D*-glucopyranoside. Sitosterol and its derivatives possess important biological activities. Sitosterol is commercially produced from soya beans for semi-synthesis of medicinal steroids such as products used to reduce blood cholesterol levels [32].

2.4. EXTRACTIVES FROM *ORNITHOGALUM TENUIFOLIUM* DELAROCHE

tenuifolium = slender leaves [1]

O. tenuifolium belongs to the subfamily Ornithogaloideae [2] and is also known as *O. virens*, *O. ecklonii*, *O. inconspicuum* and *O. pretoriense*. The common names of this plant are: common chincherinchee, bosui (Afrikaans), moretele-o-monyenyane (Southern Sotho) [4].

O. tenuifolium is widespread from South Africa to Tropical Africa and is found mainly in grassland and thickets [6]. The large bulbs look structurally like an onion but lack the characteristic odour of onions, giving rise to the common name, 'bosui' [3]. The leaves are long and slender, tapering to the tip. Flowers are white with a central green strip and are sweetly scented in some areas [6].

Repeated sheep drenching experiments by Steyn *et al.*, [28], revealed that *O. tenuifolium* was not toxic. However other southern African *Ornithogalum* species like *O. saundersiae* and *O. thyrsoides* are highly toxic [7]. 'Chincherinchee poisoning' in cattle is symptomized by severe intestinal tract inflammation and frequently with blindness [29]. This is attributed to the cytotoxicity of cholestane glycosides isolated from these species. Although extra-*FSA Ornithogalum* species like *O. boucheanum* [30] and *O. nutans* [31] produce cardiac glycosides with digitalis-like activity, no such compounds have yet been found in the southern African Hyacinthaceae. Zulu people use *O. tenuifolium* as a charm by means of which an enemy can cause one's cow to miscarry or to go dry [7].

Two collections were made. The first specimen was purchased from the Warwick Triangle herbal market in Durban (*Crouch 832*, NH), and the second collection was made in Ashburton, Kwa-Zulu Natal (*Crouch 845*, NH).

2.4.1. Structural elucidation of compounds from *O. tenuifolium.*

Only fatty acids and small amounts of sterols were found in the first specimen purchased from the herbal market. A steroidal saponin was isolated from the methanol extract of *O. tenuifolium* collected in Ashburton.

2.4.1.1. Structural elucidation of compound **10**, 25*R*,5β-spirostane-1β,3α-diol.

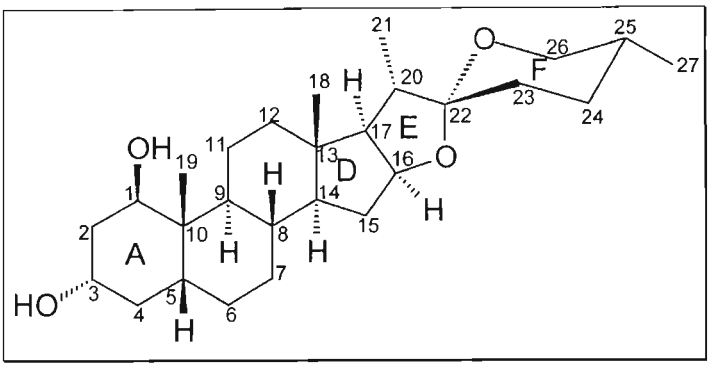


Figure 2.11. The structure of compound **10**, 25*R*,5β-spirostane-1β,3α-diol.

Compound **10** was isolated from the methanol extract of *Ornithogalum tenuifolium* (see Chapter 3, p123). The mass spectrum revealed a parent ion peak at *m/z* 432 which was consistent with the proposed molecular formula, $C_{27}H_{44}O_4$. The double bond equivalence calculated indicated the presence of six rings as no double bond resonances were present in the NMR spectra. Thus apart from the normal four rings associated with a triterpenoid skeleton, two extra rings occurred. The resonances of two tertiary methyl groups were observed as singlets at δ0.69 and δ0.99 in the ^{1}H-NMR spectrum, and were assigned to 3H-18 and 3H-19 respectively. The HMQC spectrum showed correlations between the carbon resonance at δ73.9 (CH) assigned to C-1 and the 3H-19 proton resonance. Correlations between the corresponding H-1 methine proton resonance at δ3.94 and the 2H-2 proton resonances which, in turn, correlated to another methine proton resonance at δ4.12, assigned to H-3, were observed in the COSY spectrum. Hydroxyl groups were thus placed at C-1 and C-3. The

NOESY spectrum showed correlations between the 3H-19 resonance and the H-1, H-5β and H-8β resonances. The H-5β resonance in turn correlated to the H-3β resonance and revealed that the hydroxyl group at C-3 must be α-orientated. A model showed that a correlation between the 3H-19 and H-1 resonances was possible in the NOESY spectrum, whether H-1 was α- or β-orientated. However, X-ray analysis confirmed that the hydroxyl group at C-1 was β-orientated.

The methine carbon resonance at δ62.2 was assigned to C-17 and correlated to the 3H-18 resonance and the 3H-21 doublet at δ0.89 in the HMQC spectrum. An acetal carbon resonance at δ109.2 also showed a correlation to the 3H-21 resonance in the HMQC spectrum and was assigned to C-22. The COSY spectrum showed correlations between the 3H-21 resonance and H-20 (δ1.84), between H-20 and H-17 (δ1.74), and between H-17 and H-16 (δ4.40). The chemical shift of the H-16 resonance indicated that an oxygen group was attached to C-16. The two extra rings required by the molecular formula could be explained by a spirostane skeleton with C-16, C-22 and C-22, C-26 ether linkages. The C-22 resonance showed correlations to the methylene proton resonances at δ3.39 and δ3.47 assigned to 2H-26 in the HMQC spectrum. The chirality at C-22 is fixed during biosynthesis by the stereospecificity in the formation of the ketal [32].

Correlations occurred between the 2H-26 resonances (δ3.47 and δ3.39) and the H-25 resonance at δ1.62 were seen in the COSY spectrum. The doublet integrating to three protons at δ0.81 (J=6.2) was assigned to 3H-27 and also showed correlations to the H-25 resonance in the COSY spectrum.

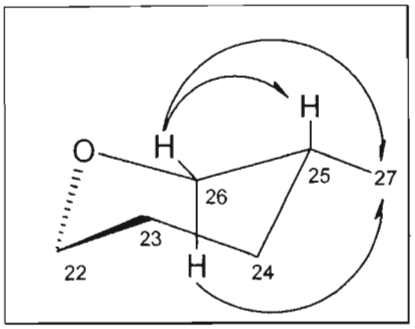
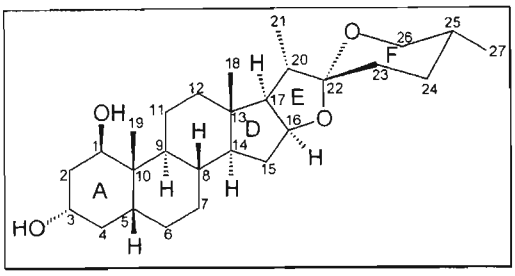


Figure 2.12. NOESY correlations observed in ring F of compound **10**

The NOESY spectrum showed correlations between the H-26 resonance at δ3.47 and the 3H-27 and H-25 resonances. The H-26 resonance at δ3.39 correlated only with 3H-27. This indicated that H-26 (δ3.47) and 3H-27 are possibly equatorial protons. If 3H-27 is equatorial, then H-25 and H-26 (δ3.39) must be *trans-diaxial*. The X-ray data confirmed the deductions made from the NOESY spectrum.

Table 2.19. NMR data for compound **10**, 25*R*,5β-spirostane-1β,3α-diol.



	^{1}H NMR data for compound 10 *	^{13}C NMR data for compound 10**	HMQC correlations C→H	NOESY correlations	COSY Correlations
1	3.94 *t*-like (2.8)	73.9 (CH)	19	11, 19	2α, 2β
2	1.59α[1]	36.9 (CH_2)	-	-	1, 3
	1.95β[1]			1α	1, 3
3	4.12 *m*	66.5 (CH)	4	2β, 5	2α, 2β, 4α, 4β
4	1.55β[1]	35.8 (CH_2)	-	3β, 5	3, 5
	1.80α[1]			-	3, 5
5	1.64[1]	35.5 (CH)	19	6β, 3	6α
6	1.19α[1]	26.1 (CH_2)	-	-	5
	1.47β[1]			5	-
7	1.63α[1]	31.3 (CH_2)	-	-	8
	1.71β[1]			6 β	-
8	1.89[1]	35.4 (CH)	14	6β	9, 14
9	1.42[1]	41.8 (CH)	19	12α	8
10	-	38.9 (C)	19	-	-
11	1.33[1]	20.6 (CH_2)	-	1α	12α, 12β
12	1.12 α[1]	40.1 (CH_2)	18	17	11
	1.70 β[1]			-	11
13	-	40.3 (C)	14, 18	-	-
14	1.15[1]	56.1 (CH)	15β, 18	9	8, 15α, 15β
15	1.29β[1]	31.7 (CH_2)	14	18β	14, 16
	2.01α[1]			16	14, 16
16	4.40 *q*-like (7.5,7.7)	80.7 (CH)	15α, 15β	15α, 17	15α, 15β, 17
17	1.74[1]	62.2 (CH)	15α, 18, 21	16	16, 20
18	0.69 *s*	16.4 (CH_3)	12, 14	8β	-
19	0.99 *s*	18.3 (CH_3)	9	1α, 8, 5	-
20	1.84[1]	41.6 (CH)	21	-	21, 17
21	0.89 *d* (6.9)	14.4 (CH_3)	20	20, 17	20
22	-	109.2 (C)	20, 21, 23ax, 25, 26ax,26eq	-	-
23	1.46eq[1]	28.7 (CH_2)	25, 27	24eq	-
	1.66ax[1]			-	-
24	1.32ax[1]	26.5 (CH_2)	-	-	25
	1.76eq[1]			23eq	25
25	1.62[1]	30.2 (CH)	27	26eq, 21α	24(ax,eq),26(ax,eq)
26	3.47eq *t*-like (10.9)	66.8 (CH_2)	27	27eq, 25ax	25
	3.39 *ax br d* (10.7)			27eq	25
27	0.81 *d* (6.2)	17.1 (CH_3)	24	25ax	25

*NMR data obtained in $CDCl_3$/CD_3OD, 400MHz

**NMR data obtained in $CDCl_3$ / CD_3OD, 100MHz , [1]Overlapping resonances

Crystals of compound **10** (crystallized from methanol, space group P1), were amenable to X-ray crystallography. The structure and relative configuration of the compound could be established unambiguously.

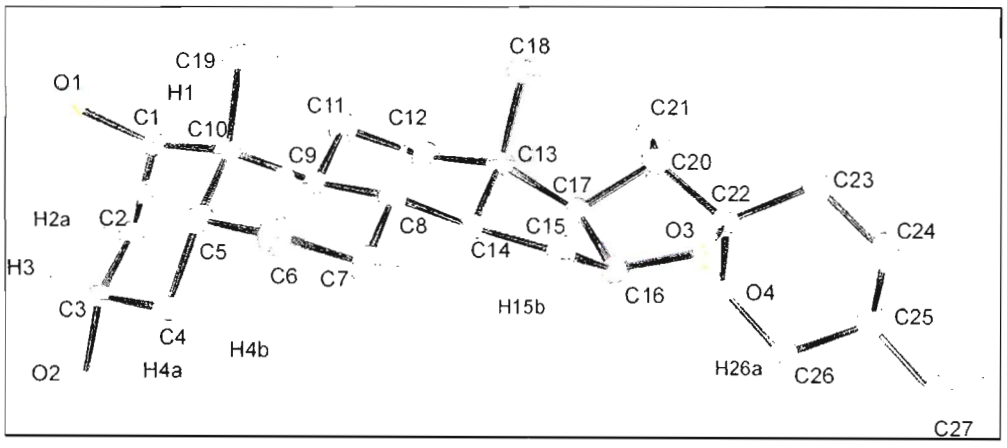


Figure 2.13. Labelled ORTEP diagram (30% probability ellipsoids) of compound **10**

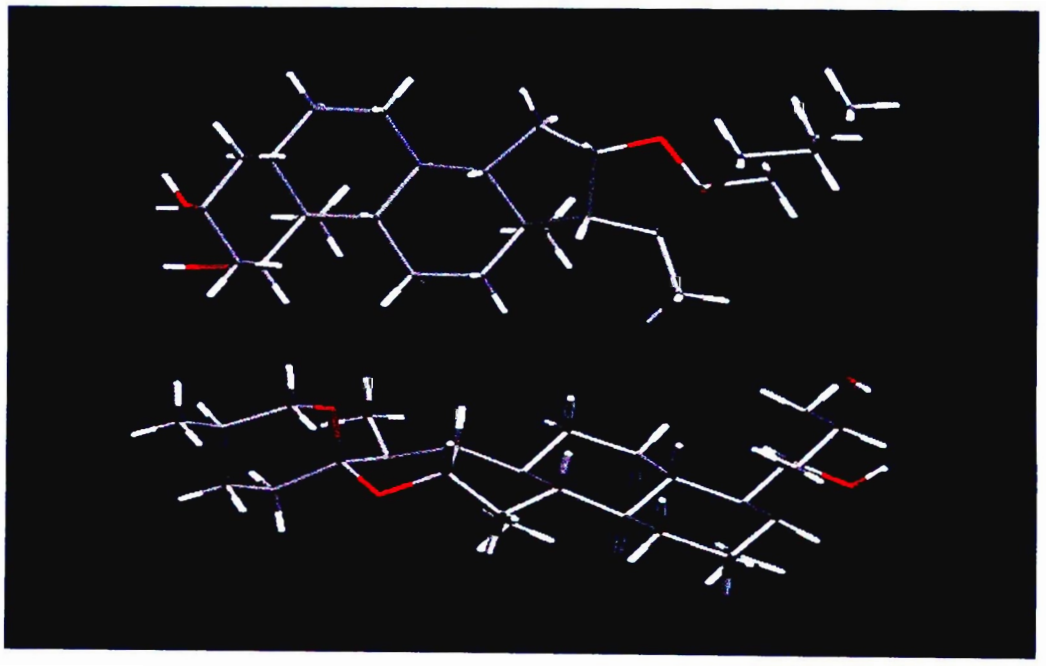


Figure 2.14. Asymmetric unit from the crystal of compound **10** (two independent molecules)

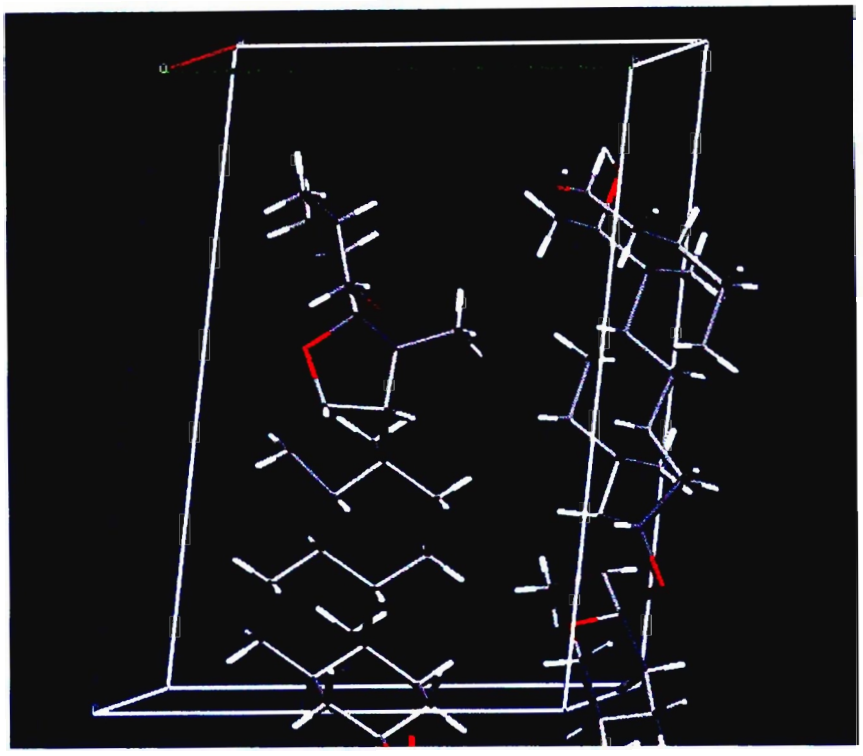


Figure 2.15. Unit cell contents

Accurate information regarding the size of the dihedral angles in compound **10** was available from the crystal structure. The Karplus equation was used to calculate exact coupling constants.

Karplus equation: $J_{AB}= 8.5(\cos^2\theta)-0.28$ for angles $<90°$

$J_{AB}= 9.5(\cos^2\theta)-0.28$ for angles $>90°$

Where: J is the coupling constant and

θ the dihedral angle.

Unfortunately, overlapping of peaks in the ^{1}H-NMR spectrum of compound **10** made the determination of coupling constants difficult.

Table 2.20. Correlation between *J* values obtained from X-ray data and NMR data of compound **10**.

	Dihedral angle θ(deg) from x-ray data	*J*(calc)(Hz)	*J*(obs)(Hz) from NMR data
H(1)H(2b)	57.5	2.2	2.8
H(15a)H(16)	154.1	7.4	7.5
H(16)H(17)	-2.8	8.1	7.7
H(25)H(26a)	180	9.2	10.9

The aim of the correlation of *J*-values obtained from NMR- and X-ray data was to determinate whether compound **10** adopted the same conformation in solution and the solid state. The *J*-values correlated reasonably well and compound **10** probably adopts a similar conformation in both states. It must be remembered that these equations are mere estimates and not 100% accurate [33].

Patil and coworkers [34], published the X-ray data of 25*R*, 5α-spirostane-1β,3β-diol.

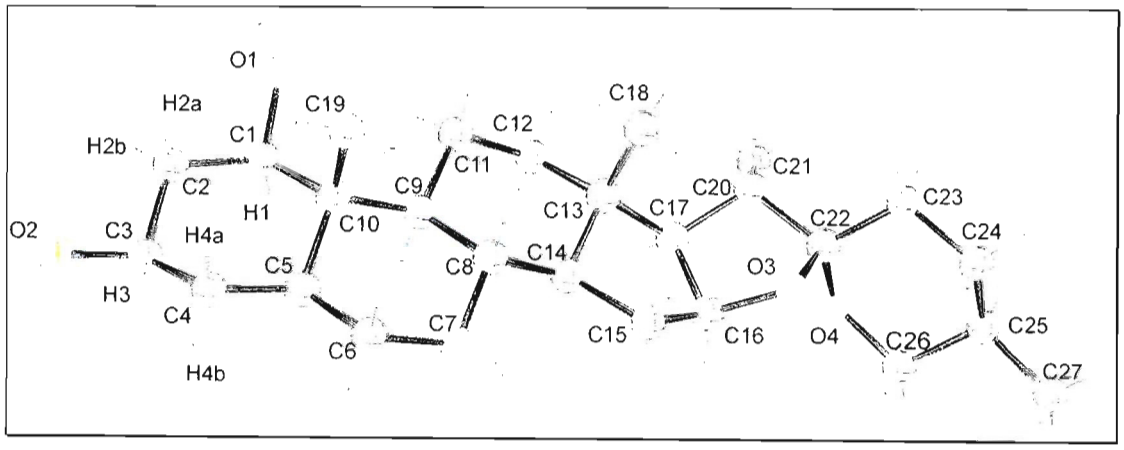
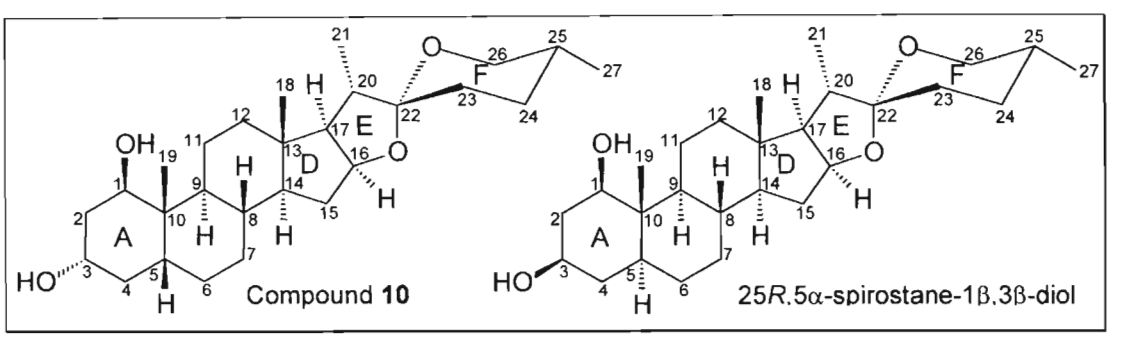


Figure 2.16. The X-ray structure of 25*R*, 5α-spirostane-1β,3β-diol, isolated by Patil *et al.* [34]

25*R*,5α-spirostane-1β,3β-diol [34] compound **10**

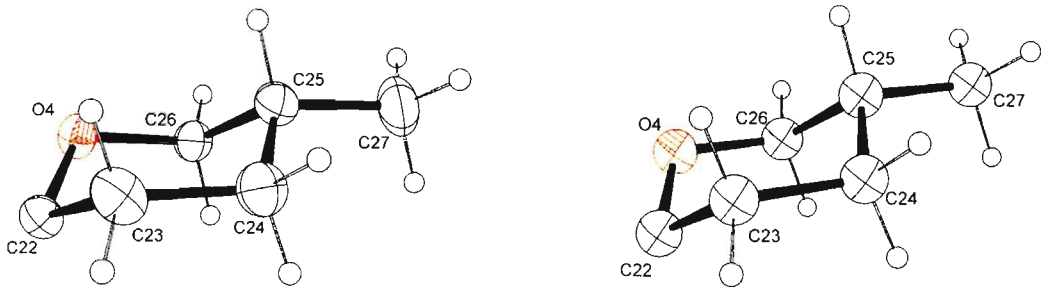
Figure 2.17. Comparison of the basic structures of 25*R*,5α-spirostane-1β,3β-diol [34] and compound **10**.

Table 2.21. Selected dihedral angles (deg) for compounds **10** and 25*R*,5α-spirostane-1β,3β-diol [34].



Dihedral angles(deg)	Compound 10	25R,5α-spirostane-1β,3β-diol [34]
H(1)C(1)C(2)C(2a)	-60.4	-177.6
H(1)C(1)C(2)C(2b)	57.5	-59.0
H(2a)C(2)C(3)H(3)	-57.3	175.4
H(2b)C(2)C(3)H(3)	-175.2	57.4
H(3)C(3)C(4)H(4b)	172.0	-59.0
H(3)C(3)C(4)H(4a)	54.8	-178.4
H(4b)C(4)C(5)H(5)	-55.7	-174.5
H(4a)C(4)C(5)H(5)	-172.9	66.3
H(5)C(5)C(10)C(19)	50.2	-178.9
H(8)C(8)C(9)H(9)	-177.1	-177.1
C(18)C(13)C(14)H(14)	173.6	171.6
C(21)C(20)C(22)O(4)	-50.2	-42.8
C(17)C(20)C(22)O(4)	-148.5	9.4
H(16)C(16)C(17)H(17)	-2.8	9.1

The dihedral angle H(5)C(19) in compound **10** (50.2°) differed from the angle in 25*R*,5α-spirostane-1β,3β-diol [34] (-178.9°). The difference suggested a *cis*-fusion of rings A and B in compound **10** (H-5 acquired the β-orientation) as opposed to the *trans*-fusion in 25*R*,5α-spirostane-1β,3β-diol [34]. The X-ray data supports the NMR data except for the orientation of H-1 which seems to be β according to the NOESY correlations of the H-1 resonance with 3H-19. Although H-1 adopts the α-orientation in both compounds, the dihedral angles suggest that H-1 is equatorial in compound **10** and axial in 25*R*,5α-spirostane-1β,3β-diol [34]. Thus the orientation of H-1 in compound **10** makes interactions with protons that are β-orientated possible. The orientation of H-3 is β in compound **10** and α in 25*R*,5α-spirostane-1β,3β-diol [34].



compound **10** 25*R*,5α-spirostane-1β,3β-diol [34]

Figure 2.18. Comparison of F rings of compound **10** and 25*R*,5α-spirostane-1β,3β-diol [34]

The above figure shows the chair conformations adopted by the F rings in both compounds. The methyl groups of both compounds are equatorial and indicated that the compounds had the 25*R* configuration. Again, the NMR data was supported by the equatorial and axial positions that the hydrogens adopted in ring F. The conclusion was made that compound **10** was 25*R*,5β-spirostane-1β,3α-diol. The X-ray data is new and has not been published previously.

2.5. EXTRACTIVES FROM *URGINEA LYDENBURGENSIS* R.A. DYER.

U. lydenburgensis belongs to the subfamily Urgineoideae [2] and is also known by the common names gifbol (A) [3], bergtulp (Afrikaans) and slangkop (Afrikaans) [7].

The plants only occur in the Lydenburg district [7]. Much confusion exists between the *Urginea* and the closely related *Drimia* genus, partly because of an attempt to unite the genera [35]. *U. lydenburgensis* R.A. Dyer [=*Drimia delagoensis* (Baker) Jessop] has a red bulb and long slender leaves [7]. Leaves are usually produced after flowers and flowers are dense on a long erect stem resembling a snake, hence its common name 'slangkop' [6].

U. lydenburgensis forms part of a highly toxic indigenous genus that is only occasionally cultivated [36]. It came under suspicion as a poisonous plant in South Africa in 1937 because of stock poisoning [37]. In drought years the plants sprout during the September to November period, drawing on their own reserves [38]. Hungry animals eating the green sprouts experience anorexia, tympanites, duiresis and fairly severe diarrhoea. Poisoning with these plants is usually fatal [7].

The bulbs of *Urginea* species growing in Zululand and Transkei are irritating to the skin. Some produce such irritation that they are used by small boys in endurance battles. The plants are also used as anthelmintics, for bronchial asthma, heart conditions, fevers and during pregnancy [5].

A collection was made in Nelspruit, Mpumalanga (*Crouch 864*, NH)

2.5.1. Structural elucidation of compounds from *U. lydenburgensis.*

Two bufadienolides were isolated from the methylene chloride extract of *Urginea lydenburgensis.* The other extracts contained fatty acids, sugars and small amounts of sterols.

2.5.1.1. Structural elucidation of compound **11**, 16β-acetoxyberscillogenin.

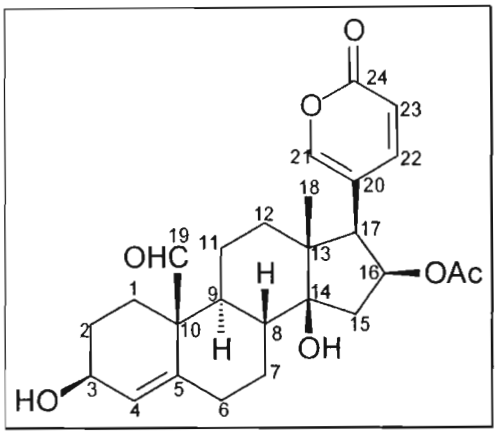


Figure 2.19. The structure of compound **11**, 16β-acetoxyberscillogenin.

The mass spectrum of compound **11** gave the highest peak at *m/z* 456 corresponding to the proposed structure, and other peaks at *m/z* 396 and *m/z* 378 indicating the loss of a molecule of acetic acid and a molecule of water. Compound **11** was characterized as a bufadienolide by the proton and carbon resonances of the δ-pyrone ring. Two doublets at δ7.21 (*J*=2.5) and δ6.17 (*J*=9.8) were seen to be coupled to a double doublet at δ7.95 (*J*=2.5, 9.8) in the ^{1}H NMR spectrum and were assigned to H-21, H-23 and H-22 respectively.

The HMBC spectrum showed correlations between the C-21 and C-22 resonances and a doublet at δ2.84 (*J*=8.8) ascribed to H-17. The corresponding C-17 resonance showed correlations to a singlet integrating to three protons at δ0.77 which was ascribed to 3H-18. A quaternary carbon resonance in the δ80-

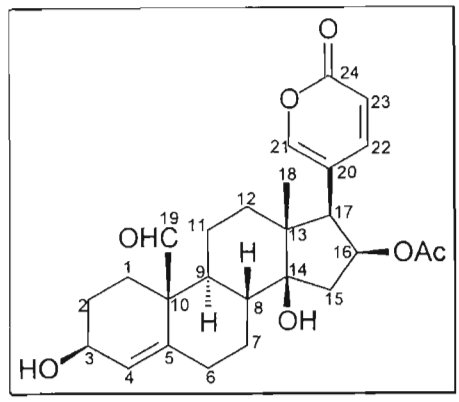
85 region of the ^{13}C-NMR spectrum usually indicates C-14 when a 14β-hydroxyl group is present – a characteristic feature of most known bufadienolides [32,39]. The correlation between the resonance at δ83.8 (C) ascribed to C-14, and the methyl group proton resonance ascribed to 3H-18 in the HMBC spectrum, confirmed the presence of the hydroxyl group at C-14. The C-14 resonance showed a correlation to the doublet of triplets at δ5.47 (*J*=1.3, 8.8), ascribed to H-16 in the HMBC spectrum, which in turn, was seen to be coupled to the doublet at δ2.84 (*J*=8.8), ascribed to H-17, in the COSY spectrum. The downfield shift of the H-16 proton at δ5.47, as well as a correlation between the acetate carbon resonance at δ169.9 and H-16 resonance in the HMBC spectrum indicated that an acetate group must be placed at C-16. A correlation between the H-16 resonance and the H-17 resonance in the NOESY spectrum indicated that H-16 is α-orientated.

The HMBC spectrum showed correlations between the C-14 resonance and the resonance at δ1.82 ascribed to H-8. Positive correlations were also observed between the H-8β resonance and the 3H-18 resonance in the NOESY spectrum. The H-8 resonance was seen to be coupled to the H-9 and 2H-7 resonances and the 2H-7 resonances, in turn, was seen to be coupled to the 2H-6 resonances in the COSY spectrum. An alkene proton resonance at δ5.71 in the ^{1}H NMR spectrum, ascribed to H-4, was seen to be coupled to a methine resonance at δ4.15 ascribed to H-3. The COSY spectrum indicated the presence of two other sets of methylene protons in the same spin system and they were assigned to 2H-1 and 2H-2. A hydroxyl group was placed at C-3 and the double bond at C-4. The quaternary carbon resonance at δ53.1, ascribed to C-10, showed correlations to the 2H-1 resonance as well as the resonance at δ9.75 which was assigned to the proton of an aldehyde group at C-10. The NMR data for compound **12** correlated well with that of other bufadienolides containing aldehydes in the same position [39].

The IR data obtained for compound **11** was consistent with the proposed structure. Peaks were observed at 3456 cm^{-1} (OH stretching), 2934 cm^{-1} (aliphatic stretching), 1714 cm^{-1} (C=O stretching), 1533 cm^{-1} (C=C stretching), 1086 cm^{-1} (C-O stretching) (see Chapter 3, p124).

Taking all this information into account compound **11** was identified as 16β-acetoxy-3β,14β-dihydroxy-19-formyloxo-bufa-4,20,22-trienolide (16β-acetoxyberscillogenin). The 3β,16β-diacetate derivative of this compound was obtained by acetylation of berscillogenin isolated from *Bersama abyssinica* by Kupchan *et al.*, [40] but compound **11** has not been described previously. No NMR data of similar structures in a suitable solvent system was available for comparison with the NMR data of compound **11**.

Table 2.22. NMR data for compound **11**, 16β-acetoxyberscillogenin.



	^{1}H NMR data for compound 11 *	^{13}C NMR data for compound 11**	HMBC correlations C→H	NOESY correlations	COSY Correlations
1	2.34[1]	32 6 (CH_2)	4	19, 4	2α, 2β
2	1.33β[1]	29.5 (CH_2)	4	19	1, 3
	2.08α[1]			3	1, 3
3	4.15 *br s*	66.5 (CH)	1, 2α, 2β	2α	2α, 2β, 4
4	5.71 *s*	129.9 (CH)	2α, 6β	1, 3	3, 6β
5	-	137.3 (C)	1, 6β	-	-
6	1.18α[1]	27.72 (CH_2)	-	7α	7α, 7β
	2.40β[1]			-	4, 7α, 7β
7	1.12β[1]	28.8 (CH_2)	-	-	6α, 6β, 8
	2.18α[1]			9α	6α, 6β, 8
8	1.82[1]	43.3 (CH)	-	18, 19	7α, 7β,9
9	1.22[1]	49.4 (CH)	6α	7α	8, 11a, 11b
10	-	53.1 (C)	1, 2α, 4, 19	-	-
11	1.50a[1]	21.4 (CH_2)	-	-	9, 12α
	1.88b[1]				9, 12α
12	1.24α[1]	40.0 (CH_2)	18	17	11
	1.60β[1]			18	11
13	-	49.2 (C)	15, 17	-	-
14	-	83.8 (C)	8, 15, 16,17,18	-	-
15	2.46α[1]	39.7 (CH_2)	17	16α	16
	1.82β[1]			18	16
16	5.47 *dt* (1.3,8.8)	73.2 (CH)	15, 17	15α, 17	15α, 15β,17
17	2.84 *d* (8.8)	56.8 (CH)	15, 18	12α, 16α, 21	16
18	0.77 *s*	16.3 (CH_3)	-	8, 21, 22	-
19	9.75 *s*	202.9 (CH)	1, 2α, 2β	1, 8, 2β	-
20	-	116.5 (C)	17, 21, 23	-	-
21	7.21 *d* (2.5)	151.0 (CH)	17	17	22, 23
22	7.95 *dd* (2.5,9.8)	148.8 (CH)	17, 21	18, 23	21, 23
23	6.17 *d* (9.8)	113.2 (CH)	-	22	22
24	-	161.8 (C)	21, 23	-	-
16-OCO**C**H_3	1.84 *s*	20.9 (CH_3)	-	18	-
16-O**C**OCH$_3$	-	169.9 (C)	16, 16-OCO**C**H_3	-	-

*NMR data obtained in $CDCl_3$, 400MHz

**NMR data obtained in $CDCl_3$, 100MHz

[1]Overlapping resonances

2.5.1.2. Structural elucidation of compound **12**, lydenburgenin.

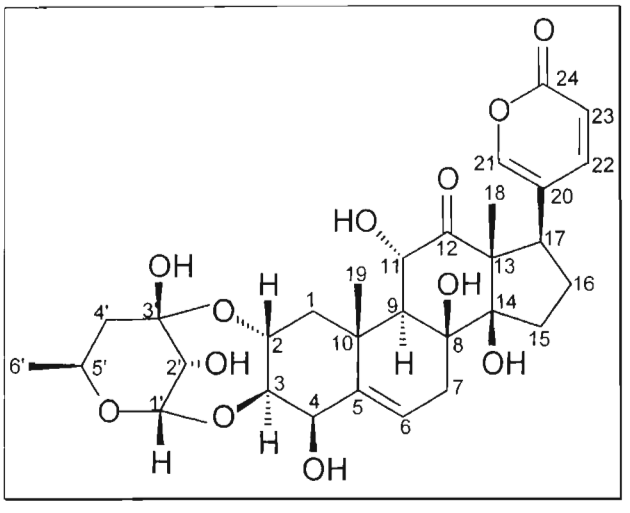


Figure 2.20. The structure of compound **12**, lydenburgenin

Use was made of ^{1}H, ^{13}C, HSQC, HMBC, COSY, TOCSY and NOESY spectra to determine the structure of compound **12** and to assign ^{1}H and ^{13}C NMR resonances (Table 2.23). The ^{1}H NMR spectrum of compound **12** indicated the presence of a bufadienolide side-chain with doublets at δ7.51 (*J*=2.5Hz) and δ6.31 (*J*=9.7Hz) and a doublet of doublets at δ7.95 (*J*=2.5, 9.7Hz) assigned to H-21, H-23 and H-22 respectively. The NOESY spectrum showed correlations between C-21 and a doublet of doublets at δ4.14 ascribed to H-17 of which the corresponding carbon resonance at δ41.5 correlated to the 3H-18 singlet at δ1.11 in the HMBC spectrum.

Six HC-O resonances were indicated by methine resonances at δ83.1, δ77.9, δ71.6, δ70.4, δ69.1 and δ65.5 in the ^{13}C NMR spectrum and corresponding resonances at δ3.65, δ4.28, δ5.03, δ4.28, δ4.57 and δ4.28 in the ^{1}H NMR spectrum. Quaternary C-O carbon resonances at δ100.2, δ86.1 and δ74.4 were also observed. The resonance at δ86.1 (C) was ascribed to C-14 on the basis of HMBC correlations with the 3H-18 resonance and a hydroxyl group was placed

at C-14. The TOCSY spectrum confirmed this as it showed that apart from H-17 the spin system only contained the 2H-16 and 2H-15 protons. The carbonyl resonance at δ213.9 was ascribed to a carbonyl group at C-12 as it showed correlations to the 3H-18 resonance in the HMBC spectrum. Correlations were also observed between the C-12 resonance and a methine proton resonance at δ5.03 ascribed to H-11 in the HMBC spectrum, and a hydroxyl group was placed at C-11. The resonance ascribed to H-11 gave a correlation with 3H-18 as well as with the methyl group proton singlet at δ1.68 ascribed to 3H-19 in the NOESY spectrum indicating that H-11 adopted the β-configuration. The COSY spectrum showed that the H-11 doublet at δ5.03 was only coupled with a resonance at δ1.63 ascribed to H-9 and that H-9 was coupled to no other protons. A hydroxyl group was thus placed at C-8. The tertiary carbon resonance at δ74.4 (C), ascribed to C-8, showed correlations to the 2H-7 protons at δ2.32 in the HMBC spectrum, which, in turn, were coupled only to the H-6 proton resonance at δ5.72 in the COSY spectrum, and confirmed that a hydroxyl group was present at C-8. The stereochemistry at C-8 and C-14 could not be confirmed but a literature search showed that bufadienolides containing these groups have them in the β-configuration.

A trisubstituted alkene double bond was indicated by resonances at δ124.1 (CH) and δ139.8 (C) in the ^{13}C NMR spectrum. The corresponding proton resonance at δ5.72 was ascribed to H-6 previously. The tertiary carbon resonance at δ139.8, ascribed to C-5, was seen to correlate with the 3H-19 resonance as well as with the methine proton resonance at δ4.28 ascribed to H-4 in the HMBC spectrum. The alkene group was placed at C-5 and a secondary hydroxyl group at C-4. The doublet of doublets (J=3.8, 7.6 Hz) at δ3.65, ascribed to H-3, was seen to be coupled with H-4 as well as with the double doublet at δ4.57 (J=13.6, 2.9, 7.2 Hz) ascribed to H-2 in the COSY spectrum. The HMBC spectrum showed correlations between the C-2 resonance and the H-1 resonance at δ3.00 and between C-1 and the methyl group proton resonance, ascribed to 3H-19.

The stereochemistry at C-2, C-3 and C-4 was determined from the NOESY spectrum. The H-9 resonance which was assigned as α-orientated on biosynthetic grounds, gave a positive correlation with the H-4 resonance in the NOESY spectrum, indicating that it was α-orientated, and the H-4α resonance, in turn, showed a correlation with the H-3 resonance indicating that it was α-orientated. The resonance which was assigned to H-2 showed a positive NOESY correlation with the 3H-19 resonance indicating that H-2 adopted the β-configuration. The stereochemistry of H-2β and H-3α was confirmed by the coupling constant of ≈7 Hz [41].

Two resonances which appeared to be due to hemiacetal and hemiketal carbons were observed at δ99.7 (CH) and δ100.2 (C) in the ^{13}C NMR spectrum suggesting the presence of a sugar moiety. Use was made of HSQC, HMBC and COSY spectra and four carbon resonances at δ65.5 (CH), δ36.9 (CH_2), δ70.4 (CH) and δ20.7 (CH_3) could be ascribed to the sugar. Correlations between C-3 and the hemiacetal proton resonance ascribed to H-1' in the HMBC spectrum as well as positive correlations in the NOESY spectrum between H-2 and a resonance at δ4.28 which forms part of the sugar moiety indicated that the sugar was attached to C-2 and C-3. Overlapping of resonances at δ4.28 made it difficult to determine the nature of the sugar and compound **12** was thus acetylated to form compound **13**.

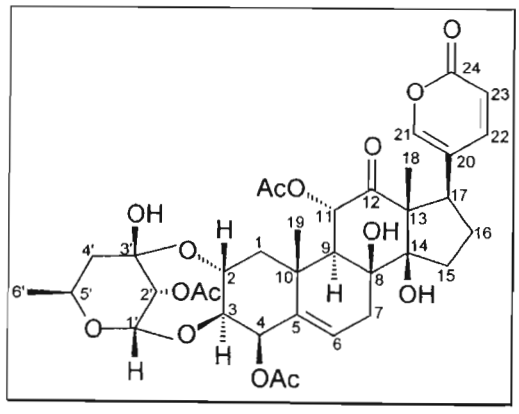


Figure 2.21. The structure of compound **13**, the acetylation product of compound **12**.

A tri-acetate had been formed and acetylation had occurred at the secondary hydroxyl groups at C-4, C-11 and C-2' as can be seen from the downfield chemical shifts of the resonances. Correlations seen in the COSY spectrum between the resonances of H-1' (hemiacetal carbon at δ98.6) and H-2', H-5' and 2H-4' as well as H-5' and 3H-6' enabled the assignment of all the glycoside resonances. The COSY spectrum also made it possible to differentiate between two spin systems in the sugar moeity containing the H-1' and H-2' protons and the 2H-4', H-5' and 3H-6' protons respectively. NOESY correlations were observed between the H-2'β proton resonance and the resonances of H-1'β and H-2β. The hemiketal carbon resonance at δ99.7 (C) was assigned to C-3' which showed correlations to the H-1' and H-2' resonances in the HMBC spectrum. Because of the necessity of a *cis*-fusion in bridged six-membered rings, the hydroxyl group at C-3' should adopt the β-orientation. Finally NOESY correlations between the proton resonance ascribed to H-5'α at δ4.33 and H-3α allowed the assignment of the β-orientation to the methyl group in position 6'. The sugar moiety was identified as a 2'-epimer of 4'-deoxyrhamnose.

The mass spectrum of compound **13** gave the highest peak at *m/z* 429, but the NMR spectroscopy indicated that the molecular formula is $C_{36}H_{44}O_{15}$ (see Chapter 3, p125). This could be explained by the loss of two molecules of acetic acid and the acetylated sugar moiety. Compound **12** was identified as a novel bufadienolide related to rubellin which had been identified as the main toxic compound of *Urginea rubella* [41]. The NMR data of rubellin was not compared to that of compound **12**, as the solvent system used was not suitable.

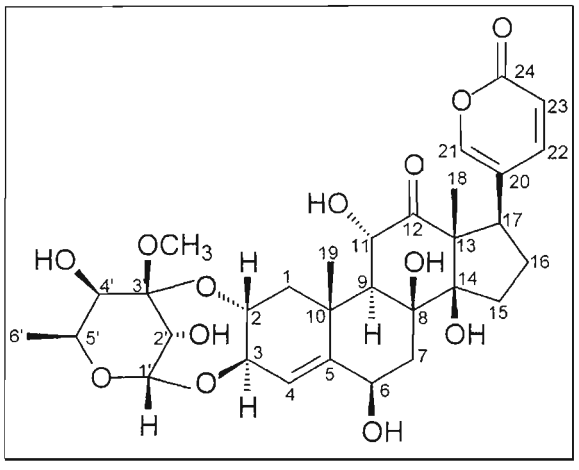
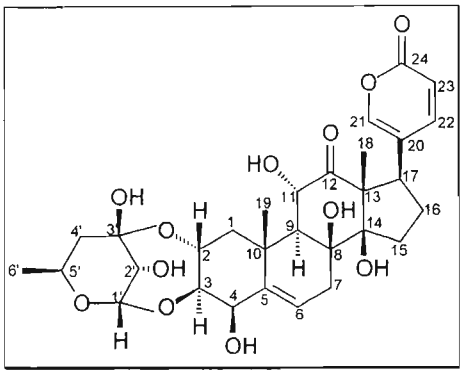


Figure 2.22. The structure of rubellin [41].

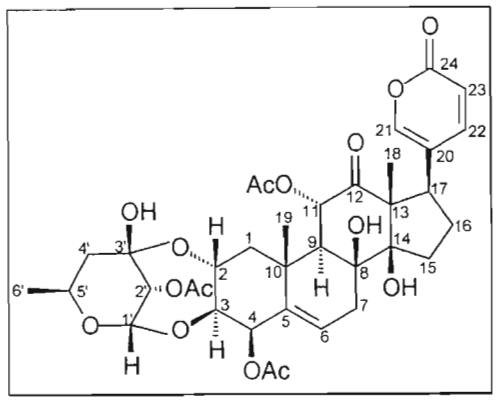
Table 2.23. NMR data for compound **12**, lydenburgenin.



	^{1}H NMR data for compound 12 *	^{13}C NMR data for compound 12**	HMBC correlations C→H	NOESY correlations	COSY correlations
1	1.07α[2]	46.9 (CH_2)	9, 19	1β, 3, 9	2
	3.00β[2]			1α	2
2	4.57 *ddd* (13.6,2.9,7.2)	69.1 (CH)	1β, 3, 4	2', 19	1α, 1β, 3
3	3.65 *dd* (3.8, 7.6)	83.1 (CH)	1α, 4, 1'	4	2, 4
4	4.28[1]	77.9 (CH)	6	3	3
5	-	139.8 (C)	1β, 4, 7, 19	-	-
6	5.72[1]	124.1 (CH)	4, 7	4	7
7	2.32[1]	35.1 (CH)	-	6	6
8	-	74.4 (C)	7	-	-
9	1.63[2]	51.9 (CH)	7, 11, 19	4	11
10	-	39.4 (C)	1β, 4, 9,11,19	-	-
11	5.03 *d* (11.9)	71.6 (CH)	9	18, 19	9
12	-	213.9 (C)	11, 18	-	-
13	-	62.6 (C)	18	-	-
14	-	86.1 (C)	18	-	-
15	1.39[1]	34.87 (CH_2)	-	-	16α, 16β
16	1.67β[1]	27.8 (CH_2)	-	16α	15, 17
	2.00α[1]			17	15, 17
17	4.14 *dd* (7.5, 9.3)	41.5 (CH)	18	16α, 21	16α, 16β
18	1.11 *s*	18.8 (CH_3)	-	11, 22	-
19	1.68 *s*	19.22 (CH_3)	9	-	-
20	-	122.1 (C)	21, 23	-	-
21	7.51 *d* (2.5)	150.3 (CH)	-	17	22
22	7.95 *dd* (2.5, 9.7)	147.9 (CH)	-	18, 23	21, 22, 23
23	6.31 *d* (9.7)	114.7 (CH)	-	22	22
24	-	163.3 (C)	21, 23	-	-
1'	5.20[1]	99.7 (CH)	2'	-	2'
2'	4.28[1]	65.5 (CH)	1'	-	1'
3'	-	100.2 (C)	2'/ 5'	-	-
4'	1.61β[1]	36.9 (CH_2)	6'	1', 4'α	5'
	1.82α[1]			4'β	5'
5'	4.28[1]	70.4 (CH)	1', 4a', 6'	-	4', 6'
6'	1.18 *d* (6.2)	20.7 (CH_3)	4b'	2'	5'

*NMR data obtained in CD_3OD, 400MHz
**NMR data obtained in CD_3OD, 100MHz
[1] overlapping peaks
[2] obscured by solvent peaks

Table 2.24. NMR data for compound **13**, acetylation product of compound **12**.



	^{1}H NMR data for compound 13 *	**^{13}C NMR data for compound 13****	**HMBC correlations C→H**	**NOESY correlations**	**COSY correlations**
1	1.28α[1]	47.5 (CH_2)	19	1β	2
	2.69β[1]			1α	2
2	4.61 *m*	70.4 (CH)	1α, 1β, 3, 4	2'	1α, 1β, 3
3	3.86*dd* (3.9, 9.7)	82.4 (CH)	1', 4	4, 5'	2, 4
4	5.61 *d* (3.8)	79.2 (CH)	-	3, 6	3
5	-	136.6 (C)	4, 7, 19	-	-
6	5.91 *m*	128.9 (CH)	4, 7	4	7
7	2.44[1]	35.9 (CH)	-	-	6
8	-	75.5 (C)	7	-	-
9	2.09[1]	48.6 (CH_2)	11, 19	-	11
10	-	40.3 (C)	1α, 4, 9,19	-	-
11	6.03 *d* (12.4)	76.2 (CH)	-	19, 18	9
12	-	211.7 (C)	15, 18	-	-
13	-	64.4 (C)	18	-	-
14	-	87.0 (C)	15, 18	-	-
15	1.50[1]	30.7 (CH_2)	-	-	16α, 16β
16	1.98α[1]	29.0 (CH_2)	-	-	15, 17
	1.77β[1]				15, 17
17	4.05 *m*	42.4 (CH)	18	21	16α, 16β
18	1.15 *s*	19.4 (CH_3)	-	11	-
19	1.45 *s*	20.0 (CH_3)	-	2, 11	-
20	-	123.1 (C)	-	-	-
21	7.48 *d* (2.5)	151.5 (CH)	-	17	22
22	7.92 *dd* (2.5, 9.6)	148.9 (CH)	21	23	21, 23
23	6.30 *d* (9.6)	115.9 (CH)	-	22	22
24	-	164.4 (C)	21, 23	-	-
1'	5.07 *s*	98.6 (CH)	-	2'	2'
2'	5.48 *s*	67.5 (CH)	1'	1', 2	1'
3'	-	99.7 (C)	1', 2'	-	-
4'	1.69[1]	36.1 (CH_2)	6'	-	5'
5'	4.33 *m*	71.4 (CH)	1', 6'	3, 6'	4', 6'
6'	1.20 *d* (6.0)	21.8 (CH_3)	4'	5'	5'

*NMR data obtained in CD_3OD, 400MHz

**NMR data obtained in CD_3OD, 100MHz

[1] overlapping peaks

[2] obscured by solvent peaks

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CHAPTER 3

EXPERIMENTAL

3.1 FOREWORD

3.1.1. Melting points

The melting points for crystalline compounds isolated were determined on a Reichert hot-stage apparatus or on an electro-thermal digital melting point apparatus and are uncorrected.

3.1.2. Optical rotations

Optical rotations were recorded at room temperature on a Perkin-Elmer 241 polarimeter. The concentrations of solutions are expressed in g/100ml.

3.1.3. Infrared spectroscopy (IR)

Spectra were recorded on a Shimadzu FTIR-4300. Use was made of KBr discs and NaCl windows. The spectrometer was calibrated against air before spectra were run.

3.1.4. Ultra-violet spectroscopy (UV)

All ultra-voilet absorption spectra were recorded on a Perkin-Elmer Lamda 45 UV-visible spectrophotometer except for compound **4** of which the spectrum was recorded on a Varian CARY 50 Probe UV-visible spectrometer. The long wave aborption maxima was determined – it appears between 358 and 367 nm in unsaturated and between 285 and 297nm in saturated systems. The NaOAc and

$AlCl_3$ solutions used for bathochromic shift tests were prepared by dissolving 0.5 g of each salt (anhydrous) in 100 ml redistilled methanol.

3.1.5. Mass spectrometry

GC-MS, unless otherwise stated, was performed on a MAT CH7A mass spectrometer (Finnigan MAT, Bremen) coupled with a Varian type 1700 gas chromatograph at Natal University, Durban.

Mass spectra for compounds **7** and **9** were performed by Dr. Louis Fourie at the Potchefstroom University on a Micromass 70-70E double focusing magnetic sector mass spectrometer. This instrument is equipped with an Ion Teck B70N saddle field FAB gun. Xenon was used as bombardment gas and *m*-nitrobenzylalcohol as matrix.

High resolution mass spectra for compounds **11** and **13** were performed by Mr. John Hill at Kent Mass Spectrometry in London on a VG (now Waters) 70-SE magnetic sector mass spectrometer by direct insertion probe using an accelerating voltage of 8KV and a mass range of 3000. Electron Impact (EI) used an ionizing potential of 70eV, a 100μA trap current and a source temperature of 200°C.

3.1.6. Nuclear Magnetic Resonance Spectrometry (NMR)

NMR spectra were recorded at room temperature on a Varian Inova 500 MHz spectrometer by Mr M. Watson and Mr C. Grimmer at the University of Natal, Pietermaritzburg, and on a Varian Inova 400 MHz by Mr D. Jagjivan at the University of Natal, Durban. HMQC spectra were recorded on the 500 MHz instrument, and HMBC spectra on the 400 MHz instrument. Deuteriomethanol (CD_3OD) and deuteriochloroform ($CDCl_3$) were used as solvents. All δ values are expressed in ppm relative to TMS.

3.1.7. Chromatography

3.1.7.1. Column chromatography

Use was made of glass columns (1-3 cm in diameter) packed with silica gel 60 (particle size 0.040–0.063 mm, 230-400 mesh ASTM, Merck Art 9385). Repeated chromatography, flash or under gravity, was performed to isolate pure compounds in extracts. A variety of solvent systems were used as eluents, mostly ethyl acetate, dichloromethane, hexane and methanol.

3.1.7.2. Thin Layer chromatography

Precoated, aluminium backed silica gel 60 plates (thickness 0,2 mm; Merck Art 5533) which contained a fluorescent indicator (F254), were used in the separation process to detect the composition of different fractions collected from a column. After developing the plates in an appropriate solvent (usually ethyl acetate, dichloromethane, hexane and methanol), they were sprayed with anisaldehyde spray agent. The anisaldehyde reagent was made by mixing anisaldehyde, concentrated sulphuric acid and methanol in the ratio 1:2:80. Spots were observed after heating the developed and sprayed plate with a heat gun. If compounds were UV active they could be detected under the light of a UV lamp set at 254 nm.

3.1.7.3. Chromatotron chromatography

Chromatotron chromatography runs were performed on a Harrison Research (Model 7924T) instrument with silica coated plates (Merck 60, PF_{254}, 7749), using UV light to detect compounds. Solvents used were ethyl acetate, dichloromethane, hexane and methanol.

3.2. EXTRACTIVES FROM *EUCOMIS COMOSA* (HOUTT.) WEHRH.

Bulbs of *E. comosa* (Houtt) Wehrh. were purchased from the Warwick Triangle herbal market in Durban and a specimen was grown to flowering (*Crouch 940*, NH). The air dried fresh bulbs (3.9 kg) were chopped into small pieces and extracted in methanol (2.5 l) at room temperature with continuous agitation for approximately 48 hours. The waxy extract was concentrated under reduced pressure and extracted with ethyl acetate (4 x 250ml). The removal of the solvent left a residue (210 g), which was subjected to column chromatography starting with hexane/ethyl acetate (1:1) as mobile phase and then introducing an ethyl acetate gradient. Fractions collected gave mixtures which were purified by repeated chromatography making use of the chromatotron and preparative thin layer chromatography. From the hexane / ethyl acetate (1:1) fraction the homoisoflavanones, punctatin, (*Z*)-eucomin and (*E*)-eucomin were isolated. From the hexane / ethylacetate (2:1) fraction 3,9-dihydropunctatin was isolated and from the ethyl acetate / hexane (4:1) fraction 5-methoxy-7-hydroxy-3-(4'-hydroxybenzyl)-4-chromanone was isolated.

3.2.1. Physical data for compound 1, (*E*)- 5,7-dihydro-8-methoxy-3-(4'-hydroxy-benzylidene)-4-chromanone, punctatin

Yield: 9 mg

Physical description: orange needles

Melting point: 190 °C, lit value: 189 – 190 °C [1]

Infra-red: ν_{max}[KBr](cm^{-1}) 3395 (OH stretching), 2928, 2835 (aliphatic stretching), 1638 cm^{-1} (carbonyl stretching), 1512 (aromatic C=C stretching), 1400, 1259 (CH_2 and CH_3 bending), 1177 (C-O stretching).

Ultra-violet: λ_{max} nm 363 (log ε 4.42), lit value: 368 (log ε 4.49) [1]
Bathochromic shifts: 395 (+ $AlCl_3$), 398 (+NaOAc)

Mass spectrum: EIMS *m/z* 314 $[M]^+$, 183, 182, 132

Data for ^1H and ^{13}C NMR spectroscopy (in $CDCl_3$) is presented in Table 2.2 (Chapter 2). ^1H and ^{13}C NMR data were also run in CD_3OD.

^1H NMR: δ_H (ppm), CD_3OD
3.73 (3H, s, 8-OCH_3), 5.38 (2H, d, *J*=1.3, H-2), 5.93 (1H, s, H-6), 6.88 (2H, d, *J*=8.4, H-3' and H-5'), 7.27 (2H, d, *J*=8.4, H-2' and H-6'), 7.74 (H-1, br s, H-9).
^{13}C NMR: δ_C (ppm), CD_3OD
61.4 (CH_3, 8-OCH_3), 68.8 (CH_2, C-2), 97.7 (CH, C-6), 103.2 (C, C-4a), 116.8 (CH, C-3'), 116.8 (CH, C-5'), 129.8 (C, C-3), 130.1 (C, C-8), 132.3 (C, C-1'), 133.5 (CH, C-2'), 133.5 (CH, C-6'), 138.1 (CH, C-9), 153.8 (C, C-8a), 154.7 (C, C-4'), 160.7 (C, C-5), 161.8 (C, C-7), 186.2 (C, C-4).

3.2.2. Physical data for compound 2, (*Z*)-5,7-dihydroxy-3-(4'-methoxybenzylidene)-4-chromanone, (*Z*)-eucomin

Yield: 12 mg

Physical description: yellow needles.

Melting point: 143° , lit value: 143 – 145°C [2]

Infra-red: ν_{max}[KBr](cm^{-1}) 3133 (OH stretching), 2925, 2853 (C-H aromatic and aliphatic stretching), 1637 (carbonyl stretching), 1509 (aromatic C=C stretching), 1400, 1259 (CH_2 and CH_3 bending), 1177 (C-O stretching).

Ultra-violet: λ_{max} nm: 357 (log ε 4.32), lit value: 363 (log ε 4.39) [2]

Bathochromic shift: 394 (+ $AlCl_3$), 378 (+ NaOAc)

Mass spectrum: EIMS *m/z* 298 $[M]^+$, 283, 153, 152 and 146.

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.4 (Chapter 2).

3.2.3. Physical data for compound 3, (*E*)-dihydroxy-3-(4'-methoxybenzylidene)-4-chromanone, (*E*)-eucomin

Yield: 3 mg

Physical description: yellow needles.

Melting point: 196-197 °C, lit value: 194-196 °C [3]

Infra-red: ν_{max}[KBr](cm^{-1}) 3134 (OH stretching), 2928, 2855 (C-H aromatic and aliphatic stretching), 1638 (carbonyl stretching), 1514 (aromatic C=C stretching), 1399, 1260 (CH_2 and CH_3 bending), 1160 (C-O stretching).

Mass spectrum: EIMS *m/z* 298 $[M]^+$, 283, 153, 152 and 146.

Data for 'H and ^{13}C-NMR spectroscopy is presented in Table 2.7 (Chapter 2).

3.2.4. Physical data for compound 4, 5,7-dihydroxy-8-methoxy-3-(4'-hydroxy-benzyl)-4-chromanone, 3,9-dihydropunctatin

Yield: 27 mg

Physical description: yellow crystals

Melting point: 205 °C, lit. value 204 - 206°C [1]

Optical rotation: $[\alpha]_D$ = -37° (c=0.25g / 100ml, dioxan), lit value: -37° (c=0.3, dioxan) [1]

Infra-red: ν_{max}[KBr](cm^{-1}) 3384 (OH stretching), 3015, 2930 (C-H aromatic and aliphatic stretching), 1638 (carbonyl stretching), 1514 (aromatic C=C stretching), 1383, 1259 (CH_2 and CH_3 bending), 1160 (C-O stretching).

Ultra-violet: λ_{max} nm: 290 (log ε 4.40), lit value: 293 (log ε 4.53) [1]
Bathochromic shift: 317 (+$AlCl_3$), 329 (+NaOAc)

Mass spectrum: EIMS *m/z* 316 $[M]^+$, 209, 183, 182, 107

Data for ^1H and ^{13}C NMR spectroscopy (in $CDCl_3$) is presented in Table 2.9 (Chapter 2). ^1H and ^{13}C NMR data were also run in CD_3OD.

^1H NMR: δ_H (ppm), CD_3OD
2.65 (1H, dd, *J* = 10.2, 13.5, H-9b), 2.81 (1H, m, H-3), 3.10 (1H, dd, *J* = 4.4, 13.8, H-9a), 3.71 (3H, s, 8-OCH_3), 4.15 (1H, dd, *J* = 7.1, 11.3, H-2b), 4.30 (1H, dd, *J* = 11.3, 4.1, H-2a), 5.92 (1H, s, H-6), 6.73 (2H, d, *J*=8.6, H-3' and H-5'), 7.05 (2H, d, *J*=8.3, H-2' and H-6').
^{13}C NMR: δ_C (ppm), CD_3OD
33.0 (CH_2, C-9), 48.0 (CH, C-3), 61.4 (CH_3, 8-OCH_3), 70.5 (CH_2, C-2), 97.0 (CH, C-6), 199.4 (C, C-4), 102.8 (C, C-4a), 116.3 (CH, C-3'), 116.3 (CH, C-5'), 129.6 (C, C-8), 130.0 (C, C-1'), 131.1 (CH, C-2'), 131.1 (CH, C-6'), 155.6 (C, C-8a), 157.2 (C, C-4'), 161.0 (C, C-5), 161.2 (C, C-7).

3.2.5. Physical data for compound 5, 5-methoxy-7-hydroxy-3-(4'-hydroxy-benzyl)-4-chromanone

Yield: 9 mg

Physical description: colourless crystals

Melting point: 194 °C, lit. value: 196-197 °C [1]

Optical rotation: $[\alpha]_D$ = -38° (c, 0,06g/100ml, CH_3OH), lit value: -38° (dioxin) [1]

Infra-red: ν_{max}[KBr](cm^{-1}) 3186 (OH stretching), 2927, 2857 (C-H aromatic and aliphatic stretching), 1655 (carbonyl stretching), 1516 (aromatic C=C stretching), 1399, 1253 (CH_2 and CH_3 bending), 1165 (C-O stretching).

Mass spectrum: EIMS *m/z* 300 $[M]^+$, 193, 167, 166 and 107

Data for ^1H and ^{13}C NMR spectroscopy is presented in Table 2.10 (Chapter 2).

3.3. EXTRACTIVES FROM *GALTONIA PRINCEPS* (BAK.) DECNE.

The collection was made by Dr Neil Crouch in Richmond, Kwa-Zulu Natal (*Crouch 847*, NH). Air dried fresh bulbs of *Galtonia princeps* (Bak.) Decne (3,5 kg) were chopped into small pieces and extracted in methanol (2,5 l) at room temperature for approximately 48 hours. The extract was concentrated under reduced pressure. The residue was submitted to column chromatography starting with the solvent system hexane / ethyl acetate (1:1) as mobile phase and then introducing and ethyl acetate gradient. When 100% ethyl acetate as mobile phase was reached, a methanol gradient was introduced. Five fractions were collected. Compound **6** was isolated from the hexane/ethyl acetate (2:8) fraction (fraction 2) and purified using dichloromethane / ethyl acetate / hexane (2:2:1) as

mobile phase. From the ethyl acetate / methanol (95:5) fraction (fraction 4) compound **7** and compound **9** were isolated and purified employing ethyl acetate / methanol (95:5) as mobile phase.

3.3.1. Physical data for compound 6, 7-*O*-methyleucomol

Yield: 6 mg

Physical description: amorphous solid

Melting point: amorphous solid

Optical rotation: $[\alpha]_D$ = -35° (c, 0,06g/100ml, CH_3OH), lit value: -31° (chloroform) [3]

Infra-red: ν_{max}[NaCl](cm^{-1}) 3142 (OH stretching), 2927, 2855 (C-H aromatic and aliphatic stretching), 1640 (carbonyl stretching), 1512 (aromatic C=C stretching), 1377, 1291 (CH_2 and CH_3 bending), 1158 (C-O stretching).

Mass spectrum: EIMS *m/z* 330 $[M]^+$, 312, 209, 166, 167, 121

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.12 (Chapter 2).

3.3.2. Physical data for compound 7, 3β,17α-dihydroxycholest-5-en-22-one 16β-*O*-α-*L*-arabinopyranoside.

Yield: 32 mg

Physical description: amorphous white powder.

Melting point: amorphous solid

Optical rotation: $[\alpha]_D$ = -26.1 ° (c, 0.046 g/100ml, $CHCl_3/CH_3OH$, 8:1)

Infra-red: λ_{max} [KBr](cm^{-1}) 3442 (O-H stretching), 2924, 2853 (C-H stretching), 1642 (C=O stretching), 1384, 1325, 1260 (CH_2 and CH_3 bending), 1022 (C-O stretching).

Mass spectrum: Molecular ion not observed
Positive-ion FAB-MS *m/z* 416 (40) [M-arabinose] $^+$, 267 (6), 147 (67), 123 (53), 109 (100).

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.13 (Chapter 2).

3.3.2.1. Acetylation of compound **7**.

Compound **7** (10 mg) was dissolved in pyridine (3 ml) and acetic anhydride (3 ml) was added. The mixture was heated on a steam bath (60°C) for 30 minutes, covered and left overnight. Water was added and the mixture extracted with ethyl acetate (4x10 ml). The ethyl acetate extract was dried and the acetylated product (compound **8**) purified employing column chromatography and hexane / ethyl acetate (3:2) as mobile phase.

Yield: 13 mg

Physical description: amorphous powder

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.15 (Chapter 2).

3.3.3. Physical data for compound 9, sitosterol 3-*O*-β-*D*-glucopyranoside.

Yield: 14 mg

Physical description: white powder

Melting point: 286 °C, lit. value: 284 °C [4]

Infra-red: λ_{max} (KBr) 3419 (O-H stretching, 2918, 2850 (C-H stretching), 1642 (C=C stretching), 1383, 1321 (CH_2 and CH_3 bending), 1026 (C-O stretching) cm^{-1}.

Optical rotation: $[\alpha]_D$ = -38.6° (c, 0.03 g/100ml, $CHCl_3/CH_3OH$, 8:1), lit value: -40.2° (pyridine) [4]

Mass spectrum: Positive-ion FAB-MS $[M+H]^+$ *m/z* 577.447643 (21) ($C_{35}H_{61}O_6$ requires 577.446815), 414 (11) $[M\text{-glucose}]^+$, 289 (9), 154 (100), 136 (83), 107 (48).

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.17 (Chapter 2).

3.4. EXTRACTIVES FROM *ORNITHOGALUM TENUIFOLIUM* DELAROCHE.

Two collections were made and the same procedure was followed for both the collections. The first specimen was purchased from the Warwick Triangle herbal market in Durban (*Crouch 832*, NH), and the second collection was made in Ashburton, Kwa-Zulu Natal (*Crouch 845*, NH). Fresh bulbs of *Ornithogalum tenuifolium* Delaroche. (567.3 g) were cut into pieces and extracted at room temperature with methanol for approximately 48 hours. The methanol extract was concentrated under reduced pressure and extracted with methylene chloride. Removal of the solvent left 6.7 g of residue. The residue was subjected to column chromatography using hexane / ethyl acetate (8:2) as mobile phase introducing an ethyl acetate gradient to 100% ethyl acetate and then introducing a methanol gradient. Five fractions were collected. Fraction 5 collected in hexane / ethyl acetate (9:1), was purified by silica gel column chromatography

with ethyl acetate / hexane (8:2) and compound **10** (12 mg), a steroidal glucoside was isolated.

3.4.1. Physical data for compound 10, 25*R*,5β-spirostane-1β,3α-diol.

Yield: 12 mg

Melting point: 195 °C

Infra-red: λ_{max} [KBr](cm^{-1}) 3428 (O-H stretching, 2928, 2868 (C-H stretching)1240 (CH_2 and CH_3 bending), 1061 (C-O stretching)

Mass spectrum: EIMS *m/z* 432 $[M]^+$ (4), 139 (100), 289 (10).

Physical description: colorless crystals

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.19 (Chapter 2).

X-Ray structure determination: $C_{27}H_{43.50}O_4$, fw = 432.12 amu, a = 6.9958(19) Å, b = 11.4271(10) Å, c = 15.189(3) Å, α = 98.055(11)°, β = 90.17(2)°, γ = 90.011(15)°, V = 1202.2(4) $Å^3$, triclinic, $P\tau$, Z=2, D_c = 1.194g cm^{-3}, μ = 0.078 mm^{-1}, T = 293(2) K, R_1 (wR_2) = 0.0437 (0.1232) for 5362 unique data with I > 2σ(I), R_1 (wR_2) = 0.0483 (0.1273) for all 5471 data (R_{int} = 0.0081).

3.5. EXTRACTIVES FROM *URGINEA LYDENBURGENSIS* R.A. DYER.

A collection was made in Nelspruit, Mpumalanga by Dr Neil Crouch (*Crouch 864*, NH). The fresh bulbs of *Urginea lydenburgensis* RA Dyer (3 kg) were cut into pieces and extracted firstly with methylene chloride and then with methanol at room temperature. These extracts were concentrated under reduced pressure.

Methylene chloride extract: 24 g
Methanol extract: 98 g

The crude methylene chloride extract was subjected to column chromatography using ethyl acetate / hexane (2:3) as starting mobile phase and then an ethyl acetate gradient was introduced. Five fractions were collected. Fraction 5 (ethyl acetate / hexane (4:1) was purified by column chromatography starting with 100% ethyl acetate as mobile phase introducing a methanol gradient. Nine fractions were collected. Two bufadienolides (compound **11** and **12**) were isolated. Pure compound **11** was isolated from fractions 2 and 3 in ethyl acetate / methanol (95:5) and compound **12** from fraction 7 in ethyl acetate / methanol (8:2). Compound **12** was further purified employing the chromatotron and ethyl acetate / dichloromethane / methanol (5: 4.5:0.5) as mobile phase.

3.5.1. Physical data for compound 11, 16β-acetoxyberscillogenin

Name: 16β-acetoxyberscillogenin

Yield: 6mg

Physical description: white crystalline powder

Melting point: 150 °C

Optical rotation: $[\alpha]_D$ = +18.6 ° (c, 0.043 g/100ml, $CHCl_3$)

Infra-red: λ_{max} [KBr](cm^{-1}) 3456 (O-H stretching, 2934, 2857 (C-H stretching), 1714 (C=O stretching), 1533 (aromatic C=C stretching), 1374, 1245 (CH_2 and CH_3 bending), 1086, 1039 (C-O stretching).

Mass spectrum: EIMS *m/z* 456 $[M]^+$ (30), 396 $[M^+-CH_3COOH]$ (25), 378 $[M^+-CH_3COOH-H_2O]$ (8), 368 (7), 350 (12), 307 (23), 91 (100)

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.22 (Chapter 2).

3.5.2. Physical data for compound 12, lydenburgenin

Name: 4β,8β,11α,14β-tetrahydroxybufa-5,20,22-trienolide-12-one 2α,3β-*O*-glycoside (lydenburgenin)

Yield: 4.2 mg

Physical description: white crystals

Melting point: 217- 219°C

Optical rotation: $[\alpha]_D$ = +47.6 ° (c, 0.042, $CHCl_3$)

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.23 (Chapter 2).

3.5.2.1. Acetylation of compound **12**

An acetylation was performed to enable the assignment of all the peaks in the 'H-NMR. See acetylation process in section 3.3.2. Compound **13** was formed during acetylation.

Yield: 6.7 mg

Physical description: colourless oil

Mass spectrum: EIMS *m/z* 429 [M^+-glycoside-2.CH_3COOH] (1), 149 (28), 84 (97), 49 (100).

Data for ^1H and ^{13}C-NMR spectroscopy is presented in Table 2.21 (Chapter 2).

3.6. REFERENCES

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2. Heller, W., Andermatt, P., Schaad, W.A., Tamm, C., 1976. Homoisoflavanone IV. Neue Inhaltsstoffe der Eucomin-Reihe von *Eucomis bicolor*. Helvetica Chimica Acta 59, 2048-2058.

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4. Misra, A.N., Tiwari, H.P., 1973. Mass spectral studies of phytosterolins and a ketone from *Trianthema pentandra*. Phytochemistry 12, 393-395.

5. Faizi, S., Ali, M., Saleem, R., Bibi, S., 2001. Complete ^1H and ^{13}C NMR assignments of stigma-5-en-*O*-β-glucoside and its acetyl derivative. Magnetic Resonance in Chemistry 39, 399-405.

CHAPTER 4

ANTI-INFLAMMATORY SCREENING OF HOMOISOFLAVANONES AND RELATED STRUCTURES

4.1. INTRODUCTION

Vast differences exist between societies in the perceptions of disease. Modern medicine is rationalistic in approach, whilst traditional medicine ascribes a spiritual dimension to illness and religious practices play a role in the healing process. Early remedies combined natural products with witchcraft, mysticism and astrology [1]. Co-operation between traditional and modern medicine is jeopardized by views that traditional medicine has no scientific basis [2].

However, these views have little impact on the popularity of traditional medicine. In South Africa alone, up to 60% of the population consult traditional healers, and a large part of the medicines used by healers are derived from plants [3]. Traditional healers are also more accessible in rural areas than western doctors [4]. The high demand for popular ethnomedicinal or "muthi" plants may cause them to be exploited to extinction [5].

Investigations of renowned medicinal plants led to the discovery of biologically active compounds such as the alkaloids which include morphine, atropine and codeine. These compounds are still the cornerstones of many aspects of drug discovery [6].

It is important, from a social and pharmacological perspective to validate the medicinal uses of plants scientifically before the knowledge is lost due to urban expansion and breakdown of traditional society structures through which this knowledge is transmitted [1].

4.1.1. Medicinal properties of plants and compounds utilized in this investigation

The biological screening of homoisoflavanones and other compounds (norlignans, chalcones and coumarins) made it possible to rationalize some of the traditional medicinal uses of the plants from which these compounds were isolated. All the plants belong to the Hyacinthaceae except *Tachiadenus longiflorus* Griseb. which belongs to the Gentianaceae. Medicinal uses of the plants investigated are listed in Table 4.1.

Table 4.1. Medicinal uses of plants from which test compounds were isolated

Plants	Medicinal uses	Reference
Eucomis humilis Bak.	None documented	-
Eucomis pole-evansii NE Brown	Mental disease	[7]
Eucomis comosa (Houtt) Wehrh.	Anti-rheumatic Enema for teething infants	[7] [8]
Drimiopsis maculata Lindl.	Enema for young children with stomach ailments	[8]
Drimiopsis burkei Bak.	None documented	-
Urginea delagoensis Bak	Protective cream Protect animal skins from dogs	[8] [7]
Albuca fastigiata (L.f) Dryand	Treatment of people poisoned by jealous rivals Against evil spirits	[8] [7]
Ledebouria ovatifolia (Bak.) Jessop	Enema for gastro-enteritis Influenza, Backache	[8]
Ledebouria zebrina (Bak.) S. Venter	Purgatives in general	[8]
Merwilla natalensis (Planchon) Speta	Powdered bulbs are rubbed into sprains and fractures (Sotho) Boils and veld sores (Swati)	[7]
Tachiadenus longiflorus Griseb.	None documented	-

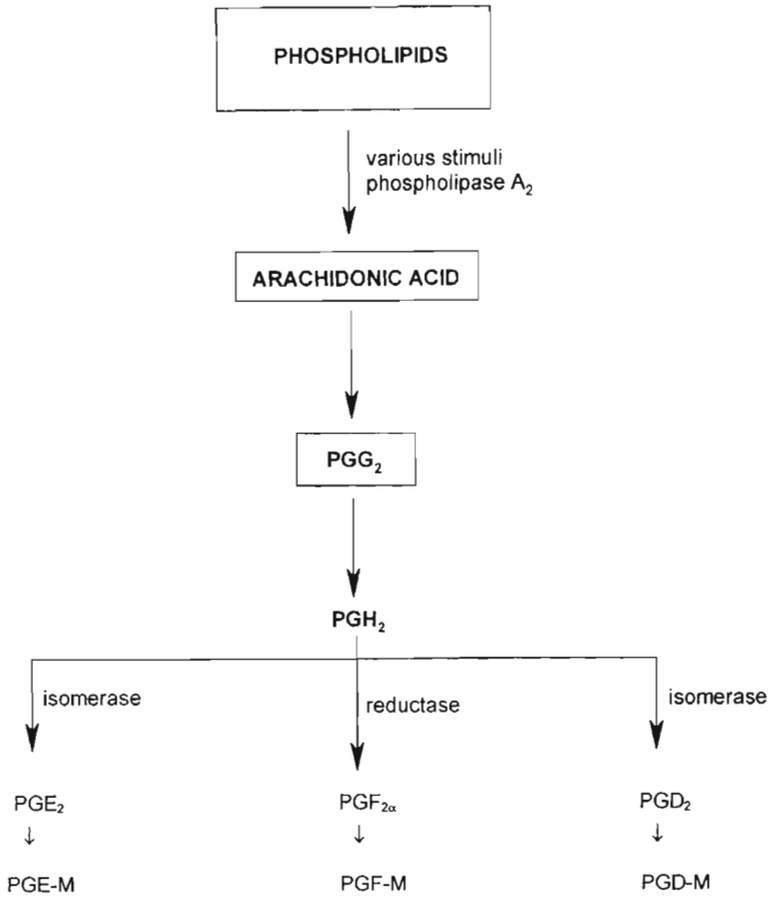
The pharmacological value of homoisoflavanones is insufficiently known. Limited studies on these compounds showed anti-inflammatory, antibacterial, antihistaminic, antimutagenic and angioprotective properties as well as their being potent phosphodiesterase inhibitors [9,10,11]. 3-Benzyl-4-chromanone, 3-benzyl-3-hydroxy-4-chromanone, 3-benzylidene-4-chromanone and scilla-scillin types of homoisoflavanones as well as other structurally related compounds like chalcones, coumarins (aesculetin, scoparone, scopoletin and

isoscopoletin) and norlignans, were included in this investigation to ensure a variety of structural features which would make structure-activity studies more informative. In this section the anti-inflammatory activity of homoisoflavanones and related compounds isolated from plants are investigated.

4.1.2. The inflammatory process

The inflammatory process can be evoked by different stimuli like antigen-antibody interactions, infectious agents and thermal or physical injury. Signs of inflammation are erythema, oedema and pain [12]. The inflammatory process is necessary for survival against pathogens and injury, but sometimes the inflammatory response is aggravated and sustained without benefit. Inflammation is a complex process and many different mediators are involved [12]. For the purpose of this study only a part of this process is emphasized.

Arachidonic acid is released from the cell membrane by chemical and mechanical stimuli and converted by the cyclooxygenase enzymes (COX-1/ COX-2) to the unstable prostaglandin intermediates PGG_2 and PGH_2 (Scheme 4.1). The fate of the cyclooxygenase products, PGG_2 and PGH_2, differs from tissue to tissue depending on the metabolizing enzymes present [12]. The arachidonic acid cascade results in the formation of prostaglandins PGE_2, PGD_2, $PGF_{2\alpha}$, depending on the tissue, that can be regarded as pro-inflammatory mediators responsible or partly responsible for symptoms like vasodilation and increased blood vessel permeability [14]. If the enzymes responsible for the formation of prostaglandins are inhibited, pain and inflammation will also be inhibited.



Scheme 4.1. Part of the inflammatory process important for this study, modified [13]

4.1.3. Cyclooxygenase enzymes and their inhibitors

There are three isoforms of cyclooxygenase enzymes, cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2) [15] and the recently discovered cyclooxygenase 3 (COX-3) [16]. COX-1 is found in most normal tissues and cells but COX-2 is induced in settings of inflammation and not constitutively expressed in normal cells and tissues [12]. COX-3 enzymes were found to be expressed in the canine cerebral cortex and in lesser amounts in other tissues analyzed [16].

The constitutively expressed COX-1 is responsible for the formation of prostaglandins that maintain gastrointestinal mucosa and renal blood flow [17]. COX-1 inhibitors will therefore exhibit gastric side-effects whilst gastric toxicity is markedly reduced with selective inhibitors of COX-2. COX-2 inhibitors can therefore be used on a chronic basis. Evidence suggests that COX-2 is upregulated in colon tumors, COX-2 inhibitors could thus be useful as chemopreventive agents for sporadic colon cancer [12].

This information has led to a new search for selective COX-2 inhibitors. It must however be mentioned that selective COX-2 inhibitors share the side-effect of alterations in renal function with COX-1 inhibitors and also lack the cardio-protective effects of COX-1 inhibitors [12]. All the adverse effects are not known as yet and the side-effect profile will be completed with time.

Substantial progress has been made in elucidating the mechanism of action of nonsteroidal anti-inflammatory drugs (NSAIDs). Generally inhibition of cyclooxygenase (COX) is thought to be the major mechanism of action [13].

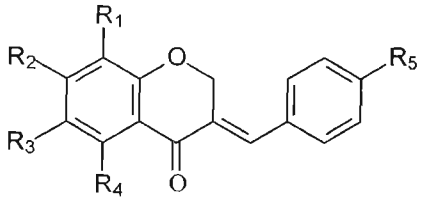
4.2. METHODOLOGY

No definite model covering all aspects of inflammation exists [18]. The pathway from arachidonic acid *via* PGG_2 to PGE_2, is the favoured one in vesicular glands from which microsomal cells are extracted. The assay on microsomal cells is however not mechanism based as other substrates and enzymes associated with the cells may influence the results. The very active endoperoxide isomerase and COX-1 are both microsomal enzymes [19]. However, isolated COX-1 and COX-2 enzyme assays are highly specific – any compound with another mechanism will be bypassed [20]. Compounds were screened against microsomal cells and isolated COX enzymes. Methods described by White and Glassman [21], were implemented with slight modifications by Jäger *et al.* [22] for COX-1 screening. The COX-2 assay was

done according to the method of Noreen *et al.* [23], also with minor modifications, but the basic protocols for these enzyme assays are the same.

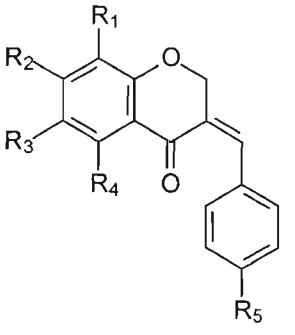
4.2.1. Test compounds

All the compounds used in this study were isolated by the Natural Products Research Group at the Chemistry Department of the University of Natal, Durban. The compounds are described in Scheme 4.2 and Table 4.2. Many of these compounds are novel and no biological studies have previously been performed.

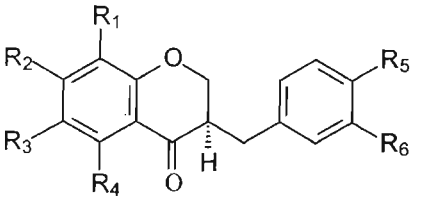


(**1**) $R_1 = OCH_3$, R_2, R_3, R_4, $R_5 = OH$

(**3**) $R_1 = OCH_3$, R_2, R_4, $R_5 = OH$, $R_3 = H$



(**2**) R_2, $R_4 = OH$, R_1, $R_3 = H$, $R_5 = OCH_3$



(**4**) R_1, R_3, $R_6 = H$, R_2, R_4, $R_5 = OH$

(**5**) R_1, R_3, $R_6 = H$, R_2, $R_4 = OCH_3$, $R_5 = OH$

(**6**) R_1, $R_3 = H$, R_2, R_4, $R_5 = OH$, $R_6 = OCH_3$

(**7**) R_1, $R_6 = H$, $R_2 = OCH_3$, R_3, R_4, $R_5 = OH$

(**8**) R1, R2 = OCH_3, R3, R6 = H, R4, R5 = OH

(**9**) R_1, R_3, $R_6 = H$, R_2, $R_5 = OH$, $R_4 = OCH_3$

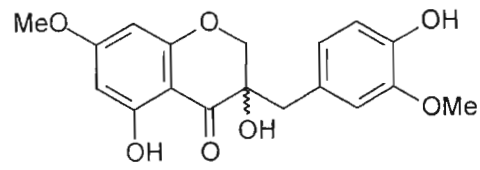
(**10**) $R_1 = OCH_3$, R_2, R_4, $R_5 = OH$, R_3, $R_6 = H$

(**11**) $R_1 = H$, R_2, $R_6 = OCH_3$, R_3, R_4, $R_5 = OH$

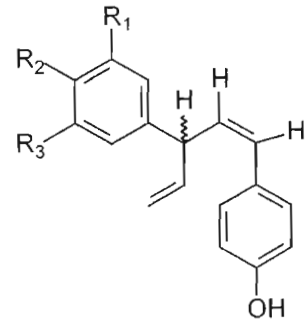
(**12**) $R_1 = H$, R_2, R_4, $R_6 = OCH_3$, R_3, $R_5 = OH$

(**13**) R_1, $R_6 = H$, R_2, R_4, $R_5 = OH$, $R_3 = OCH_3$

(**14**) $R_1 = H$, R_2, R_4, $R_6 = OH$, R_3, $R_5 = OCH_3$

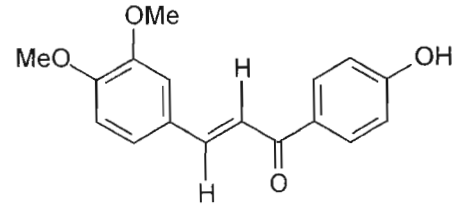


(**15**)

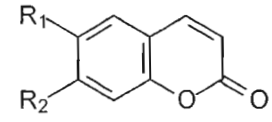


(**16**) R_1, R_3 = H, R_2 = OH

(**17**) R_1, R_2 = OCH_3, R_3 = OH



(**18**)

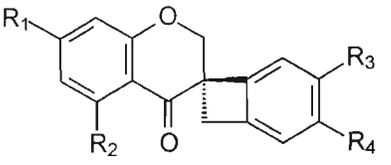


(**19**) R_1, R_2 = OH

(**20**) R_1, R_2 = OCH_3

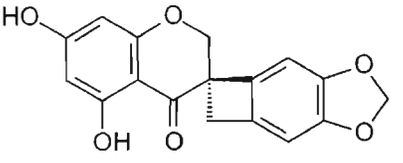
(**21**) R_1 = OCH_3, R_2 = OH

(**22**) R_1 = OH, R_2 = OCH_3



(**23**) R_1, R_2, R_4 = OH, R_3 = OCH_3

(**24**) R_1, R_3 = OCH_3, R_2, R_4 = OH



(**25**)

Scheme 4.2. Homoisoflavanones and related compounds tested for biological activity.

Table 4.2. Names, plant sources and references of compounds employed in the biological screening.

Compound number	Plant source	Reference
1	*Eucomis pole-evansii*	Unpublished results
2	*Eucomis comosa*	[24]
3	*Eucomis comosa*	[25]
4	*Drimiopsis maculata*	[26]
5	*Drimiopsis burkei*	Unpublished results
6	*Urginea delagoensis*	Unpublished results
7	*Drimiopsis maculata*	Unpublished results
8	*Drimiopsis maculata*	Unpublished results
9	*Eucomis comosa*	[25]
10	*Eucomis comosa*	[25]
11	*Ledebouria zebrina*	[27]
12	*Ledebouria zebrina*	[27]
13	*Merwilla natalensis*	[28]
14	*Merwilla natalensis*	[28]
15	*Albuca fastigiata*	Unpublished results
16	*Drimiopsis burkei*	[29]
17	*Ledebouria ovatifolia*	Unpublished results
18	Synthetic	Unpublished results
19	Synthetic	[30]
20	Synthetic/*Tachiadenus longiflorus*	[30]
21	*Tachiadenus longiflorus*	[30]
22	Synthetic	[30]
23	*Drimiopsis maculata*	[31]
24	*Drimiopsis maculata*	[31]
25	*Eucomis humilis*	[32]

All compounds were isolated from members of the Hyacinthaceae family except for **18**, **19**, **20** and **22** which are synthetic, and **21** which was isolated from a member of the Gentianaceae family. Compounds were dissolved in DMSO or ethanol (10 mg/ml). These solvents can be used up to a concentration of 2% for solubilization without any influence on the test results [14]. Since biological work was done on purified compounds, limitations in terms of availability of compounds were experienced. Therefore all biological assays do not necessarily contain every compound mentioned above. On the other hand, this procedure ensured that pitfalls of testing extracts were bypassed as only the pure compound could be responsible for the results. Other compounds giving false positive results like tannins were excluded [33].

4.2.2. Enzyme assays

Sheep seminal vesicles were homogenized in potassium phosphate buffer with 1mM EDTA (on ice). The homogenate was centrifuged and the cell debris discarded. The supernatant was then centrifuged at 100 000 g for 1 hour. The microsomal pellet was resuspended in 0.1M K-Phosphate, 3mM $MgCl_2$ buffer (pH 7.4) and a standard protein assay was used to determine enzyme concentration. Standardized aliquots were stored at -70°C.

The standardized enzyme preparation (10 µl/sample) and co-factor solution (50 µl/sample), were preincubated for 15 minutes on ice. This solution (60 µl) was added to the test solution (2.5 µl compound solution and 17.5 µl water) and preincubated for 5 minutes at room temperature. ^{14}C- Arachidonic acid (20 µl) was added to the enzyme-test compound mixture and incubated for 8 minutes in a water bath at 37°C. The reaction was then terminated by adding 10 µl 2N HCl to samples.

Purified human recombinant COX-2 enzyme (purity 70%) was purchased from Sigma. Three units of enzyme was activated with co-factor solution (50 µl) on

ice for 5 minutes. The enzyme solution (60 µl) and compound solution (2.5 µl solution and 17.5 µl water) were preincubated for 5 minutes at room temperature. ^{14}C-Arachidonic acid (20 µl) was added to the solutions and the samples were then incubated for 10 minutes in a water bath at 37°C. The reaction was terminated with 2N HCl (10 µl).

4.2.3. Determination of IC_{50} values

The IC_{50} value was determined by preparing a dilution series of four different concentrations of the active compound. Regression analysis of the results made calculation of the IC_{50} value possible (see p. 144).

4.2.4. Controls

Four controls were run for each test. Two were solvent blanks and two backgrounds. HCl was added to the backgrounds to inactivate the enzyme before addition of ^{14}C-arachidonic acid. Indomethacin standards (5 µM for microsomal cells, 12,5 µM for the COX-1 assay and 200 µM for the COX-2 assay) were included as a positive control. All experiments were performed in duplicate.

4.2.5. Separation of prostaglandins

Unlabelled prostaglandin carrier solution (4 µl per sample) was added to the reaction mixture. The ^{14}C-prostaglandins, synthesized in the assay, were then separated from unmetabolized arachidonic acid by column chromatography. Silica gel (Kieselgel 60, Particle size 0.063-0.200 mm, 70-230 mesh ASTM) in eluent 1, was used to pack Pasteur pipettes stoppered with glass wool to a height of 3 cm. Eluent 1 (1 ml) was added to the assay mixture and applied to the column. This was followed by an additional 4x1 ml eluent 1 to elute the unreacted arachidonic acid. The eluent was then discarded. Eluent 2 (3x1 ml)

was used to elute prostaglandins into scintillation vials. Scintillation fluid (4 ml) was added and the radioactivity counted after 30 minutes in the dark. A Beckman LS3801 scintillation counter was used.

4.2.6. Solutions prepared for assays

- Co-factor solution for COX-1 assay: 0.003 g l-adrenalin/l-epinephrine and 0.003 g reduced glutathione in 10 ml 0.1 tris buffer, pH 8.2
- Co-factor solution for COX-2 assay: 0.006 g l-adrenalin/l-epinephrine and 0.003 g reduced glutathione as well as 1 µM hematin in 10 ml 0.1 tris buffer, pH 8.0
- ^{14}C-Arachidonic acid: 16 Ci/mole, 3 mM
- Prostaglandin carrier solution containing 0.2 mg/ml of unlabelled prostaglandins (PGE_2:PGF_2 in the ratio 1:1)
- Eluent 1: hexane:1,4-dioxan:acetic acid (350:150:1 v/v/v)
- Eluent 2: ethyl acetate: methanol (85:15 v/v)

4.2.7. Calculation of inhibition

All samples were tested in duplicate. Percentage inhibition of formation of radioactive PGE_2 with reference to an untreated sample (solvent blank) was obtained as follows:

$$\%\text{inhibition} = \left[1 - \left[\frac{(DPM_{compound} - DPM_{background})}{(DPM_{solvent\ blank} - DPM_{background})} \right] \right] \times 100$$

The radioactivity (DPM) of the control (background) was subtracted from the radioactivity of both the sample and the blank.

4.2.8. Method alterations

To aid the activity of the COX enzyme it is necessary to add cofactors to the enzyme preparation [23]. Enzymatic activity of endoperoxidase isomerase (involved in the conversion of PGG_2 into PGE_2) was stimulated by the addition of reduced glutathione [19]. Low levels of hydroperoxide and the absence of reducing substrates cause the enzyme to undergo inactivation [34].

Formation of PGE_2 (and PGD_2) increases in the presence of l-epinephrine [23]. When screening phenolic compounds, it is essential to add l-adrenalin/l-epinephrine as co-factors. Phenolic compounds can act as electron donors and may be co-oxidized in the hydroperoxidase step of prostaglandin biosynthesis. This will result in activation instead of inhibition of COX [14]. Hematin is added as co-factor in COX-1 and COX-2 assays to stabilize the enzymes. Time dependent inhibitors can be detected by implementing a pre-incubation step of the compounds and enzyme before arachidonic acid is added [35]. High yields of PGE_2 in the control reaction (blank) increase the efficiency of the assay.

4.3. RESULTS AND DISCUSSION

Anti-inflammatory assays were conducted with cell microsomal fractions, with isolated COX-1 enzymes and with isolated COX-2 enzymes.

Table 4.3. The % inhibition of prostaglandin synthesis by pure compounds isolated from different plants in cell microsomal fractions (significant activity printed in bold).

Compound number	Plant isolated from	Inhibition (%)
1	*E. pole-evansii*	29 ± 3.0
4	*D. maculata*	61 ± 3.6
5	***D. burkei***	**81 ± 8.9**
6	*U.delagoensis*	25 ± 8.8
7	*D. maculata*	60 ± 2.2
8	***D. maculata***	**83 ± 6.3**
10	*E. comosa*	28 ± 9.0
11	***L. zebrina***	**70 ± 3.8**
12	*L.zebrina*	46 ± 0.7
13	*M. natalensis*	68 ± 8.9
14	***M. natalensis***	**70 ± 0.1**
15	*A. fastigiata*	56 ± 2.4
16	***D. burkei***	**100 ± 2.2**
17	***L. ovatifolia***	**72 ± 4.5**
18	Synthetic	23 ± 8.9
19	Synthetic	27 ± 2.6
20	Synthetic	4 ± 4.4
21	*T. longiflorus*	7 ± 6.4
22	Synthetic	21 ± 0.7
23	*D. maculata*	65 ± 4.7
24	*D. maculata*	47 ± 1.2
25	*E. humilis*	34 ± 1.7
Indomethacin	-	70-80

*activity of 70% and above = significant

*activity between 70% and 40% = moderate

*activity below 40% = low

Unspecific inhibition occurs when plant extracts are tested. Therefore, exclusion criteria for activity were set, making use of the levels of activity defined by Tunon *et al.* [36]. Using a test concentration of 200 µg/ml, activity between 70% and 100% was considered high, activity between 40% and 70% as moderate and activity between 20% and 40% as low [36]. During this investigation, a test concentration of 250 µg/ml was used and an activity of 70% and above was considered significant and between 40% and 70% as moderate. Six compounds (**5**, **8**, **11**, **14**, **16**, **17**), showed significantly high levels of anti-inflammatory activity. Seven compounds (**4**, **7**, **12**, **13**, **15**, **23**, **24**) showed moderate activity and nine compounds showed very low activity (Table 4.3).

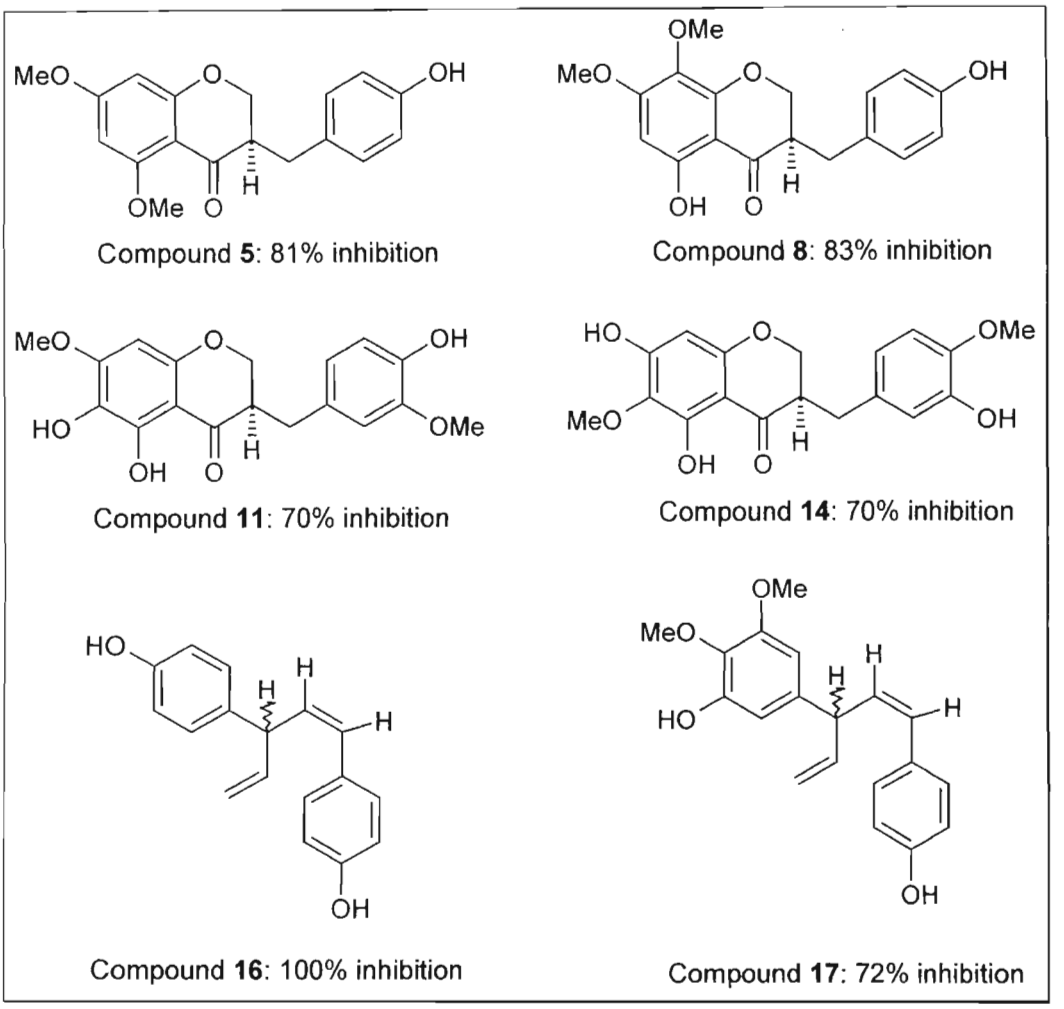


Figure 4.1. Compounds with significant anti-inflammatory activity (inhibition ≥ 70%).

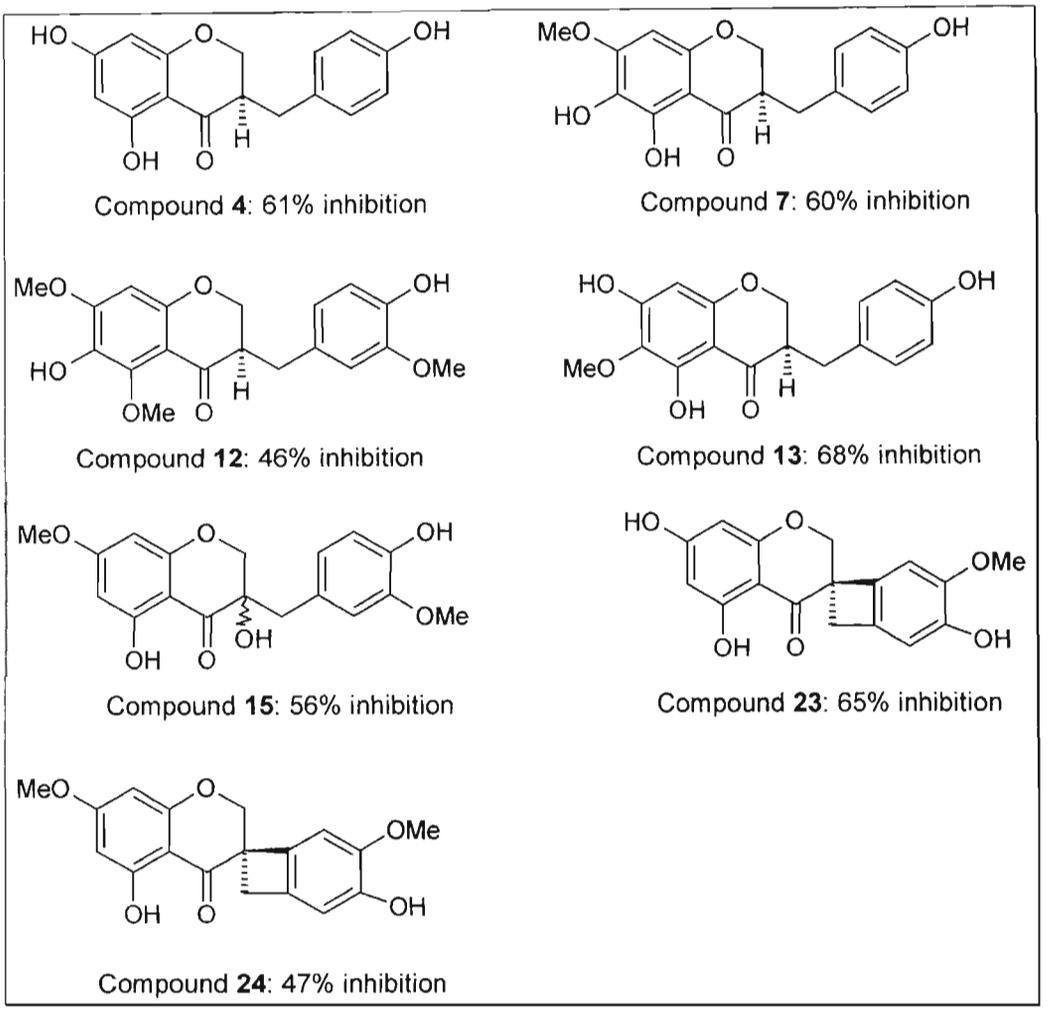


Figure 4.2. Compounds with moderate anti-inflammatory activity (% inhibition between 40 and 70%)

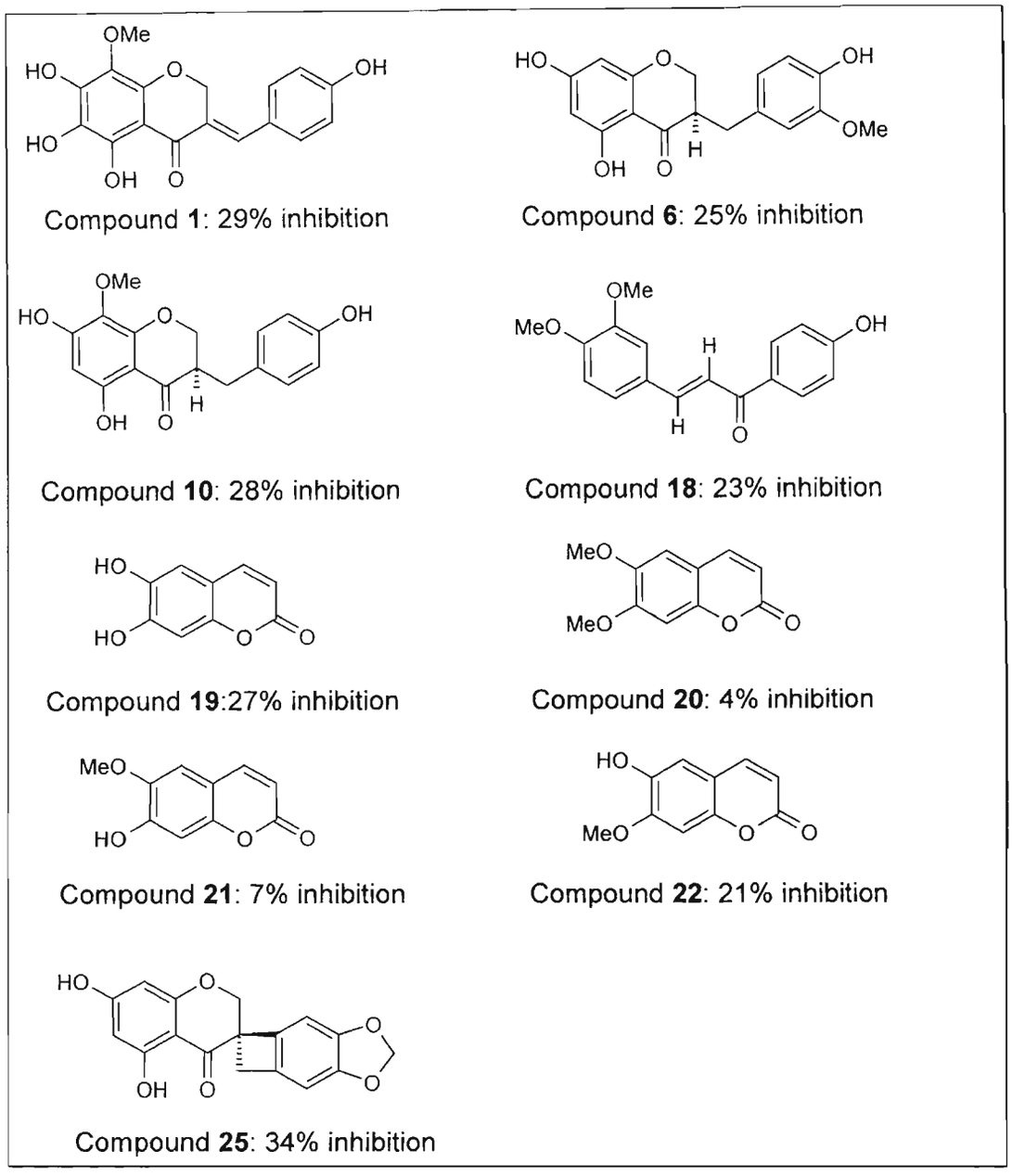


Figure 4.3. Compounds exhibiting anti-inflammatory activity lower than 40%

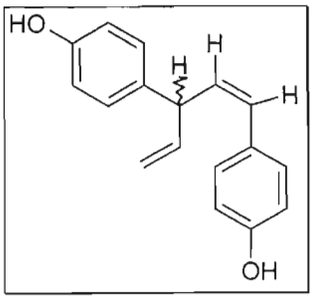
This assay was repeated on purified COX-1 and COX-2 enzymes (Table 4.4).

Table 4.4. The % inhibition of COX-1 enzymes by isolated compounds

Compound number	Inhibition of COX-1 (%)	Inhibition of COX-2 (%)
2	-	-
3	-	17 ± 7.1
4	-	
5	43 ± 2.1	-
7	-	-
8	26 ± 8.9	-
10	-	2.5 ± 2.2
11	24 ± 6.7	-
13	35 ± 9.0	14 ± 6.4
14	21 ± 2	12 ± 4.7
16	100 ± 0.5	19 ± 2.4
17	-	23 ± 8.9
18	-	-
19	-	-
21	-	-
23	-	-
24	-	-
25	-	-
Indomethacin	60-70	60-70

Only one compound (**16**) showed high levels of anti-inflammatory activity and one (**5**) moderate levels. The activity was significantly lower than that measured with cell microsomal fractions. Potencies of compounds to inhibit purified enzyme have been found to be different compared to inhibition of enzymes contained in cells previously [37]. Studies on isolated enzymes are highly informative, but do not always mimic the *in vivo* situation. *In vitro* conditions do not take factors such as binding of compounds to plasma/proteins and the possibility that several substrates, inhibitors and co-factors are active in the cell into account [13]. Isolated enzyme assays are, as mentioned before, mechanism based, but since cell microsomal fractions were used, other mechanisms of action for the test compounds are possible. Very low COX-2 inhibitory activities (between 0-23%) were observed for all the compounds. Thus COX-1 inhibitory activity was much higher than COX-2 inhibitory activity.

Dose-response curves were drawn for compound **16**, a norlignan.



From this data the IC_{50} value was calculated making use of regression analysis.

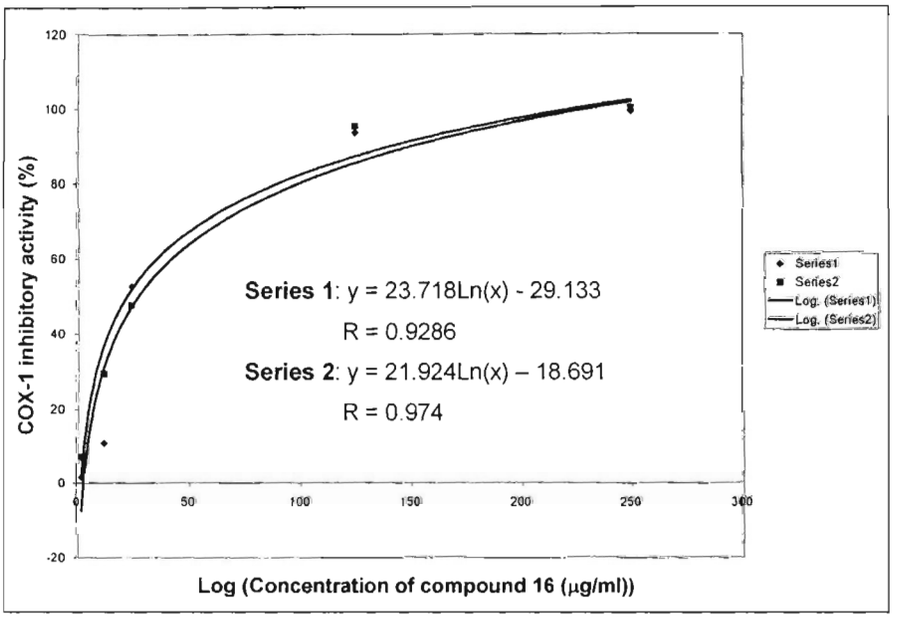


Figure 4.4. Logarithmic concentration versus inhibitory activity curves of compound **16** isolated from the bulbs of *Drimiopsis burkei* tested in the COX-1 assay

The IC_{50} value of compound **16** was calculated to be 0.101 ± 0.01mM. This is a relatively low IC_{50} value and it emphasizes the pharmacological potential of the compound. Both the norlignans (compounds **16** and **17**) exhibited

significant activity in cell microsomal fractions, whilst the chalcone (compound **18**) exhibited very low activity.

The only documentation of anti-inflammatory activity of all the tested compounds was that of compound **4** which inhibited croton oil-induced dermatitis (40% against indomethacin 60%) in the mouse ear in a dose dependent way [9].

Of the compounds exhibiting significant anti-inflammatory activities employing microsomal cells (≥70%), three were isolated from *Drimiopsis* species, two from *Ledebouria* species and one from *Merwilla natalensis*. This information validates the ethnomedicinal use of these homoisoflavanone-containing plant species. Powdered bulbs of *M. natalensis* are rubbed on sprains and fractures by the Southern Sotho [7] and *L. ovatifolium* is used for influenza and backache [8]. All these conditions are associated with pain, inflammation and/or fever.

Of the compounds isolated from *Drimiopsis maculata*, one compound (**8**) exhibited very high anti-inflammatory activity and four compounds (**4**, **7**, **23**, **24**) moderate activity. *Drimiopsis maculata* is used by traditional healers as medicine for stomach ailments in young children, and the effectiveness has been ascribed to the mucilage produced [8]. However, the fact that prostaglandin E_2 acts mainly as spasmogenic in the intestinal tract [38] and that prostaglandins exhibit diarrheogenic properties [39] is of significant importance here. Inhibition of prostaglandin synthesis by the compounds isolated from *Drimiopsis maculata* will relieve spasms of the smooth muscle of the intestinal tract and will stop diarrhoea. This validates the ethnomedicinal usage of this plant for stomach disorders. In addition, stomach ailments in children are also often accompanied by fever which would be controlled by using the compounds (**4**, **7**, **8**, **23**, **24**) with anti-inflammatory activity isolated from *Drimiopsis maculata*.

Taylor and Van Staden [40, 41] tested bulb extracts from different *Eucomis* species including *E. comosa-punctata, E. humulis and E. pole-evasii* for anti-inflammatory activity using microsomal cells. Crude ethanolic bulb extracts (using screening concentrations of 250 μg/ml), produced high COX-1 (>70%) and COX-2 (>70%) inhibitory activity. This investigation of the anti-inflammatory activity of purified compounds did not reveal similar high inhibitory results by homoisoflavanones isolated from the bulbs of the *Eucomis* species (see Tables 4.3 and 4.4). No significant COX-2 inhibitory activity could be found for the test compounds.

It is known that chemical content varies within plant populations and the possible reasons are to aid in adaptation for survival and growth within changing ecosystems. Steyn [42] found that toxicity in plants varies with soil and cultivation, climatic conditions, nature and intensity of light, season, stage of development and parts of the plants used. The state of the plants, whether fresh or dried plant material is used, also plays an important role in toxicity. Taylor and Van Staden [40] however, found that COX-1 inhibitory levels did not differ significantly in specimens harvested during summer or winter and that COX-1 inhibitors were relatively stable over time, both in solution (ethanol) and in dried plant material. The *Eucomis* species are also widely used in different parts of South-Africa with different soil and climatic conditions by Southern Sotho, Tswana, Xhosa and Zulu people mainly for pain and inflammation [43].

The best explanation of the discrepancy between activities found by Taylor and Van Staden [41] and in this investigation was that the crude extracts of bulbs of *Eucomis* species must contain other compounds (known or unknown), than the homoisoflavanones tested in this study, with high COX-2 inhibitory activity. The difference between COX-1 and COX-2 inhibition levels in various plant parts tested by Taylor and Van Staden [41] also implied that different active compounds were present. This warrants a re-investigation at the biological activity of compounds isolated from the *Eucomis* species.

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CHAPTER 5

ANTIBACTERIAL SCREENING OF HOMOISOFLAVANONES AND RELATED STRUCTURES

5.1. INTRODUCTION

The development of antimicrobial agents for clinical use has brought unquestionable benefit to individuals and society. Infectious diseases that were formerly often fatal became curable [1]. However, mankind is now confronted with new and re-emerging infections for which no effective treatments are available [2]. In contrast to other types of medication, antibiotics ultimately lose their effectiveness as they are used over time and resistant strains of bacteria develop [1]. Antibiotic resistance is of crucial importance since several pathogens are currently undergoing rapid evolution. One example is that of the Gram-positive bacteria, methicillin-resistant *Staphylococcus aureus*. Incidence figures in some hospitals have shown that more than 40% of strains of *S. aureus* are resistant to methicillin [1,3]. There is an urgent need to identify novel, active chemotypes as leads for drug development [2] and natural products will play a crucial role in meeting this demand [4]. Of the drugs approved between 1983 and 1994 by either the United States Food and Drug Administration (FDA) or comparable entities in other countries, drugs of natural origin predominate (78%) in the area of antibacterials [2].

Plants exhibit a high resistance to bacterial diseases, and depend on many defense mechanisms. One of these defense mechanisms is the synthesis of antimicrobial compounds [5]. The presence of antibacterial activity in plants is important from an ecological as well as pharmacological viewpoint.

5.2. METHODOLOGY

5.2.1 Test compounds

The compounds used in this study are discussed in Chapter 4. They are homoisoflavanones and structurally related compounds isolated from different plant sources. Most of these compounds are new or have never been investigated before.

5.2.2 Bacteria

The test compounds were screened against *Staphylococcus aureus* (ATCC 12600). *Staphylococci* are natural inhabitants of the body. However, as pathogens they cause many suppurative infections ranging from boils, carbuncles and abscesses to fatal septicaemias. They are also often secondary invaders in bronchitis, cystitis, meningitis and other conditions [6,7]. *Staphylococcus* infections are of great variety and an enourmous range of severity.

5.2.3. Bioassays

5.2.3.1. Bioautographic method

Test compounds were spotted on thin layer chromatography (TLC) plates (Merck, Kieselgel 60 F254). The plates were then developed in ethyl acetate/ hexane (80:20) and left to dry overnight. An overnight culture of *S. aureus* was grown in Mueller-Hinton (MH) broth (Oxoid), in a water bath at 37°C. The culture was then centrifuged at 3000 g for 10 minutes. The supernatant was decanted, the pellet was resuspended in 10 ml of MH broth and sprayed onto the TLC plate. The plate was then placed on damp tissue in a metal tray covered with plastic and incubated at 37°C in an oven at 100% humidity for 18

hours. Plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT) (Sigma). Bacteria reduce the tetrazolium salt through dehydrogenase activity and produce an intensely coloured formazan [8]. Inhibition of growth was indicated by clear zones against a dark pink background (see figure 5.1).

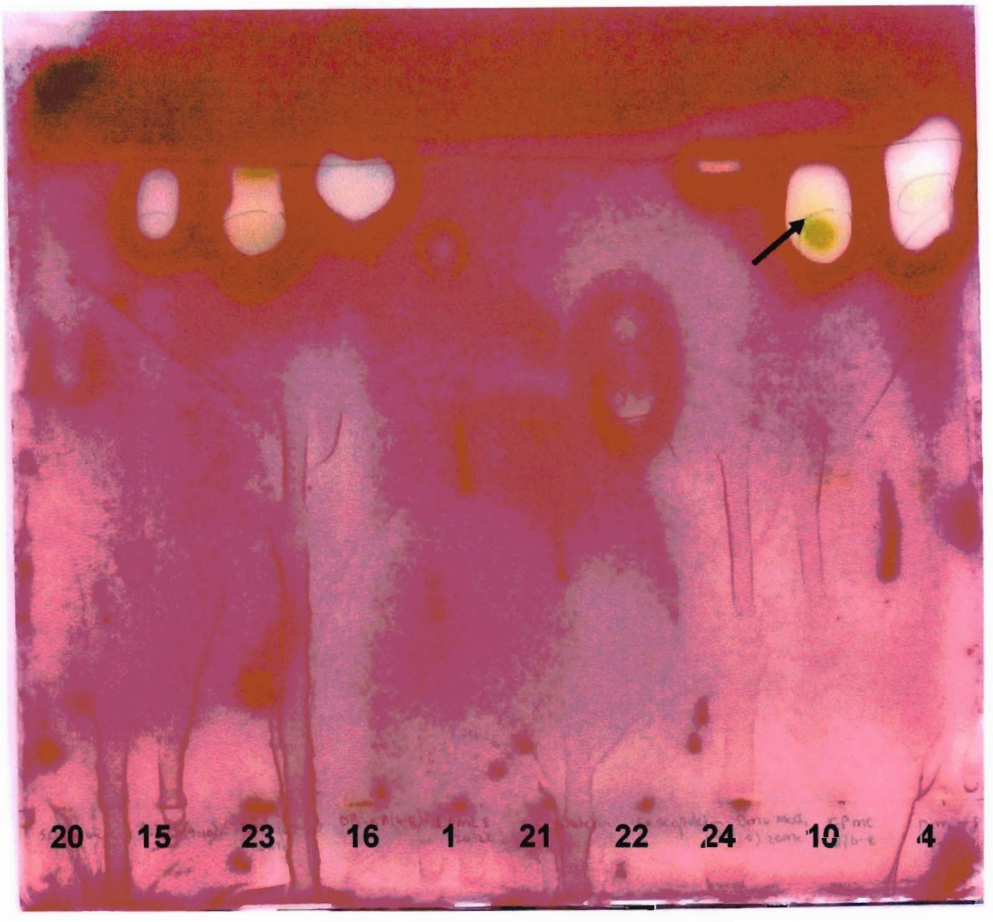

Figure 5.1. An example of the bioautographic assay. The background of the TLC plate is an intense pink colour and the inhibition zones clear as indicated by the arrow. Compound numbers were added at the bottom of the plate. The solvent system was ethyl acetate/ hexane (80:20).

5.2.3.2. The microplate antibacterial method

Compounds that showed antibacterial activity with the bioautographic method (see table 5.1), were dissolved to a concentration of 10 mg/ml in ethanol or DMSO. Sterile water (100 µl) was added to each well of the microplates. For each dissolved compound a two-fold serial dilution was made down the microplate starting at a concentration of 5.0 mg/ml. Overnight bacterial culture was diluted (1:100) in MH broth and 100 µl added to each well. This gives a further dilution to 2.5 mg/ml. Microplates were covered with plastic and incubated overnight at 37°C. The following day 40 µl of 0.2 mg/ml INT was added to each well and incubated at 37°C for 30 minutes. With INT the bacterial suspension turned red where bacterial growth was not inhibited. Where bacterial growth was inhibited, the suspension in the well remained clear. Bacteriostatic activity caused the wells to become a faint pinkish brown colour (see figure 5.2). Neomycin (50 µg/ml) was used as a positive control and solvent and bacteria free wells were used as negative controls. Experiments were repeated twice.

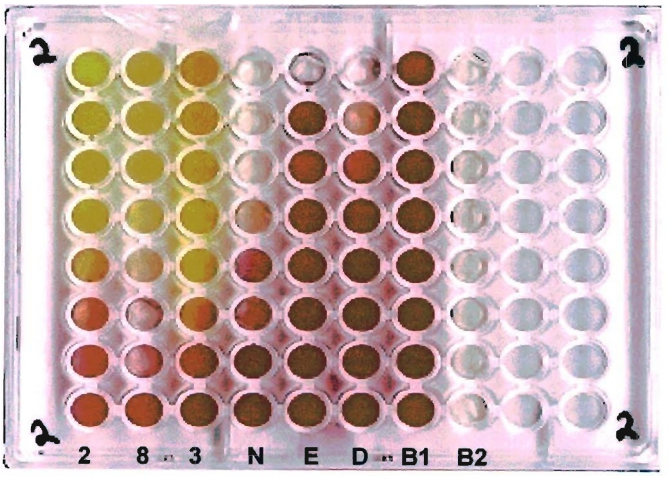

Figure 5.2. An example of the microplate method. Bacterial growth can be seen as dark pink wells. Wells in which inhibition of bacterial growth occurred are a yellowish colour and a light pink colour indicates bacteriostatic activity. Compound numbers are indicated at the bottom of the plate as well as the controls, neomycin (N), ethanol (E), DMSO (D), Bacteria (B1) and Broth (B2)

5.3. RESULTS AND DISCUSSION

The bioauthographic method was successfully employed to determine which compounds exhibited antimicrobial activity.

Table 5.1. Inhibition of *S.aureus* growth by different compounds employing the bioauthographic method.

Compound number	Inhibition	Compound number	Inhibition
1	-	**15**	+
2	+	**16**	+
3	+	**17**	-
4	+	**18**	-
5	+	**19**	-
6	+	**20**	-
7	+	**21**	-
8	+	**22**	-
9	+	**23**	+
10	+	**24**	+
11	-	**25**	+
12	-		

- no inhibition of bacterial growth

+ inhibition of bacterial growth

The synthetic compounds and those isolated from the Gentianaceae family (**18**, **19**, **20**, **21**, **22**) as well as compound **1** (isolated from *E. pole-evansii*) were inactive against *S. aureus*.

(**19**) R1, R2 = OH
(**20**) R1, R2 = OCH_3
(**21**) R1 = OCH_3, R2 = OH
(**22**) R1 = OH, R2 = OCH_3

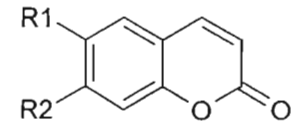

Figure 5.3. The structures of the coumarins, aesculetin (**19**), scoparone (**20**), scopoletin (**21**) and isoscopoletin (**22**)

Although Sparg [9] reported good antimicrobial activity of ethanolic and dichloromethane extracts of *Ledebouria ovatifolia* against *Staphylococcus aureus* and *Bacillus subtilis*, the compounds isolated from the *Ledebouria* species exhibited no antimicrobial activity. The activity is probably due to other compounds than those tested in this study.

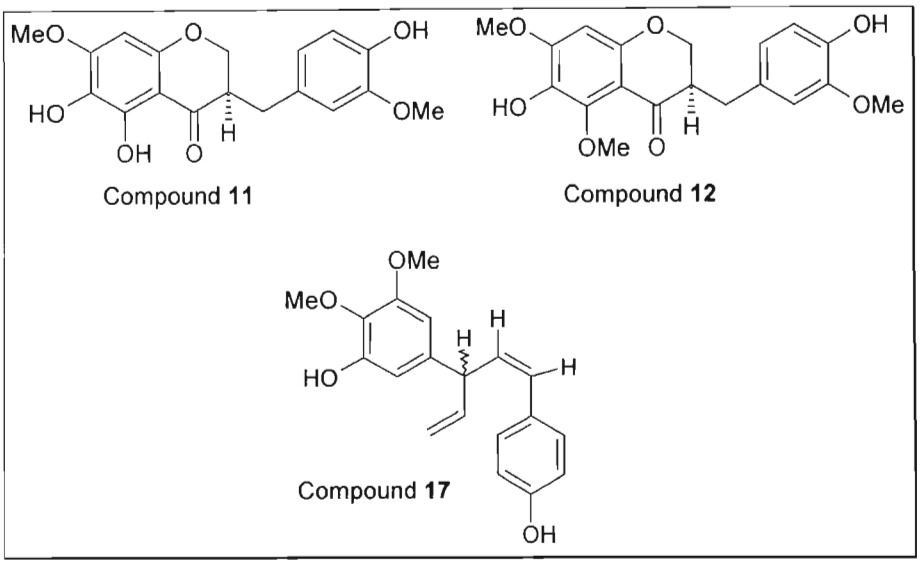

Figure 5.4. Compounds isolated from *Ledebouria zebrina* (**11** and **12**) and *Ledebouria ovatifolia* (**17**).

According to the results of the bioauthographic assay, compounds were chosen for determination of Minimum Inhibitory Concentration (MIC) and Bacteriostatic Concentration (BC) values. Only ten compounds that showed strong inhibition and were available in high enough concentrations, were investigated further employing the microplate method. Eloff [8], described the microplate method to determine MIC and BC values for compounds or extracts with antibacterial activity. The method is not expensive, requires a small quantity of sample and can be used for a large number of samples. Since cultures do not dry out after a day or two it was easy to detect bacteriostatic and bacteriocidal effects. A further advantage of this assay is that it is about 30 times more sensitive than other methods used in the literature and gave reproducible results [8].

Table 5.2. Comparison of MIC and BC (where applicable) values of compounds tested against *S. aureus* with the microplate assay.

Compound number	Plant source	MIC value in mM	BC value in mM
2	*E.comosa*	0.52	
3	*E.comosa*	0.24	
6	*U.delagoensis*	1.97	
7	*D.maculata*	3.95	
8	*D.maculata*	0.47	
9	*E.comosa*	4.15	2.07
10	*E.comosa*	0.98	
23	*D.maculata*	3.95	1.97
24	*D.maculata*	7.60	
25	*E.humilis*	0.50	
Neomycin		0.0025	

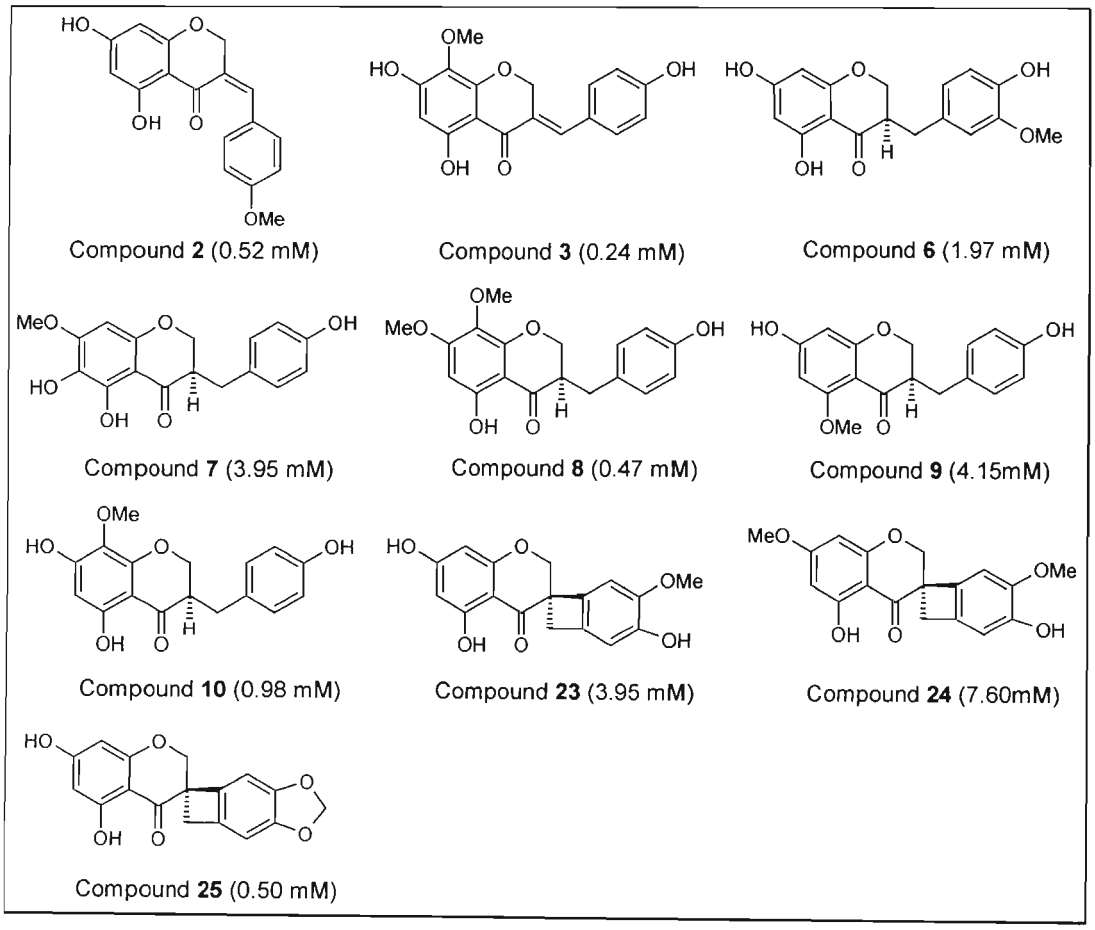

Figure 5.5. Compounds showing antimicrobial activity with the microplate assay (MIC value in mM)

Compounds **2** (isolated from *E. comosa*), **3** (isolated from *E. comosa*), **8** (isolated from *D. maculata*) and **25** (isolated from *E. humulis)* showed significant inhibitory activity against *S. aureus* with MIC values of ≤ 0.54mM. Compounds **9** and **23** were the only compounds exhibiting bacteriostatic activity. Although little is known about the antimicrobial activity of the specific *Eucomis* species from which the test compounds were isolated, the ethnomedicinal usage of all *Eucomis* species in terms of antimicrobial activity was investigated. *E. autumnalis* [10] and *E. regia* [11] are used for coughs and respiratory problems. Leaves of *E. undalata* are sometimes used as a poultice on suppurating sores and boils and the juice of the stems are used to soothe sores and rashes [12]. Secondary infections of *S. aureus* are associated with pneumonia, bronchitis and suppurative infections like boils [6,7]. The homoisoflavanones present in *Eucomis* species could be the reason for the effectiveness of some of these species against respiratory problems as well as sores and boils.

Drimiopsis maculata is used as ethnomedicine for stomach ailments in young children [10]. Although stomach ailments are not usually associated with *S. aureus*, except perhaps in food poisoning, compound **8**, isolated from this plant showed significant antimicrobial activity with a MIC value of 0.47mM. Compounds **7**, **23** and **24** isolated from *D. maculata* also exhibited antimicrobial activity. Interestingly, compounds **4**, **7**, **8**, **23**, **24** isolated from *D. maculata* inhibited prostaglandin synthesis in microsomal cells as well. The activity of the compounds isolated from this plant validates its popularity amongst traditional healers. *Drimiopsis maculata* is rated amongst the twenty-three most popular plants grown by Zulu traditional healers at their homes [13] and it is non-toxic [11].

Although the compounds were only tested against *S. aureus*, the results were encouraging and the project could be extended to other Gram-positive and Gram-negative organisms. Scientific exploration of compounds isolated from