Steroidal Glycosides of Cordyline australis

A thesis
submitted in partial fulfilment
of the requirements
for the degree of

Master of Philosophy in Chemistry

At the

University of Waikato

by

Tamar Korkashvili

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2006

ABSTRACT

The *n*-butanol extract of aerial parts of *Cordyline australis* demonstrated antifungal activity. *n*-Butanol and chloroform extracts of dried or fresh leaves of *C. australis* afforded a steroidal glycoside, which was identified as 5α-spirost-25(27)-en-3β-ol 3-*O*{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside}, saponin **1**. This spirostanol glycoside showed strong antifungal activity towards *Trichophyton mentagrophytes* and some aspecific activity and cytotoxicity against *MRC5* cell.

The chloroform extract of fresh leaves of *C. australis* yielded a second new spirostanol glycoside which was identified as 5α-spirost-25(27)-ene-1β,3β-diol 1-{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranoside}, saponin **2.** The *n*-butanol extracts of senescent leaves of *C. australis* afforded a third new spirostanol glycoside that was identified as 5α-spirost-25(27)-ene-1β,3β-diol 1-{*O*-β-D- fucopyranoside, saponin **3.**

A mixture of two isomeric flavonoid glycosides was isolated from dried leaves of *C. australis* and shown to be a *ca* 1:1 mixture of isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside}, **4** and isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside}, **5.**

Three other known steroidal glycosides, *β*-sitosterol glucoside, **6**, prosapogenin A of dioscin, **7**, and trillin, **8** were also isolated from the leaves of C. *australis*. The *n*-butanol extract of dried stems of *C. australis* afforded (25*S*)-5α-spirostane-1β,3α-diol 1-{*O*-β-D-glucopyranoside}, **9**. This spirostanol glycoside showed moderate cytotoxicity against *Herpes simplex type I virus* (ATCC VR733) and *Polio Virus Type I* (Pfiser vaccine strain).

ACNOWLEDGEMENT

I would like to take this opportunity to thank all those people who have contributed to this study and helped me complete this thesis.

Firstly I wish to thank Dr Merylin Manley-Harris, my first supervisor, who gave me the chance to undertake this thesis. Without her critical involvement and support this work would have never got started in the first place and never would have been finished.

Foremost I wish to thank my chief supervisor Professor Alistair Wilkins for suggesting to me the most beautiful plant for study, the cabbage tree, *Cordyline australis*, an icon of New Zealand. I am grateful for all his help, guiding me in GCMS and NMR experiments, for his knowledge and expertise, encouragement and supportive enthusiasm at a most difficult time, to finish this thesis on time.

I am also deeply grateful to Professor Brian Nicholson, the former Chairperson of the Department of Chemistry, who helped me with financial support for this study. Without his support and guidance this study would never have been done at Waikato University. Thanks also to the Scholarship's Committee for granting me a University of Waikato Research Study Award and an University of Waikato Doctoral Scholarship, and to the New Zealand Vice Chancellors' Committee for the award of a William Georgetti Scholarship.

Special thanks to all staff members of the Chemistry Department of the Waikato University, for their assistance, continuing support, and friendship during this study. My thanks to the Associate Professor Chris Hendy and Advanced Technical Officer Mrs Wendy Jackson, for supplying cabbage tree (*C. australis*) material for this study.

Also to Research Officer, Dr. Ralph Thomson for his technical advice in NMR spectrometry, Technical Officers Mrs Pat Gread and Wendy Jackson for assistance in ESMS and always helping me to find chemicals around the laborotories, Technical Officer Mrs Jannine Sims for her assistance in GCMS and her friendly

attitude, Senior Technical Officer Mr John Little for his prompt supply of goods and reagents, Technicians Mrs Amu Upreti and Annie Barker for their very kind assistance and encouragement, Technician Natalie Curnow for always helping me with the creation of cabbage tree images, Computer Support Consultant David Nicholls for his expertise in troubleshooting computer related problems and Administration Secretary Jacque Mackenzie for warm caring assistance in the Chemistry Department. Thanks also to Associate Professor Lindsay Main, Senior Lecturers Michele Prinsep and Tony Cartner who always offered friendly encouragement and support, Head of Department, Professor Bill Henderson, Associate Professors Peter Morris and Derek Smith, Senior Lecture Michael Mucalo from whom I always received friendly support, Science Librarian Cheryl Ward for her assistance and guidance with the Endnote program and helpful support during the study and PhD student Dougal Laird for helping me in final thesis formating.

I am deeply indebted to all my friends and colleagues from my mother country, the Republic of Georgia, Sakartvelo. Your love, friendship, support, encouragement are always with me. Great thanks to my former chief supervisor, Honored Scientist**,** Academician of the Georgian Academy of Sciences, Doctor of Pharmaceutical Sciences, former Director of Institute of Pharmacochemistry, Professor Ether Kemertelidze and all staff members of the Phytochemistry Department.

Finally, I am particularly indebted to my closest friends, my mother Nino, my father Shota, my brother Temur, my nephew Levan, my husband Levan, and my two sons Zourab and Becka for their love and support and being always together with me.

TABLE OF CONTENTS

CHAPTER TWO

CHAPTER THREE

Structural Elucidation of Steroidal and Flavonoid Glycosides from *C. australis*……..….29

CHAPTER FOUR

List of Figures

List of Tables

Abbreviations

^{1}J	One bond NMR coupling constant
^{2}J	Two bond NMR coupling constant
^{3}J	Three bond NMR coupling constant
^{4}J	Four bond NMR coupling constant
2D	two dimentional
%	percent
^{o}C	degrees Celsius
α	axial
β	equatorial
μg	microgram
μL	microlitre
AR	AnalaR grade solvent
ATCC	American Type Culture Collection
Br	broad peak
ca	approximately
cf	compared to
cm	centimetres
COSY	Correlated Spectroscopy
COSYLR	Correlation Spectroscopy Long Range
Δ	delta
d	doublet signal
dd	doublet of doublets
DEPT90	Distortionless Enhancement by Polarisation Transfer (with a 90° pulse)
DEPT135	Distortionless Enhancement by Polarisation Transfer (with a 135° pulse)
Dr	Doctor
ESMS	Electrospray Mass Spectroscopy
et al	and others
GC	Gas Chromatography

GCMS	Gas Chromatography-Mass Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
HOHAHA	Homonuclear-Hartmann-Hahn
HPLC	High Performance Liquid Chromatography
Hz	hertz
M	molar
M^+	molecular ion (positive ion detection)
M^-	molecular ion (negative ion detection)
MeOH	methanol
m.p.	melting point
MS	Mass Spectrometer
MSD	Mass Spectral Detector
m/z	mass to charge ratio
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Enhancement
NOESY	Nuclear Overhauser and Exchange Spectroscopy
NZ	New Zealand
OAc	acetate functional group
q	quartet carbon NMR signal
RF	Radio frequency
R_f	Retention factor
R_F	Response factor
RI	Refractive Index
ROESY	Rotating-frame Overhauser Enhancememt Spectroscopy
s	singlet carbon NMR signal
SIM	Selected Ion Mode
t	triplet carbon NMR signal
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
TOCSY	Total Correlation Spectroscopy
UV	Ultraviolet
V	volts

CHAPTER ONE

General Introduction

1.1 Saponins

Saponins are one of the biggest classes of compounds in natural products chemistry. The classical definition of saponins is based on their surface activity. Many saponins have detergent properties. [1-7]. 'Saponin' comes from the Latin word *sapo* meaning soap [1,2]. *Saponaria officinalis* (common name soapwort) is an example of a saponin containing plant that has been employed for hundreds of years, as a natural soap.

Saponins occur in nature as glycosides: that is they contain a sugar moiety linked to an aglycone. The aglycone or non-saccharide portion of the saponin molecule is called the genin or sapogenin or sometimes both terms are used in the same article [2,5,6,8]. Depending on the type of sapogenin or genins present, the saponins can be divided into three major classes, namely,

1. Triterpene glycosides (**Figure 1.1**)
2. Steroidal glycosides (**Figure 1.2**)
3. Alkaloid glycosides (**Figure 1.3**)

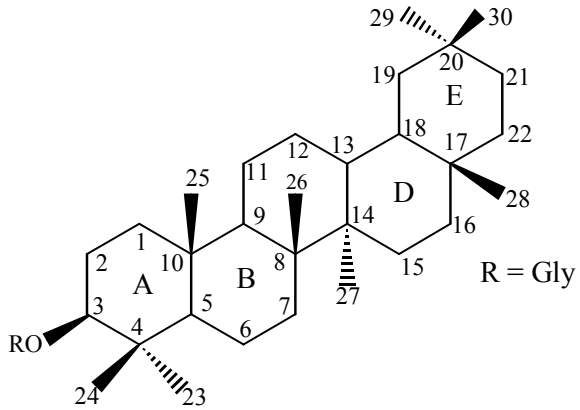


Figure 1.1 Typical structure of a triterpene glycoside

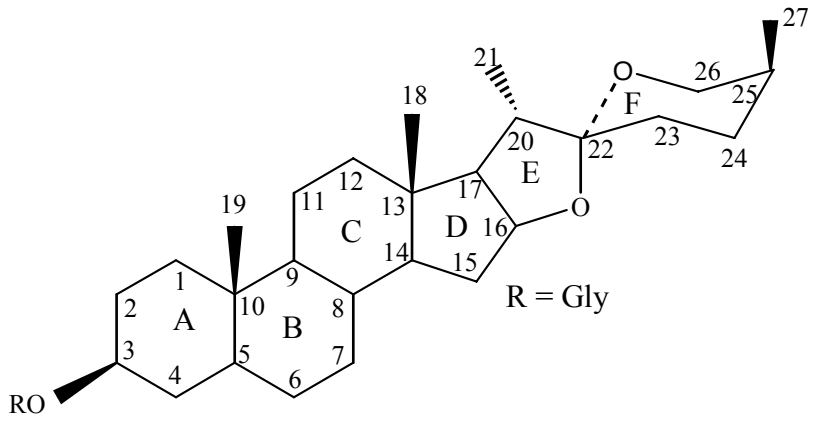


Figure 1.2 Typical structure of a steroidal glycoside

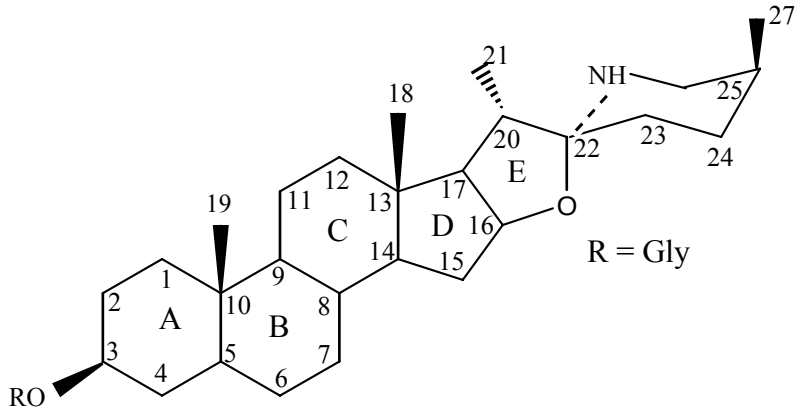


Figure 1.3 Typical structure of an alkaloid glycoside

Saponins are widely distributed in the plant and marine animal kingdoms [1-3,5,6,9-12]. A large number of saponins are biologically active [3,9-11,13-32]. Despite their diverse chemistry, saponins have some common characteristic properties. These include:

1. Bitter taste
2. Formation of stable foams in aqueous solution
3. Hemolysis of red blood cells
4. Toxicity to cold-blooded animals such as fish, snails, insects, etc.
5. An ability to interact with bile acids, cholesterol, or other 3β-hydroxysteroids in aqueous of alcoholic solution to form mixed micelles orcoprecipitates [1,2,4,6,11,12,18].

A number of plants consumed by humans (for nutrition) contain saponins. Examples of plant species which contain triterpene saponins include many of the edible beans, such as navy bean, kidney bean and green bean varieties of *Phaseolus vulgaris*, silver beet and sugar beet varieties of *Beta vulgaris*, spinach (*Spinacea olearacea*), peanuts (*Arachis hypogaeae*), quinua (*Chenopodium quinoa*), tea (*Thea sinensis*), licorice (*Glycyrrhiza glabr*a) and ginseng (*Panax spp*). Examples of plants which contain steroidal saponins include asparagus (*Asparagus officinalis*), oats (*Avena sativa*), potatoes and tomatoes (*Solanum spp*.) and garlic (*Allium sativum*) [5,12].

Recent publications have described the importance of spirostanes and furostanes (steroidal sapogenins) and their glycosides (steroidal saponins) not only as economically important raw materials convertible into various steroid hormonal drugs [33-36], but also as biologically active materials having independent value [3,9,12]. Anticancer and cytotoxicity [15,22,29,32,37-43], antitumour [21,27,30,38,44,45], antiinflamatory and antioxidant [26,46-48], antiviral [25], antifungal and antimicrobial [20,49], molluscicidal [50,51], antihypercholesteremic [28,52-55] and as a plant growth stimulant [56] activities have been reported for steroidal glycosides.

1.2 Steroidal Sapogenins and Saponins

Steroidal saponins are found in relatively limited sections of the Plant Kingdom. The order Liliales, comprising the Families Liliaceae, Amaryllidaceae and Dioscoreaceae (**Figure 1.4**) are the main plant groups in which these compounds are found. An exception is digitonin, a well known saponin found in *Digitalis lanata*, family Scrophulariaceae [7,57].

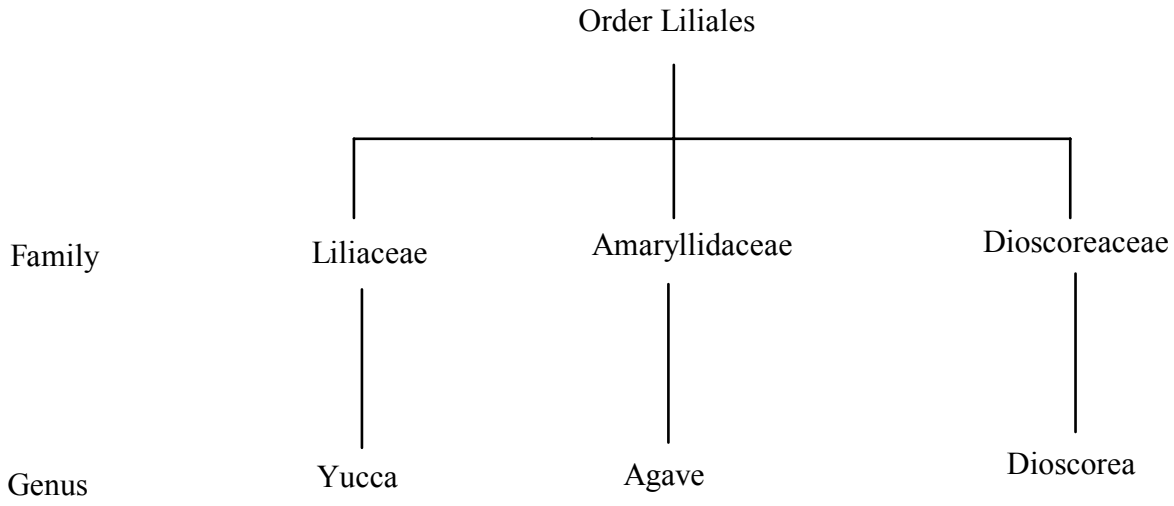


Figure 1.4 Distribution of steroidal saponins

Steroidal saponins or steroidal glycosides can be divided into three main classes, depending on what classes of steroidal sapogenins or aglycones they are derived from. Over 100 steroidal sapogenins are known and most of them possess spirostane or furostane skeletons (**Figure 1.5**). The cholestane skeleton is also found, but only rarely [1,2].

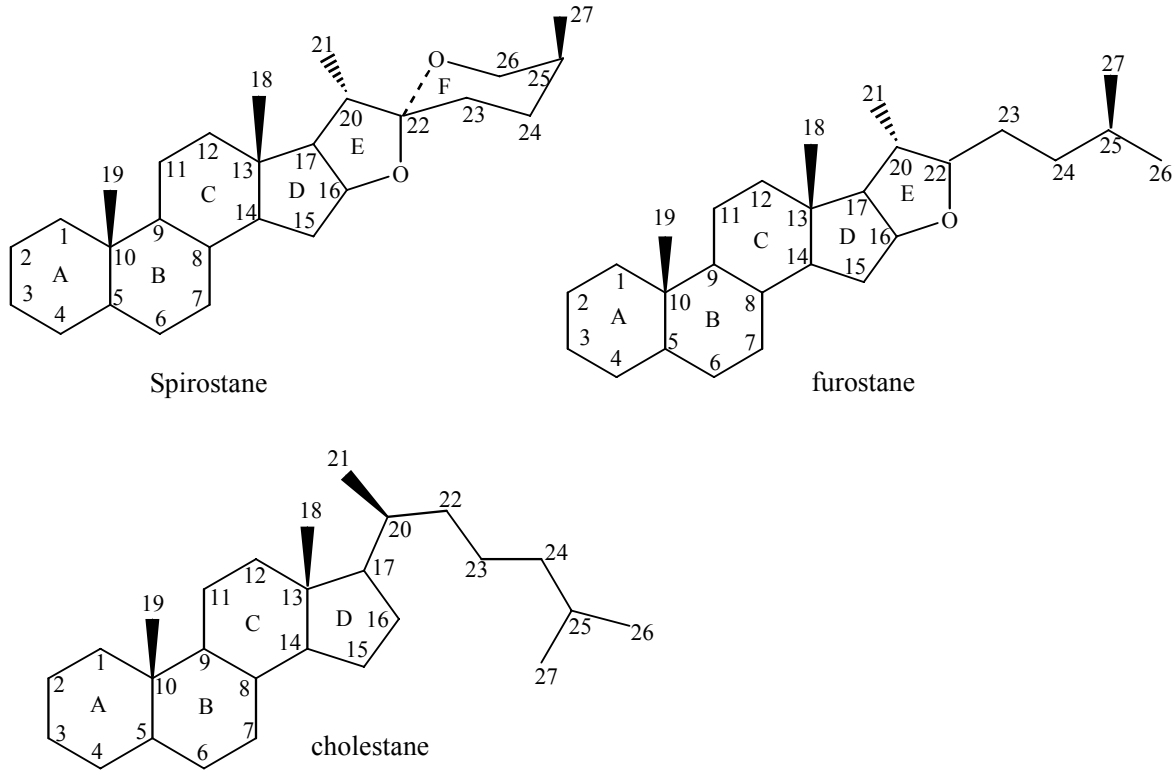
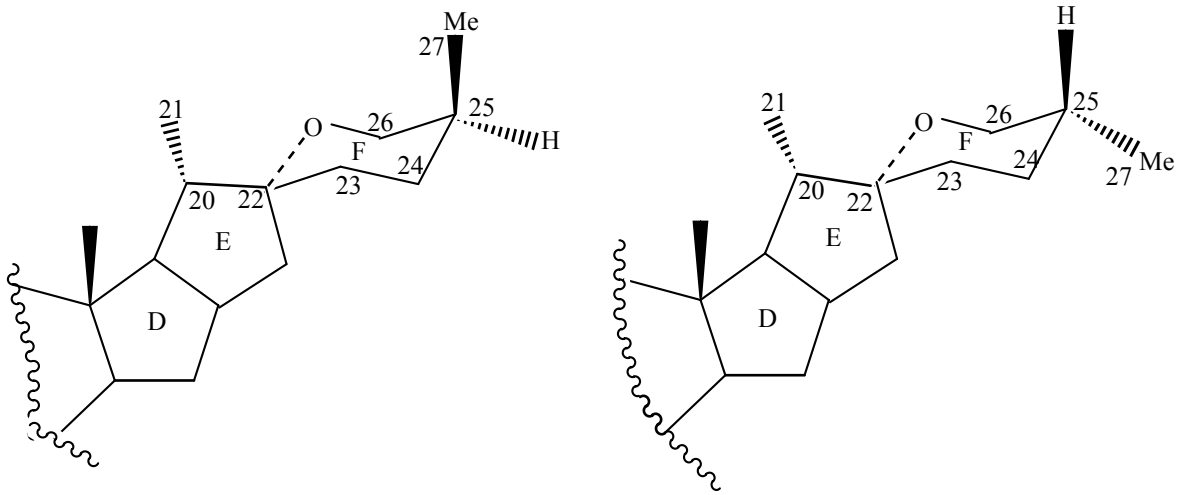


Figure 1.5 Common classes of steroidal sapogenins

In all cases the C-18 and C-19 angular methyl groups are β-orientated (on the upper face of the molecule) while the C-21 angular methyl group is α-orientated. There is sometimes a 5(6)-double bond. Sapogenins are mostly hydroxylated at C-3. There may also be hydroxyl groups at C-1, C-2, C-5, C-6, and/or C-11 [1,2,7,8].

Spirostanes are characterized by the presence of a ketospiroketal moiety (rings E/F) and may be subdivided into 25*R*- or 25*S*- series (**Figure 1.6).**



25*S*- (*neo*) 25*R*- (*iso*)

Figure 1.6 Partial structures 25*R*- and 25*S*-spirostanes

The 25*S*- (or 25β-) series and the 25*R*- (or 25α-) series were formerly referred to a *neo*-sapogenins and *iso*-sapogenins respectively. The C-25 methyl group is axially orientated in 25*R*- (or *iso*-) sapogenins and equatorially orientated in 25*S*- (or *neo*-) sapogenins [1,2,7,8,17,58].

Asymmetric centres occur at C-5, C-25, C-20, and C-22. Rings B/C and C/D are *trans* linked, while rings D/E are *cis* linked. Depending on whether rings A/B are *trans* or *cis* linked, H-5 is α- or β- oriented, as in tigogenin (5α-H) or smilagenin (5β-H) respectively [1,2,7,8,17,58] **(Figure 1.7).**

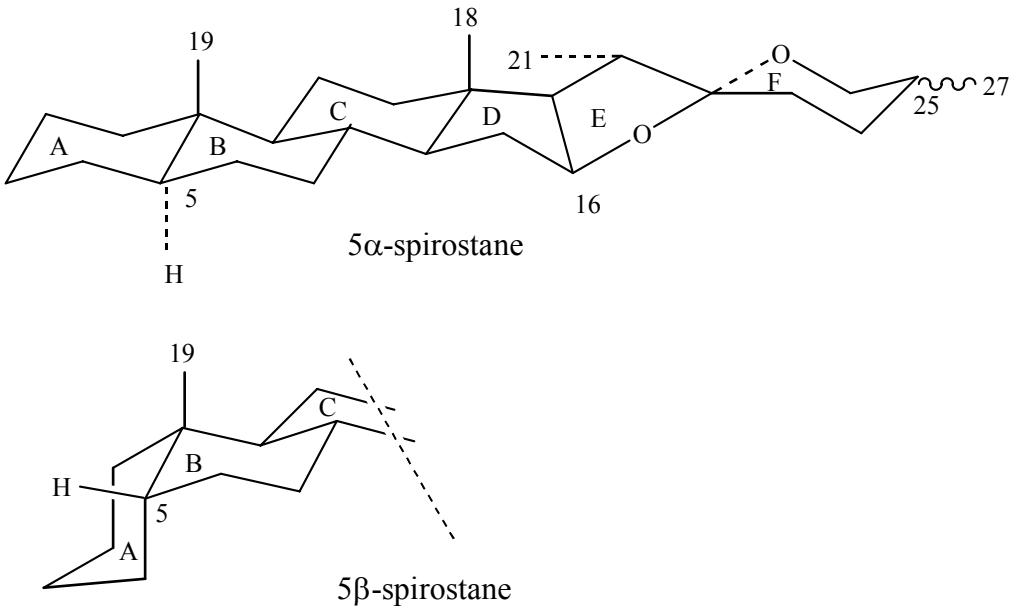


Figure 1.7 Structure (and partial structure) of 5α- and 5β-spirostanes

Furostane glycosides have an opened F ring and a sugar moiety attached at C-26. Generally furostane glycosides are bidesmodic since other glycoside residues are generally also attached to ring A (most commonly at C-3). Marker and Lopez [59] first postulated the existence of open chain glycosides of this type in 1947. Enzymatic or acid hydrolysis of the sugar in position C-26 leads to spontaneous ring closure, producing a spirostanol derivative. The reverse process, ring opening, has been observed in cell cultures [58,59].

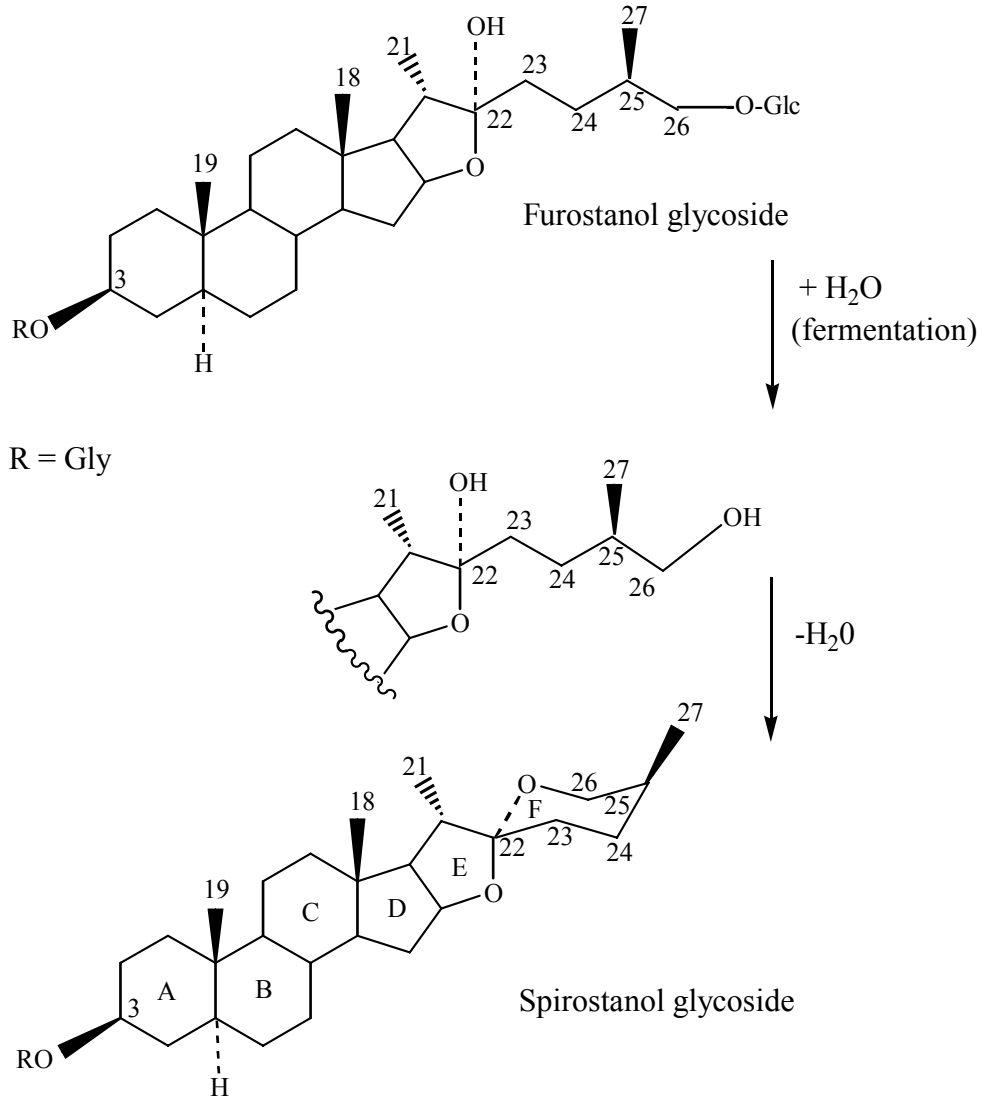


Figure 1.8 Conversion of a furostanol glycoside into a spirostane by enzymatic hydrolysis of the 26-glycoside

Isolation procedures to obtain pure saponins or glycosides are challenging. Structural elucidation of saponins is based on determinations of:

1. The structure of the aglycone.
2. The composition and sequence of the monosaccharides in the carbohydrate moiety.
3. The location of linkages between monosaccharide units.
4. The anomeric configuration of each glycosidically linked monosaccharide unit.
5. The location of the carbohydrate moiety on the aglycone.

Different physico-chemical, and instrumental methods such as liquid-solid and liquid-liquid extraction, TLC, flash chromatography, open-column chromatography IR, ^{1}H NMR and ^{13}C NMR spectroscopy, GLC, HPLC, GC and LC-MS, have been widely used for structural elucidation of sapogenins and saponins [6,60-73]. ^{13}C NMR spectroscopy is an integral part of the procedure for establishing the structure of new steroidal sapogenins and saponins. The first investigations of the ^{13}CNMR spectra of steroidal sapogenins were reported by Eggert and Djerassi [74]. Subsequently numerous authors, including Agrawal *et al* [71,75-82] have reported the ^{13}C NMR data for steroidal saponins.

1.3 The Genus *Cordyline*

The name *Cordyline* is derived from the Greek "kordule' meaning "club" to refer the form of the flower buds and was first reported by Adanson in 1768 [83]. The genus *Cordyline* comprises about 20 species and is distributed in South-east Asia, Australia, New Zealand and some parts of South America. These are evergreen, long-leaved trees and shrubs [84,85]. The New Zealand flora includes five native and one adventive representatives of the genus *Cordyline*, which are often referred to as cabbage trees. The Māori generic name for *Cordyline* species is Ti. Species listed in the Flora of New Zealand [85] are *C. terminalis (L.), C. australis* (*Forst.f.*) *Endl* (Ti kāuka [kōuka]),*C. banski* (*Hook.f.*), (forest cabbage tree, Ti ngahere or Ti parae), *C. indivisa* (*Forst.f.*), (mountain cabbage tree or broad leaved cabbage tree, Ti toii or Toi), *C. kaspar* (*W.R.B.*), (Three Kings Islands forest cabbage tree) and *C. pumilio* (*Hook f.*), (dwarf cabbage tree, Ti kohara or Ti papa) [85].

C. australis.Forester f., common names New Zealand cabbage tree, Giant Dracaena, Grass Palm, Sago Palm, Palm Lily, Ti kōuka, is found in New Zealand. This species is a tree plant which may reach 17-20 m. It may have a single trunk or several trunks which are usually branched. The bark is rough and corky. The leaves are narrow, typically 0.5-1 m long and 4-9 cm wide and the inner ones are strongly sweet scented. The fragrant white flowers occur in panicles and the berries are bluish-white [84,86-88] (**Figure 1.9).**

Figure 1.9 Cabbage tree, *C. australis*

The occurrence of steroidal sapogenins and saponins in the Genus *Cordyline* is reviewed in Section **1.3.1** below.

1.3.1 Steroidal Sapogenins from Genus *Cordyline*

The first reports of the presence of steroidal sapogenins in *Cordyline* species originated from the pioneering work of Marker *et al* [33] and Wall *et al* [34] who investigated natural plant sources of steroidal sapogenins, such as smilagenin and sarsasapogenin, as substrates for the commercial synthesis of steroidal hormones. Further studies concerning the distribution of steroidal sapogenins in the genus

Cordyline have been carried out by Jewers *et al* [89,90], Blunden *et al* [68,91-94]and Griffin *et al* [83,95]. Extracts of the leaves of *C. australis* obtained from plants grown in New Zealand and the United Kingdom gave a low yield of steroidal sapogenins [92]. The major compound was characterized as tigogenin based on TLC and IR data, while neotigogenin, diosgenin, yamogenin and brisbagenin were identified by TLC alone [92] (see **Table 1.1, Table 1.2, Figure 1.10** and **Figure 1.11).**

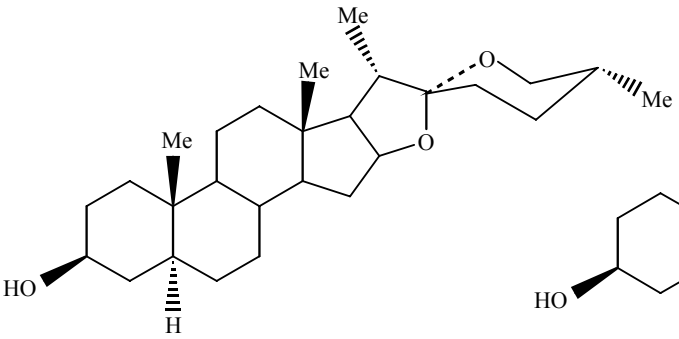
A New Zealand phytochemical survey [96] demonstrated the presence of steroidal saponins in extracts of *C. terminalis*, *C. australis* and *C. pumilio*.

Table 1.1 Steroidal sapogenins in *Cordyline spp*. [92]

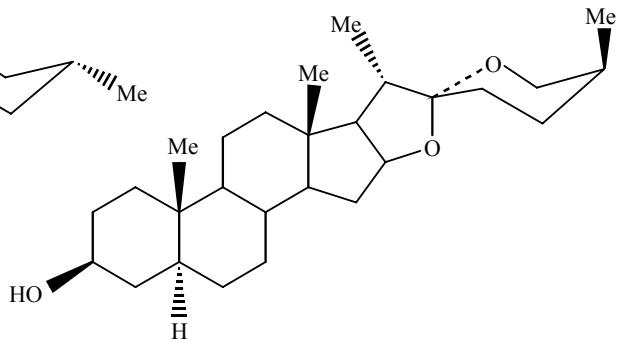
Sapogenins	*australis*	*banski*	*indiviza*	*manners/ girronie*	*pumilio*	*rubra*	*stricta*	*terminalis*
3-Epitigogenin	-	-	±	-	-	+	++	-
3-Epineotigogenin	-	-	-	-	-	±	+	-
Tigogenin	+	-	±	-	±	+	±	-
Neotigogenin	±	-	-	-	-	-	±	-
Diosgenin	±	±	-	-	-	±	±	-
Yamogenin	±	+	-	-	-	±	±	-
Smilagenin	-	-	-	-	-	-	-	++
Sarsasapogenin	-	-	-	-	-	-	-	++
Cannigenin	-	-	-	±	-	++	++	-
Cordylagenin	-	-	-	±	-	-	++	-
Brisbagenin	±	±	++	++	++	±	±	-
Ruscogenin	-	+	-	-	-	±	±	-
25*S*-Ruscogenin	-	+	-	-	-	-	-	-

Table 1.2 Structural identification data of sapogenins from *C. australis* [33,92,94,97]

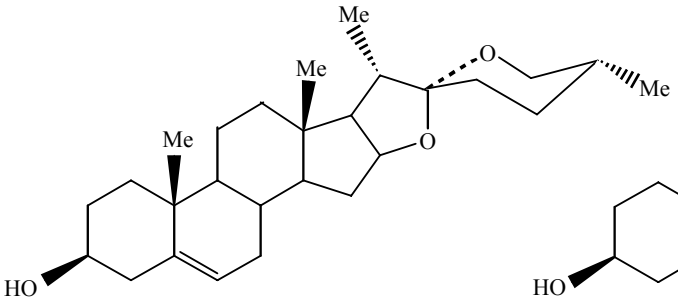
N	Name of genin		Dry plant sample
	Formula/ Trivial name	Rational name	
	$C_{27}H_{44}O_3$		
1	Tigogenin	25(*R*)-5α-spirostan-3β-ol	leaf, stem, root, fruit [92,94,97]
2	Neotigogenin	25(*S*)-5α-spirostan-3β-ol	leaf, fruit [92,94]
3	Epitigogenin	25(*R*)-5α-spirostan-3α-ol	fruit [94]
4	Epineotigogenin	25(*S*)-5α-spirostan-3α-ol	fruit [94]
5	Smilagenin	25(*R*)-5β-spirostan-3β-ol	leaf [33]
	$C_{27}H_{44}O_4$		
6	Brisbagenin	25(*R*)-5α-spirostane-1β,3β-diol	leaf, fruit [92,94]
7	Polygenin	25(*S*)-5α-spirostane-1β,3β-diol	fruit [94]
8	Gitogenin	25(*R*)-5α-spirostane-2α,3β-diol	stem, root [97]
	$C_{27}H_{42}O_3$		
9	Diosgenin	25(*R*)-spirost-5-en-3β-ol	leaf, stem, root, fruit [92,94,97]
10	Yamogenin	25(*S*)-spirost-5-en-3β-ol	leaf, fruit [92,94]
	$C_{27}H_{42}O_4$		
11	Australigenin	5α-spirost-25(27)-ene-1β,3β-diol	fruit [94]
12	Ruscogenin	Spirost-5-ene-1β,3β-diol	fruit [94]
	$C_{27}H_{40}O_3$		
13	Sceptrumgenin	Spirosta-5,25(27)-dien-3β-ol	fruit [94]



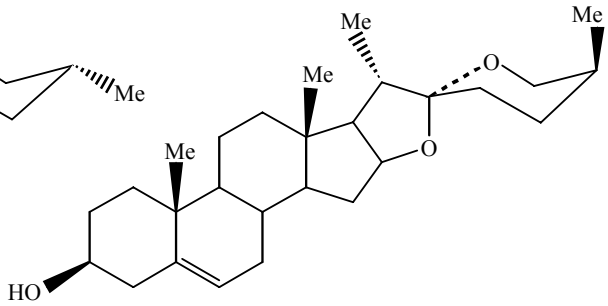
Tigogenin,
25(*R*)- sapogenin



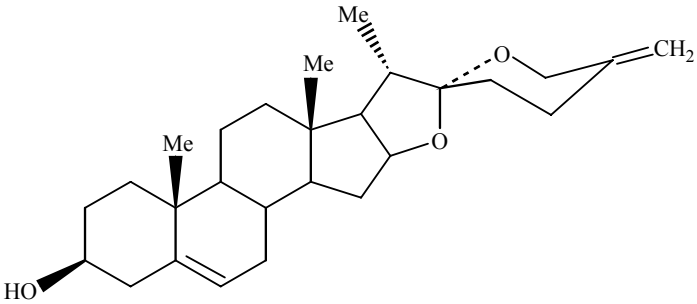
Neotigogenin,
25(*S*)-sapogenin



Diosgenin,
Δ 5(6)-, 25(*R*)-sapogenin



Yamogenin,
Δ 5(6)-, 25(*S*)-sapogenin



Sceptrumgenin
Δ 5(6)-, 25(27)-sapogenin

Figure 1.10 Monohydroxy steroidal sapogenins isolated from *C. australis*

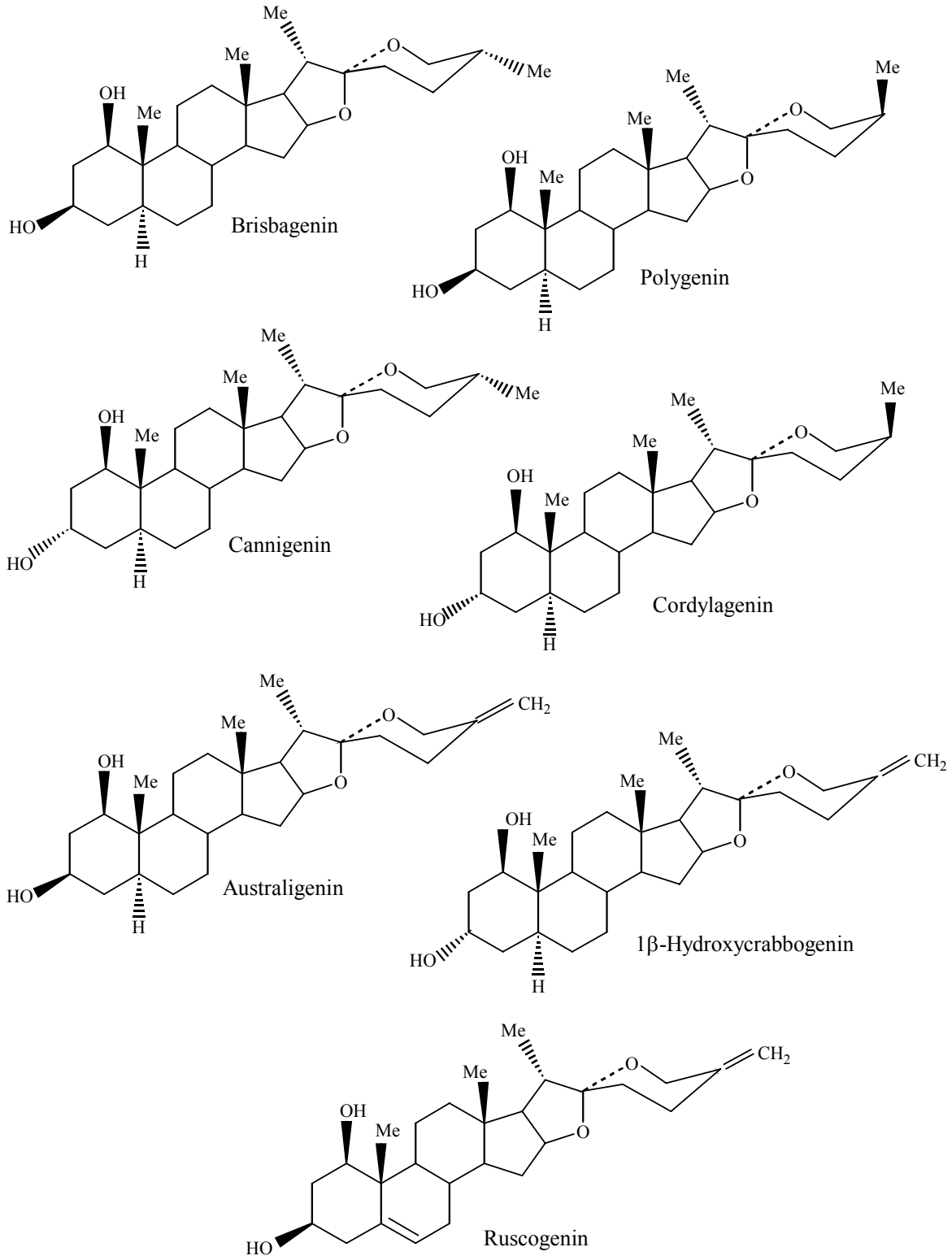


Figure 1.11 Dihydroxy steroidal sapogenins isolated from *C. australis*

The finding of these 5α-spirostanes in *C. australis* [92] (**Figure 1.10**, **Figure 1.11**)**,** contrasts with the report of the 5β-spirostane, smilagenin, in *C. australis* by Marker and Wall [33,34].

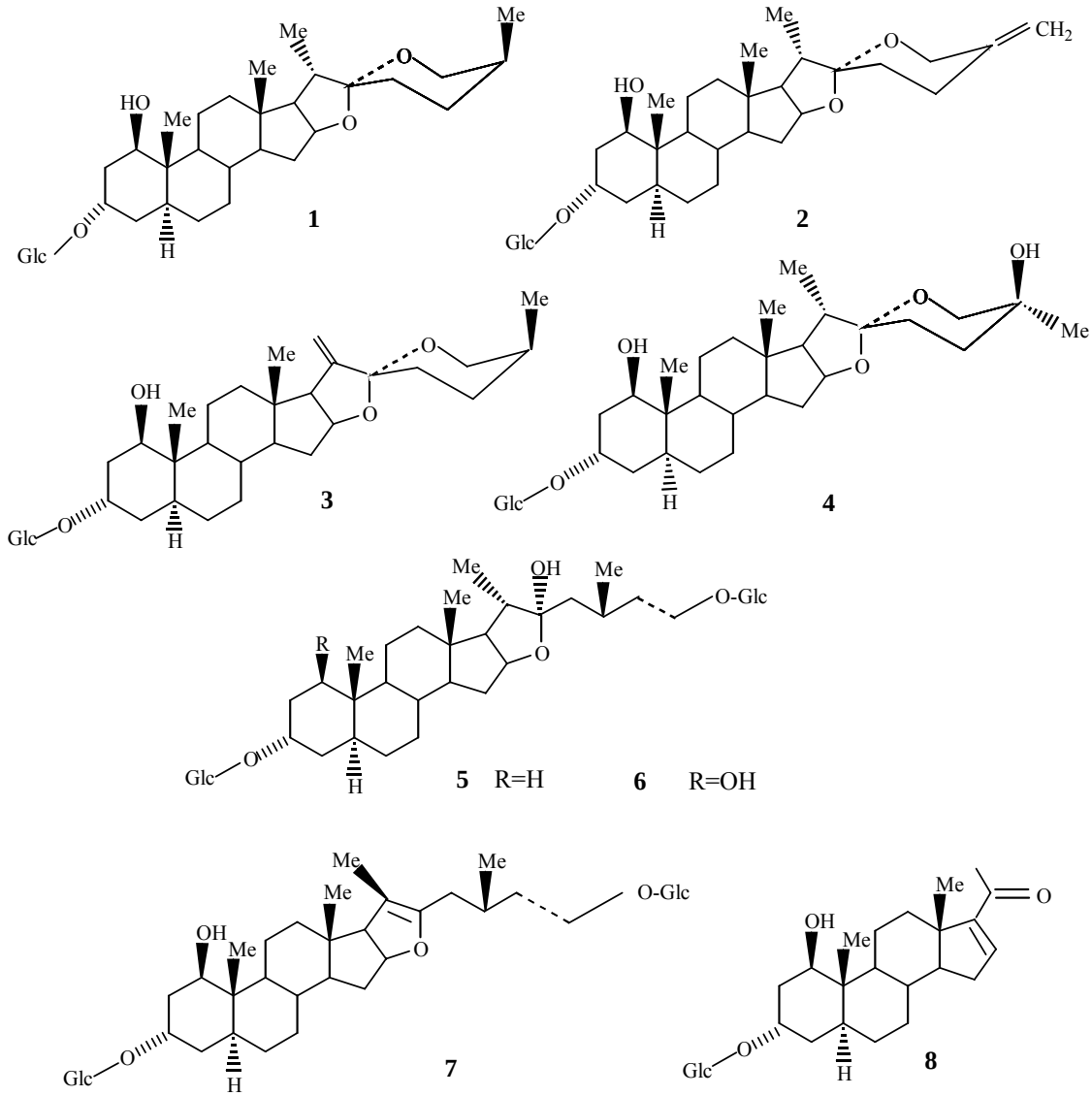
A new steroidal sapogenin was isolated from the fruits of *C. australis* and characterized by IR, ^{1}H NMR, and mass spectral data as 5α-spirost-25(27)-ene-1β,3β-diol (australigenin) [94]. (**Figure 1.11).** Thirteen other steroidal sapogenins have been identified in the fruits of *C. australis* including: polygenin, 1β-hydroxycrabbogenin and sceptrumgenin [94] (**Figure 1.10** and **Figure 1.11**)**.**

Mistry [97] has identified sapogenins in extracts obtained from the different parts of *C. australis* using GCMS and NMR methods. Dry leaves, stems and roots of young cabbage tree were first extracted with hexane to recover free sapogenins. Subsequent extraction with methanol : water (4 : 1) was employed to recover steroidal glycosides. Extracted steroidal glycosides were hydrolyzed with 1 mol/L HCl, methylated with diazomethane and acetylated using acetic anhydride-pyridine (1 : 1) to prepare them for GCMS analysis. ^{1}H and ^{13}C NMR spectra of the hexane extracts showed that only trace amounts of sapogenins were present in stem extracts. GCMS analyses showed that only the methanol : water extract of roots and the stems contained saponins, which were detected as the corresponding sapogenin acetates or diacetates. A total of six acetylated sapogenins were detected. Three were identified as the acetylated analogues of diosgenin, tigogenin and gitogenin [97].

1.3.2 Steroidal Glycosides from Genus *Cordyline*

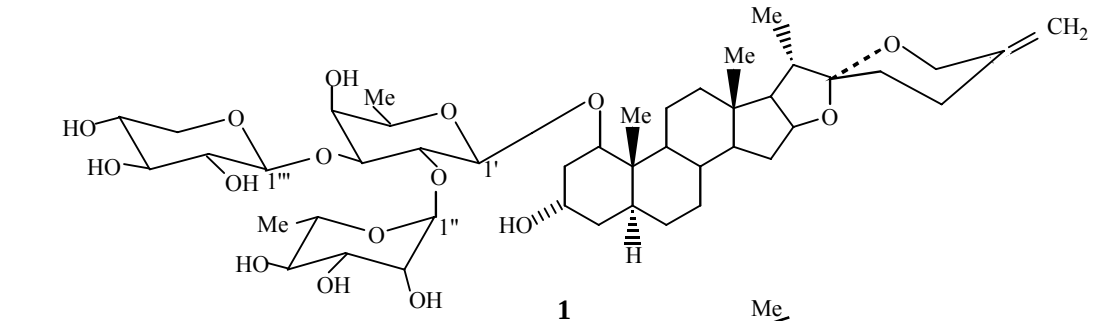
Although many steroidal sapogenins have been determined in *Cordyline spp*., no steroidal saponins or glycosides appear to have been isolated until 1997 [98]. The only reported isolation and structural determination of several saponins from *Cordyline* species, is that of Mimaki *et al* [98,99], who reported the isolation from *C. stricta* of several steroidal glycosides. Structures of four new spirostanol and three new furostanol glycosides isolated from the dry leaves of *C. stricta* were determined by NMR spectroscopy and their acid hydrolysis products. Each of the isolated compounds possesed a dihydroxy aglycone with a 3-α-*O*-glucopyranose

residue [98]. (**Figure 1.12).** Three new spirostanol saponins and two new furostanol saponins were isolated from the fresh leaves of *C. stricta*. Their structures were elucidated on the basis of spectroscopic analysis, including various 2D-NMR techniques, hydrolysis, and by comparison of spectral data determined for known compounds. Two of the isolated saponins contained a new branched triglycoside moiety assigned as *O*-α-L-rhamnopyranosyl-(1→2)-*O*-{β-D-xylopyranosyl-(1→3)}-β-D-xylopyranose with formation of an *O*-glycosidic linkage to C-1 of the aglycone [99] (**Figure 1.13**). There are no published accounts of the isolation and structural elucidation of steroidal glycosides from *C. australis.*

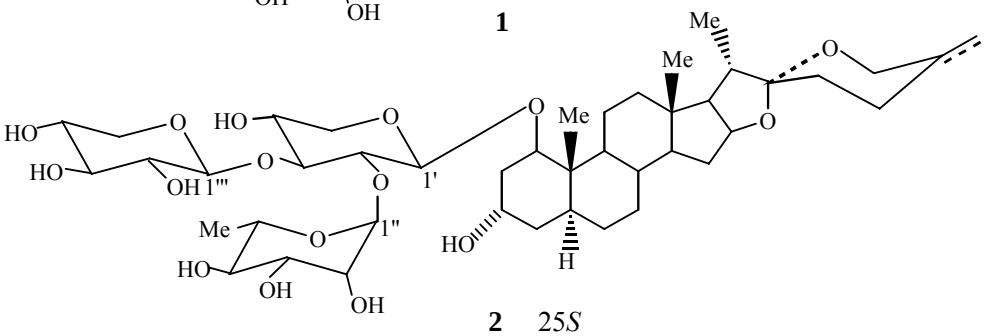


1. (25*S*)-5α-spirostane-1β,3α-diol 3-*O*-β-D-glucopyranoside
2. 25(27)-spirostene-1β,3α-diol 3-*O*-β-D-glucopyranoside
3. (25*S*)-5-spirost-20-ene-1β,3α-diol 3-*O*-β-D-glucopyranoside
4. (25R)-5α–spirostane-1β,3α, 25-triol 3-*O*-β-D-glucopyranoside
5. 26-*O*-β-D-glucopyranosyl-22-*O*-methyl (25*S*)-5α-furostane-3α,22ξ,6-triol 3-*O*-β-D-glucopyranoside
6. 26-*O*-β-D-glucopyranosyl-22-*O*-methyl-(25S)-5α-furostane-1β,3α,22ξ,26-tetrol 3-*O*-β-D-glucopyranoside
7. 26-*O*-β-D-glucopyranosyl-5α-furost-20(22)-ene-1β,3α,26-triol 3-*O*-β-D-glucopyranoside
8. 1β,3α-dihydroxy-5α-pregn-16-en-20-one 3-*O*-β-D glucopyranoside.

Figure 1.12 Steroidal saponins isolated from the dry leaves of *C. stricta*

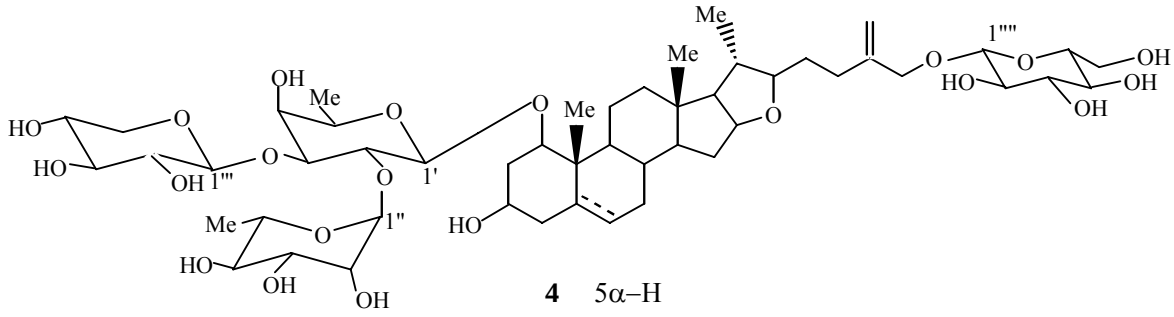


1



2 25*S*

3 $\Delta^{25(27)}$



4 5α–H

5 Δ^5

1. 5α-spirost-25(27)-ene 1β,3α-diol 1-*O*-{α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-fucopyranoside}
2. (25S)-5α-spirostane-1β,3α-diol 1-*O*-{α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-fucopyranoside}
3. 5α-spirost-25(27)-ene 1β,3α-diol 1-*O*-{α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-fucopyranoside}
4. 26-*O*-β-D-glycopyranosyl-22-*O*-methyl-5α-furost-25(27)-ene 1β,3β,22ξ,26-tetraol 1-*O*-{α-L-rhamonopyranosyl-(1→2)-*O*-{β-D-xylopyranosyl-(1→3)}-β-D-fucopyranoside}
5. 26-*O*-β-D-glycopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1β,3β,22ξ,26-tetraol 1-*O*-{α-L-rhamonopyranosyl-(1→2)-*O*-{β-D-xylopyranosyl-(1→3)}-β-D-fucopyranoside}

Figure 1.13 Steroidal saponins isolated from the fresh leaves of *C. stricta*

1.3.3 Traditional Maori Use of *Cordyline spp.*

C. australis (or Ti) was used by Maori in a variety of applications [86,87,100]. For traditional usage of *Cordyline* species for medicinal purposes, see Chapter Four. Maori commonly used three plants, flax (*Phormium tenax*), mountain flax (*P. cookianum*) and cabbage tree (*C. australis*) as sources of dye. *C. australis* invariably afforded a black dye [86,101,102].

The fiber from the leaves of Ti was used for making sandals, baskets, bird snares, sieves, thatch for roofs, rope and cord. Leaves (rau ti) or the tender young shoots were eaten raw, or roasted in the embers. Pith (commonly called Ti), was dried in the sun and cooked to make porridge [86,88,100-103]. Roots of Ti were used for making sweet drinks. Sugar was extracted by cooking in an earth oven called an umu-ti [86,104]. Large pits were used to steam the roots. The handling of Ti was always accompanied by ceremonial protocols [86,87].

1.3.4 Other Classes of Compounds from *C. australis*

The Maori tradition of recovering sugar from the roots of the cabbage tree (*Cordyline spp.*) [86-88,100-102] has been revived by Fankhauser and Brasch [104,105]. High-fructose syrup can be obtained from stems and roots of *C. australis* and the possible use of the syrup and the plant has been discussed [105]. Glucofructo-furanan comprises 60 % of the dry weight of the roots of *C. australis* [106].

Seed fats of some New Zealand and Australian monocotyledons, eg. *C. australis*, *P. tenax*, and other plants showed high level of linoleic acid [107-109]. Sterols and flavonols (quercetin and kaempherol) have been detected in the seeds and flowers of *C. indivisa* [110-112]. Fresh flowers of *C. australis* yielded 0.418% volatile oil, containing α- pinene, camphene, β-pinene, *etc* [113]. Formaldehyde, as its dimedone adduct, formaldemethone, has been detected by TLC and HPLC in *C. australis* [114].

CHAPTER TWO

Methods and Materials

2.1 General Experimental Procedures

2.1.1 Column Chromatography

Column chromatography was carried out on Merck silica gel 60 (200 - 400 mesh). TLC was carried out on Merck silica gel 60 F_{254} (0.20 mm layer) plates using $CHCl_3$ - MeOH - H_2O (65 : 35 : 7) or $CHCl_3$ - CH_3OH (4 : 1) as the developing solvents. Spots were visualised by spraying with a solution of 1% vanillin in 50% H_3PO_4 [115,116] or H_2SO_4 [117] and/or with Erlich reagent [118,119] followed by heating at 100-115^oC.

2.1.2 Melting Points

Melting points were determined using a Reichert-Jung micro-melting point apparatus and are uncorrected.

2.1.3 Nuclear Magnetic Resonance (NMR) Spectra

1D and 2D-NMR spectra (^1H, ^{13}C, DEPT135, H,H-COSY, H,H-TOCSY, H,H-ROESY, g-HSQC, g-HMBC, and NOE-difference experiments) were determined for pyridine-d_5 solutions using a Bruker Advance DRX400 spectrometer and standard XWIN-NMR pulse programs. Chemical shifts are given in δ ppm values with reference to tetramethylsilane (TMS) as an internal standard based on pyridine-d_5 (^1H at 8.72, 7.57, 7.21 ppm and ^{13}C at 149.50, 135.50, 123.50 ppm).

Typical acquisition and processing conditions for Bruker DRX 400 spectra were as follows:

^{1}H NMR	SW = 5593 Hz, 90^{o} pulse, SI = 32 K, TD = 32 K, Acq = 2.9 sec. D1 = 0.1 s, LB = 0.
^{13}C NMR	SW = 24691 Hz, 70^{o} pulse, SI = 32 K, TD = 32 K, Acq = 0.664 sec, D1 = 1 s, LB = 2.
DEPT135	SW = 24154 Hz, SI = 32 K, TD = 32 K, D1 = 1 sec, D2 = 1/2*J* = 3.8 ms, Acq = 0.664 s, LB = 2.
COSY	SW1 = SW2 = 2688 Hz, SI1 = 1 K, TD1 = 400, SI2 = 1 K, TD2 = 1 K, D1 = 0.25 s, Acq = 0.19 s, MC2 = QF, SSB1 = SSB2 = 0, WDW1 = WDW2 = SINE.
TOCSY	SW1 = SW2 = 2723 Hz, SI1 = 1 K, TD1 = 320, SI2 = 1 K, TD2 = 1K, D1 = 0.4 s, mixing time = 150 ms, Acq = 0.188 sec, MC2 = TPPI, SSB1 = SSB2 = 2, WDW1 = WDW2 = QSINE.
ROESY	SW1 = SW2 = 2874 Hz, SI1 = 1 K TD1 = 400, SI2 = 1 K, TD2 = 1K, D1 = 1 s, P15 = 200 ms (spin lock time, with PL1 = 25 db), Acq = 0.178 s, MC2 = TPPI, SSB1 = SSB2 = 2, WDW1 = WDW2 = QSINE.
HMQC	SW1 = 15092 Hz, SW2 = 2723 Hz, SI1 = 1K, TD1 = 400, SI2 = 1 K, TD2 = 1 K, D1 = 0.4 s, D2 = 1/2*J* = 3.45 ms, Acq = 0.188 s, MC2 = TPPI, SSB1 = SBS2 = 2, WDW1 = WDW2 = QSINE.
HMBC	SW1 = 19118 Hz, SW2 = 2723 Hz, SI1 = 1 K, TD1 = 160, SI2 = 1 K, TD2 = 1 K, D1 = 0.7 sec, D2 = 1/2*J* = 3.45 ms, Acq = 0.188 s, MC2 = QF, SSB1 = SSB2 = 0, WDW1 = WDW2 = SINE.

2.1.4 GCMS

SIM-GCMS analyses were performed using a 25 m x 0.22 mm id HP-5 (Hewlett Packard) column installed in a HP6890 GC interfaced to a HP5973 mass selective detector.

Experimental methods for GCMS analyses were as reported by Wilkins *et al* [70], with the following modifications of operating conditions to the oven temperature program 200°C (0.5 min) then 35°C/min to 250°C and then 10°C/min to 295°C (20 min).

2.1.5 IR Spectra

IR spectra were obtained using samples prepared as KBr discs and recorded using a Perkin Elmer, Model 1600, FT-IR spectrophotometer.

2.1.6 HPLC

Preparative HPLC was performed using three Waters Delta-Pak C18 25 x 100 mm Radial-Pak cartridges eluted with MeOH - H_2O (10 mL/min). Analytical HPLC was performed using a Waters 8 x 100 mm Radial Pak C18 column eluted with MeOH - H_2O (1 mL/min). Detection was by refractive index and by UV.

2.1.7 ESMS

The molecular formulae was determined using an LCQ Advantage Thermo Finnigan MS instrument operating with an Electrospray Ionisation Source (ESI). Sample solutions were introduced by direct infusion using a syringe pump.

2.1.8 Biological Screening

Biological screening was performed at the Department of Chemistry, University of Canterbury, New Zealand. Antimicrobial (bacterial and fungal), antiviral and antitumuor assays were carried out. *Escherichia coli, Bacillus subtilis* and *Pseudomonas aeruginosa* were used for antibacterial assays. *Candida albicans*, *Trichophyton mentagrophytes* and *Cladosporium resinae* were used for antifungal assays. The antiviral assay used BSC-1 cells (African Green Monkey kidney), infected with *Herpes simplex type 1 virus (ATCC VR 733)* or *Polio virus type 1* (Pfiser vaccine strain). *P388* (Murine Leukemia) cells were used for the antitumor cytotoxicity assay.

Parazitoze screening was performed at the Tibotec Pharmaceutical Research and Development Company in Geneva Switzerland. Antiprotozoal (antimalaria), antitrypanosoma and cytotoxicity assays were carried out. *Plasmodium falciparum* was used for antimalaria assay. *Trypanosoma brucei* (Human African Trypanosomiasis, HAT, or sleeping sickness), *Trypanosoma cruzi* (Chagas disease) and Trypanosoma *Leishmania* (Leishmaniasis) were used for antitripanosoma assays respectively. *MRC-5* Line (Human Fetal Lung Fibroblast Cells) was used for the antiviral or cytotoxicity assay.

2.2 Plant Material

Fresh and senescent leaves (from the ground beneath the tree) and stems of *C australis* were obtained from The University of Waikato campus grounds, Hamilton, New Zealand in June 2000. The trees were identified as *Cordyline australis (Forst.f.) Endl* by Professor Warwick Silvester of the Biology Department at The University of Waikato. Voucher specimens are held by the University of Waikato Herbarium (voucher number WAIK18599).

2.3 Extraction Procedure for Preliminary GSMS

2.3.1 Extraction Procedure for Obtaining Free Sapogenins for GCMS

Freeze-dried sub-samples of fresh, dried and senescent, leaves (*ca* 0.5 g) were extracted with CH_2Cl_2 for 8 h using a Soxhlet apparatus. The CH_2Cl_2 extract was evaporated and acetylated for 16 h using a 1 : 1 mixture of pyridine and acetic anhydride (1 mL), after which the mixture was diluted with HCl (1 M, 5 mL) and extracted with CH_2Cl_2 (3 x 2 mL). The CH_2Cl_2 extracts were filtered through an alumina mini-column (Brockman Grade II) packed in a Pasteur pipette. The column was washed with a further volume of CH_2Cl_2 (2 mL) and the combined filtrates, in a 10 mL glass vial, were evaporated to dryness. The dried extract was taken up in CH_2Cl_2 (2 mL) and tigogenin propionate (200 μL of a 0.094 mg/mL solution in CH_2Cl_2) was added as an internal standard. SIM-GCMS analyses were performed as described in Section **2.1.4**.

2.3.2 Extraction Procedure for Obtaining Conjugated Sapogenins for GCMS

The CH_2Cl_2 extracted plant materials (see Section **2.3.1** above) were extracted with MeOH for 8 h using a Soxhlet apparatus. The MeOH extract was evaporated to dryness using a rotary evaporator and HCl (0.5 M, 5 mL) was added and the resulting solutions were transferred to a 30 mL screw capped boiling tubes which placed in water bath (95^{o}C) and maintained at this temperature for 60 min, after which the hydrolysate solutions were cooled and extracted with CH_2Cl_2 (3 x 2 mL). The CH_2Cl_2 extracts were filtered through an alumina mini- column (Brockman Grade II) packed in a Pasteur pipette. The column was washed with a further volume of CH_2Cl_2 (2 mL) and the combined filtrates, in a 14 mL sample vial, were evaporated to dryness. The dried extract was taken up in CH_2Cl_2 (2 mL) and acetylated as described in Section **2.3.1** above. SIM-GCMS analyses were performed as described in Section **2.1.4**.

2.4 Bulk Extraction of Saponins from Dried Leaves and Stems and Senescent Leaves of *C. australis*

Powdered, air-dried leaves and stems and senescent leaves were extracted for 18 h with $CHCl_3$ using a Soxhlet apparatus to remove lipophilic compounds, after which they were extracted with MeOH - H_2O (4 : 1; 4 x 250 mL). The MeOH - H_2O extracts were concentrated to dryness using a rotary evaporator. The dry extracts were dissolved in water (*ca* 100-150 mL) and further extracted with *n*-BuOH (4 x 250 mL). The combined *n*-BuOH extracts were evaporated to dryness using a rotary evaporator to obtain a crude saponin extract.

2.5 Bulk Extraction of Saponins from Fresh Leaves of *C. australis*

Fresh leaves of *C. australis* (80 g) were ground in a blender and extracted with hot methanol (4 x 300 mL). The extract was taken to dryness under reduced pressure and the dry extract was partitioned between $CHCl_3$ (100 mL) and H_2O (150 mL). The H_2O layer was extracted with *n*-butanol (4 x 250 mL). The combined *n*-butanol extracts were taken to dryness under reduced pressure to obtain a crude saponin extract.

2.6 Isolation of Steroidal and Flavonoid Glycosides from Dried Leaves of *C. australis*

The *n*-BuOH extract (3 g) was chromatographed on a silica gel column (3 x 100 cm) using 1000 mL portions of $CHCl_3$ - MeOH (20 : 1, 15 : 1, 10 : 1, 4 : 1) and 1500 mL $CHCl_3$ - MeOH - H_2O (65 : 35 : 7); 20 x 100 mL (fractions 1-20) and 80 x 40 mL fractions (fractions 20-100) were collected. TLC analyses indicated the presence of extractives (possibly glycosides) in fractions 33-36 (bright yellow spot, R_f 0.85 and R_f 0.35 in $CHCl_3$ - CH_3OH - H_2O (65 : 35 : 7) and $CHCl_3$ - CH_3OH (4 : 1) respectively. Similarly fractions 43 - 73 gave a dark orange spot, (R_f 0.58 and 0.28)

and fractions 74-110 gave an orange-brownish spot, (R_f 0.42 and 0.20). On standing, crystalline material precipitated from fractions 33-36.

The precipitated material was identified as 5α-spirost-25(27)-en-3β-ol 3-*O*{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside}, **1**, (30 mg), m.p. 253-256°C, IR λ^{KBr}_{max} 3422 (OH), 2930 (CH), 1649, 1449, 1372, 1233, 1172, 1133, 1044, 922, 877, 833, 811 cm^{-1}. ^1H NMR (400 MHz) δ 0.83 (s, H_3-18), 0.87 (s, H_3-19), 1.12 (d, *J* = 7 Hz, H_3-21), 5.05 (1H, d, *J* = 7.6 Hz, Glc H-1'), 6.33. (1H, d, *J* = 1.6 Hz, Rha H-1''), other ^1H and ^{13}C NMR signals, see **Table 3.3.** ESMS (-20 V) *m/z* 1443 $[M_2\text{-}H]^-$ (55), 1479 $[M_2+^{35}Cl]^-$ (100), 1481 $[M_2+^{37}Cl]^-$ (90), 721 $[M\text{-}H]^-$ (83), 757 $[M+^{35}Cl]^-$ (14), 767 $[M+COOH]^-$ (18), 781 (18, $[M+CH_3COOH]^-$(18).

Evaporation of fractions 43-73 afforded a light yellow amorphous solid, a portion of which (0.8 g) was rechromatographed on a silica gel column (2 x 60 cm) using 3 x 1000 mL portions of mixture of $CHCl_3$ - MeOH - H_2O (65 : 35 : 7) as a mobile phase. 30 x 100 mL fractions were collected. TLC analyses showed the presence of a UV active substance (or substances) in fractions 7-20.

Preparative HPLC of fractions 7-20 afforded a 3 : 4 mixture of isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside} (isorhamnetin rutinosa) [120,121], **4**, and isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside} (isorhamnetin robinobiosa) [120,121], **5**, as a yellow, amorphous solid, m.p. 180-189°C, λ_{max}(MeOH) 257, 369 nm. Repeated efforts to separate **4** and **5** by HPLC were not successful.

Isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside} **4** had ^1H NMR (pyridine-d_5, δ) 3.93 (3H, s, 3'-OMe), 3.76 (1H, d, *J*= 2.1 Hz, 6-H), 6.73 (1H, d, *J* = 2.0 Hz, 8-H), 7.30 (1H, d, *J* = 8.4 Hz, 5'-H), 7.91 (1H, dd, *J* = 8.4 Hz, 6'-H), 8.38 (1H, d, *J* = 2.1 Hz, 2'-H), 6.16 (1H, d, *J* = 7.5 Hz, Glc, H-1'), 5.25. (1H, d, *J* = 1.2 Hz, Rha H-1''). ^{13}C NMR data is reported in **Table 3.13.**

Isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside} **5** had ^1H NMR (pyridine d_5, δ ppm) 4.03 (3H, s, 3'-OMe), 3.78 (1H, d, *J* = 2.1 Hz, H-6), 6.74 (1H, d, *J* = 2.0 Hz, 8-H), 7.27 (1H, d, *J* = 8.4 Hz, H-5'), 7.82 (1H, dd, *J* = 8.4 Hz,

6'-H), 8.61 (1H, d, *J* = 2.1 Hz H-2'), 6.22 (1H, d, *J* = 7.8 Hz, Gal H-1'), 5.33 (1H, d, *J* = 1.3 Hz, Rha H-1"). ^{13}C NMR data is reported in **Table 3.13.**

2.7 Isolation of Steroidal Glycosides from Fresh Leaves of *C. australis*

The $CHCl_3$ extract (6 g) of fresh leaves of *C.australis* was chromatographed on a silica gel column (4 x 100 cm) using 2000 mL portions of mixtures of $CHCl_3$ - MeOH (20 : 1, 15 :1 , 10 : 1) as a mobile phase. 60 x 100 mL fraction were collected and analysed by TLC Fractions 36-50 (0.9 g) were rechromatographed on a silica gel column (2 x 50 cm) using $CHCl_3$ - MeOH (5 : 1) as the mobile phase. 50 x 15 mL fractions were collected.

Fraction 33 afforded 5α-spirost-25(27)-ene-1β,3β-diol 1-*O*{α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranoside} (**2**) (15 mg) as a white amorphous solid, TLC R_f 0.34 $CHCl_3$ - CH_3OH (4 : 1), ^{1}H NMR (400 MHz) δ 0.81 (s, H_3-18), 1.21 (s, H_3-19), 1.02 (d, *J* = 6.9 Hz, H_3-21), 4.68 (1H, d, *J* = 7.8 Hz, Fuc H-1'), 6.31 (1H, d, *J* = 1.6 Hz, Rha H-1"), other ^{1}H and ^{13}C NMR, see **Table 3.6**. ESMS (+20 V) m/z 1467 $[M_2Na]^+$ (100); MS^2 (from m/z 1467) m/z 745 $[MNa]^+$; MS^3 (from m/z 745) m/z 599 $[MNa\text{-}Rha]^+$. Fraction 36 afforded saponin **1** (60 mg), TLC R_f 0.3 $CHCl_3$ - CH_3OH (4 : 1). The structure of **1** was confirmed by ^{13}C NMR data reported in **Table 3.3**.

2.8 Isolation of Steroidal Glycosides from Senescent Leaves of *C. australis* (Route 1)

The *n*-BuOH extract (2 g) of senescent leaves of *C. australis,* prepared as described above, was chromatographed on a silica gel column (3 x 80) using 500 mL portions of mixture of $CHCl_3$ - CH_3OH (25 : 1), 2 (10 : 1), 5.5 (4.5 : 1) and $CHCl_3$ - CH_3OH - H_2O (32 : 18 : 4). 100 x 20 mL fractions and 60 x 50 mL fractions were collected.

Fractions 32-39 afforded β-sitosterol glycoside **6**; m.p. 284°C (decomp) [122], R_f 0.68 $CHCl_3$ - CH_3OH (4 : 1), IR λ^{KBr}_{max} 3400 (OH), 2900 (CH), 1640 (C = C) cm^{-1}. ^1H NMR (400 MHz) 0.68 (s, H_3-18), 0.95 (s, H_3-19), 1.00 (d, *J* = 6.5 Hz, H_3-21), 0.87 (d, *J* = 6.6 Hz, H_3-26), 0.89 (d, *J* = 7.0 Hz, H_3-27), 0.91 (d, *J* = 7.5 Hz H_3-29). The ^{13}C NMR shifts of C-5, C-6, C-18, C-19, C-21, C-26, C-27, C-29 and C-1' in pyridine-d_5 occurred at 140.9, 122.0, 12.0, 19.4, 19.0, 19.2, 20.0, 12.2, and 102.6 ppm respectively. Other NMR spectral data are recorded in **Table 3.15** and were in accordance with literature [122].

Fractions 40-46 were rechromatographed on a silica gel column (1 x 30 cm) using $CHCl_3$ - CH_3OH (4 . 5 : 1) as an eluant. 20 x 20 mL fractions were collected.

Fractions 3-8 afforded 5α-spirost-25(27)-ene-1β,3β-diol 1-*O*{β-D-fucopyranoside}, **3**, (20 mg); R_f 0.67 $CHCl_3$ - CH_3OH (4 : 1), IR λ^{KBr}_{max} 3448 (OH), 2923 (CH), 1654,1457, 1045, 928, 848 cm^{-1}. ^1H NMR (400 MHz) δ 0.85 (s, H_3-18), 1.03 (s, H_3-19), 1.05 (3H, d, *J* = 6.9 Hz, H_3-21), 4.81 (1H, d, *J* = 7.5 Hz, Fuc, H-1'). ^{13}C NMR spectral data see **Table 3.9.** ESMS (+20 V) *m/z* 1175 $[M_2Na]^+$ (100).

Fractions 82-91 (60 mg) afforded 5α-spirost-25(27)-en-3β-ol 3-*O*{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside}, **1**. The structure of **1** was confirmed by ^{13}C NMR.

2.9 Isolation of Steroidal Glycosides from Senescent Leaves of *C. australis* (Route 2)

Powdered air-dried senescent leaves (100 g) were extracted with MeOH for 18 h using a Soxhlet extractor. The MeOH extract was concentrated to dryness using a rotory evaporator and partitioned between EtOAc and H_2O (3 x 200 mL, 3 : 1). The EtOAc extract was concentrated to 60 mL and filtered. The concentrated EtOAc extract (3 g) was chromatographed on a silica gel column (3 x 100 cm) using 1000 mL portions of a mixture of $CHCl_3$ - CH_3OH (20 : 1, 10 : 1 , 9 : 2, 4 : 1) and $CHCl_3$ – MeOH - H_2O (65 : 35 : 7) as eluents. 50 x 100 mL fractions were collected and

analysed using TLC. Fractions 18-40 R_f 0.78 $CHCl_3$ - CH_3OH - H_2O (65 : 35 : 7), R_f 0.35, $CHCl_3$ - CH_3OH (4 :1) were shown by NMR analyses to be mixtures of saponin **1** and diosgenin 3-*O*{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, **7**, also known as prosapogenin A of dioscin [71,123]. The H_2O layer was extracted again with diethyl ether. The ether extract (0.2 g) was chromatographed on a silica gel column (1.5 x 50 cm) using 400 mL portions of a mixture of $CHCl_3$ - CH_3OH (10 : 1, 9 : 2) as a mobile phase. 50 x 15 mL fractions were collected. Fraction 5-6 gave 25*R*, spirost-5-en-3β-ol, 3-*O*-β-D-glucopyranoside **8**, (trillin) [71,124], as a white amorphous solid, tentatively identified as trillin as reported by Agrawal *et al* [71].

2.10 Isolation of Steroidal Glycosides from Dry Stems of *C. australis*

The *n*-BuOH extract (3 g) was chromatographed on a silica gel column (5 x 140 cm) using 1500 mL of portions of $CHCl_3$, $CHCl_3$ - MeOH (9 : 1, 8 : 1, 4 : 1, 9 : 2, 4 : 1) and $CHCl_3$ – MeOH - H_2O (65 : 35 : 7), 50 x 200 mL fractions were colleted. TLC analyses indicated the presence of steroidal glycosides in all fractions.

Fraction 13-16 gave a white amorphous solid identified as (25*R*)-5α-spirostane-1β,3α-diol 1-*O*-β-D glucopyranoside, **9**, (15 mg), R_f 0.52 $CHCl_3$-CH_3OH (4:1), ^1H NMR (400 MHz) δ 0.86 (s, H_3-18), 1.03 (s, H_3-19), 1.10 (d, J = 7.1 Hz, H_3-21), 1.08 (d, J = 7.4 Hz, H_3-27), 5.00 (1H, d, J = 7.7 Hz, Glc, H-1). Other ^1H and ^{13}C NMR see **Table 3.11**. ESMS (+20 V) *m/z* 1211 $[M_2Na]^+$ (100).

CHAPTER THREE

Structural Elucidation of Steroidal and Flavonoid Glycosides from C. australis

3.1 Introduction

3.2 Preliminary GCMS Analyses of *C. australis* Leaves Extracts

Prior to the bulk extraction investigations reported in Section **2.4** preliminary GCMS analyses of three *C. australis* leaf types (fresh, dried, senescent leaves) were undertaken in order to determine whether or not free and conjugate steroidal sapogenins (saponins) were present in the extracts of the various plant parts. Leaf samples were extracted, derivatived with acetic anhydride/pyridine (see Sections **2.3.1** and **2.3.2**) and analysed using the SIM and TIC GCMS methods described in Sections **2.1.4**. SIM-GCMS analyses were performed as described by Wilkins *et al* [70]. Quantification was performed using tigogenin propionate (200 μL of a 0.094 mg/mL solution in CH_2Cl_2) as an internal standard (see Sections **2.3.1** and **2.3.2**).

Sapogenin acetates were identified from a combination of their relative retention time, mass spectral data and comparison with authentic standards where available. The results obtained are summarised in **Table 3.1**.

Three acetylated sapogenins, namely tigogenin, neotigogenin and $\Delta^{25(27)}$-tigogenin acetate were observed in the total ion chromatograms of the conjugated extracts of all leaf types. Additionally diosgenin acetate was observed in the chromatogram of the conjugated extract of dried leaves. Weak molecular ions were observed at *m/z* 458 for tigogenin (25*R*) and neotigogenin (25*S*) acetates. Both acetates showed a similar mass spectral fragmentation patterns with diagnostic ions at *m/z* 344, 329,

315, 284, 269 and 255, and a base peak at *m/z* 139 attributable to saturated ring F spirostanol structure [70].

Blunden *et al* [68] and Wilkins *et al* [70] have previously reported that the ratios of the intensities of some of the spectral fragment ions of sapogenins facilitates their identification. Wilkins *et al* [70] have reported that the ratio of three pairs of ions observed in the mass spectra of sapogenin acetates are more diagnostic than is the case for the corresponding ions of the parent sapogenins. The ion ratios which Wilkins *et al* [70] determined were *m/z* 284/*mz*/344, *m/z* 269/*m/z* 329 and *m/z* 255/m/z 315. Possible structures for the foregoing six ions are presented in **Figure 3.1**.

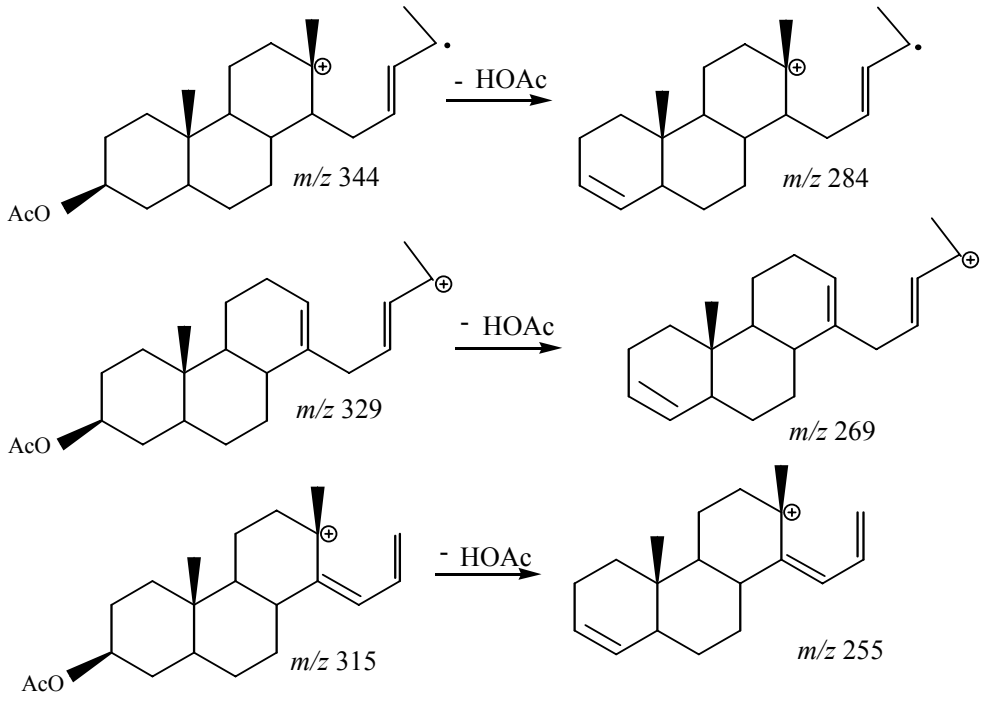


Figure 3.1 Structures of possible fragment ions

Table 3.1 Typical *m/z* 284/*m/z* 344 ratios for different sapogenin acetates [70]

Trivial name of sapogenins	Name of parent sapogenin	*m/z* 284/*m/z* 344
Tigogenin acetate	(25*R*) 5α-Spirostan-3β-ol	0.22
Neotigogenin acetate	(25*S*) 5α-Spirostan-3β-ol	0.22
Epitigogenin acetate	(25*R*) 5α-Spirostan-3α-ol	0.31
Smilagenin acetate	(25*R*) 5β-Spirostan-3β-ol	2.92

The presence in the acetylated extracts of $\Delta^{25(27)}$-tigogenin was demonstrated by a molecular ion at *m/z* 456, fragment ions at *m/z* 344, 329, 315, 284, 269 and 255, and a base peak at *m/z* 137 attributable to an unsaturated spirostenol ring F structure.

The highest observed ion in the mass spectrum of diosgenin acetate occurred at *m/z* 396 (M^+-HOAc) and 139 ions were also observed in the mass spectrum of diosgenin acetate [70]. Acetylated analogues of dihydroxygenins were also observed in the GCMS profiles but were not quantified because of the lack of suitable standards.

The only free genin detected (as the corresponding acetate) was $\Delta^{25(27)}$-tigogenin, which was found in the extract of senescent leaves.

The results presented in **Table 3.2** showed that while leaves of *C. australis* would not be a good source of free sapogenins, significant levels of conjugated sapogenins (saponins) were present in the fresh, dried and senescent leaf extracts.

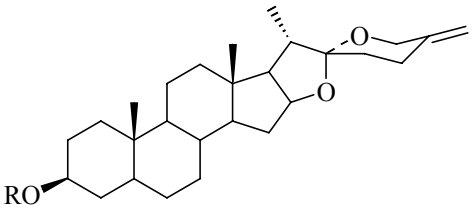
Table 3.2 Retention times and levels of acetylated sapogenins identified in *C. australis* leaf extracts

Sample	Extract	Sapogenins (mg/kg DM)[a]			
		Tigogenin	Neotigogenin	Diosgenin	$\Delta^{25(27)}$Tigogenin
Retention time (min)		15.48	15.85	15.19	16.07
Fresh Leaves	Free[b]	-	-	-	-
	Conj.[c]	10.3	27.7	-	8.4
Dried Leaves	Free	-	-	-	-
	Conj.	48.2	10.1	4.8	51.4
Senescent Leaves	Free	-	-	-	11.9
	Conj.	22.2	5.9	-	67.7

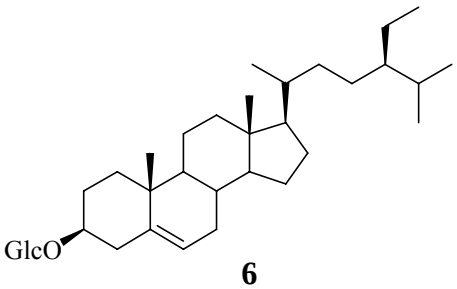
[a]average of duplicate analyses; [b]free = CH_2Cl_2; [c]conj.= conjugate extract (MeOH extract after prior extraction with CH_2Cl_2. Conjugate extracts were hydrolysed prior to GCMS analysis as the corresponding acetates).

3.3 Bulk extraction of *C. australis*

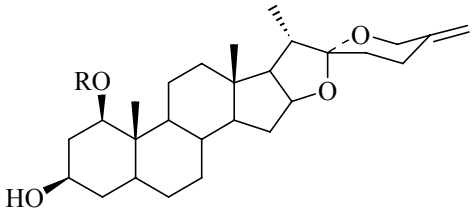
Dried leaves and stems and fresh leaves of *C. australis* were extracted as described in Chapter Two, Section **2.4** and **2.5** and that the resulting extracts were separated as described in Chapter Two, Sections **2.6, 2.7, 2.8, 2.9, 2.10.** This afforded seven steroidal glycosides, which shown to posses structures **1, 2, 3, 6, 7, 8** and **9** and an inseparable mixture of two flavanoid glycosides possessing structures **4** and **5**. The structures of these compounds were established from detailed analyses of a combination of IR, ESMS and one- and two-dimensional NMR data. The chemical structures established for compounds **1-9** are presented in **Figure 3.2.**



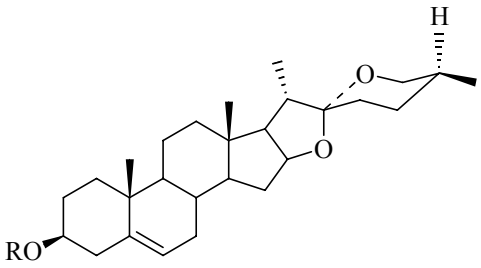
1: R = Rha-(1"→2')-Glc



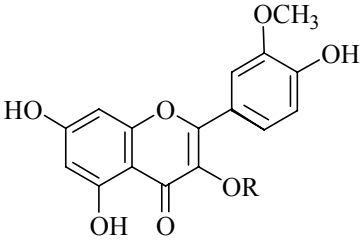
6



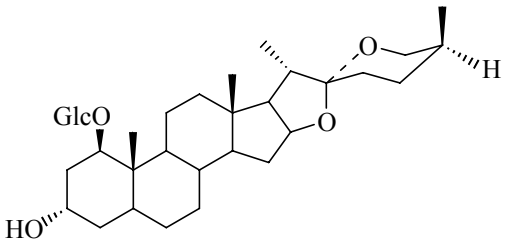
2: R = Rha-(1"→2')-Fuc
3: R = Fuc



7: R = Rha-(1"→2')-Glc
8: R = Glc



4: R = Rha-(1"→6')-Glc
5: R = Rha-(1"→6')-Gal



9

Figure 3.2 Structures of steroidal glycosides and flavonoid glycosides isolated from C. *australis*.

3.4 Structure Elucidation of Saponin 1

Saponin **1,** 5α-spirost-25(27)-en-3β-ol 3-*O*{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside}, was isolated from the n-butanol extract of the methanol extract of dried, senescent leaves and dried stems. It also obtained from the chloroform soluble residue of the methanol extract of fresh leaves.

3.4.1 IR Spectrum

The glycosidic nature of **1** was shown by a strong IR absorption at 3422 cm^{-1} (OH stretch), 1044 cm^{-1} (C-O(H) stretch) and characteristic absorptions for the spiroketal moiety appeared at 982 cm^{-1}, 920 cm^{-1}, 902 cm^{-1} and 865 cm^{-1} [60-62].

3.4.2 ESMS

The molecular formula of **1,** $C_{39}H_{62}O_{12}$, (M = 722 Da) was determined by negative ion ESMS, which showed a $[M-H]^-$ ion at *m/z* 721, along with *m/z* 757 $[M+^{35}Cl]^-$, *m/z* 767 $[M+COOH]^-$, *m/z* 781 $[M+CH_3COO]^-$**,** *m/z* 1443 $[M_2-H]^-$, *m/z* 1479 $[M_2+^{35}Cl]^-$ and *m/z* 1481 $[M_2+^{37}Cl]^-$ ions.

3.4.3 NMR Spectra

The complete assignment of the ^{1}H and ^{13}C NMR signal of saponin **1** presented in **Table 3.3** were established from detailed analyses of one and two dimensional NMR data (see following Sections).

Table 3.3. ^{1}H and ^{13}C NMR signal assignments (δ C_5D_5N) established for saponin **1**

atom	^{13}C	^{1}H	atom	^{13}C	^{1}H
1	37.0	0.81, 1.60	22	109.3	
2	29.7	1.82, 2.10	23	33.1	1.79 (2H)
3	77.1	3.99	24	28.8	2.26, 2.72
4	34.2	1.69, 1.96	25	144.0	
5	44.6	0.90	26	64.9	4.03, 4.46
6	28.9	1.17 (2H)	27	108.6	4.79, 4.82
7	32.2	0.81, 1.54			
8	35.2	1.45	1'	99.9	5.05 *d*, $J_{1',2'}$ = 7.6 Hz
9	54.2	0.53	2'	78.4	4.24, *dd* $J_{2,'3'}$ = 9.2 Hz
10	35.7		3'	79.6	4.28, *dd,* $J_{3',4'}$ = 9.7 Hz
11	21.2	1.24, 1.43	4'	71.9	4.16, *d,* $J_{4',5'}$ = 9.3 Hz
12	40.1	1.07, 1.70	5'	76.9	3.99, *t*
13	40.6		6'	62.7	4.35, 4.54 (2 x m)
14	56.4	1.05			
15	32	1.42, 2.03	1"	102.1	6.33, *d,* $J_{1",2"}$ = 1.6 Hz
16	81.3	4.54	2"	72.1	4.80, *dd,* $J_{2",3"}$ = 3.5 Hz
17	62.9	1.81	3"	72.6	4.61, *dd,* $J_{3",4"}$ = 9.4 Hz
18	16.4	0.83 (3H, s)	4"	73.8	4.37, *dd,* $J_{4"5"}$ = 9.5 Hz
19	12.3	0.87 (3H, s)	5"	69.4	4.94, *d,* $J_{5",6"}$ = 6.3 Hz
20	41.8	1.97	6"	18.4	1.76, *d*
21	14.8	1.12 (3H, d, J = 7.0 Hz)			

3.4.3.1 ^{1}H and ^{13}C NMR Spectra

The ^{1}H NMR spectrum of saponin **1** in pyridine-d_5 included three methyl protons at 0.83 (s), 0.87 (s), 1.12 (d, J = 7.0 Hz) ppm, two oxygenated methylene protons at 4.79 and 4.82 (s) ppm and two anomeric glycosidic protons at 5.05 (d, J = 7.6 Hz) and 6.33 (d, J = 1.6 Hz) ppm.

The genin portion of the ^{13}C NMR spectrum of **1** consisted of a total of 27 signals including three methyl groups, ten non-oxygenated and one oxygenated methylene groups, six non-oxygenated and two oxygenated methine groups and two non-oxygenated and one oxygenated quaternary carbons. In addition, a CH_2 signal was observed at 108.6 ppm and a C signal at 144.0 ppm consistent with the presence of 25(27)-olefinic unsaturation. A quaternary carbon (C-22) signal appeared at 109.3 ppm.

The foregoing ^{1}H and ^{13}C NMR data indicated a spirostane skeleton with an exocyclic double bond on the F ring and a single glycosidic linkage at C-3. The orientation of the C-3 glycosidic group was subsequently established to be equatorial (β) (see Sections **3.4.3.3** and **3.4.3.4.)**

The ^{13}C NMR chemical shifts of C-5, C-7, C-9 and C-19, (44.6, 32.2, 54.2 and 12.3 ppm respectively) were consistent with the presence of a 5α-spirostane steroidal skeleton (tigogenin type), rather than a 5β-spirostane skeleton [71,78]. The glycosyl portion of the ^{13}C NMR spectrum of saponin **1** contained twelve signals comprising ten oxygenated methine signals, one oxygenated methylene and a methyl group.

3.4.3.2 COSY and TOCSY Spectra

Correlations observed in COSY and TOCSY spectra, along with coupling constant data were consistent with the presence of glucopyranosyl and rhamnopyranosyl units. Starting with the signal at 5.05 ppm ($J_{1',2'}$ = 7.6 Hz, H-1') the couplings were traced around the pyranosyl ring giving $J_{2',3'}$ = 9.2 Hz, $J_{3',4'}$ = 9.7 Hz and $J_{4',5'}$ = 9.3 Hz

indicating a β-D-glucopyranosyl residue. Similarly, starting with the signal at 6.33 ppm ($J_{1",2"}$ = 1.6 Hz, H-1") coupling constants were measured around the ring giving $J_{1",2"}$ = 1.6 Hz, $J_{2",3"}$ = 3.5 Hz, $J_{3",4"}$ = 9.4 Hz and $J_{4",5"}$ = 9.5 Hz. This pattern confirms the presence of a rhamnopyranosyl unit, which was assumed to be an α-L-rhamnopyranosyl unit in common with other natural products [78].

The COSY and TOCSY spectra of saponin **1** also included correlations between H-23 (1.79 ppm), H-24α/H-24β (2.26 and 2.72 ppm) and the olefinic H-27 protons (4.79 and 4.82 ppm).

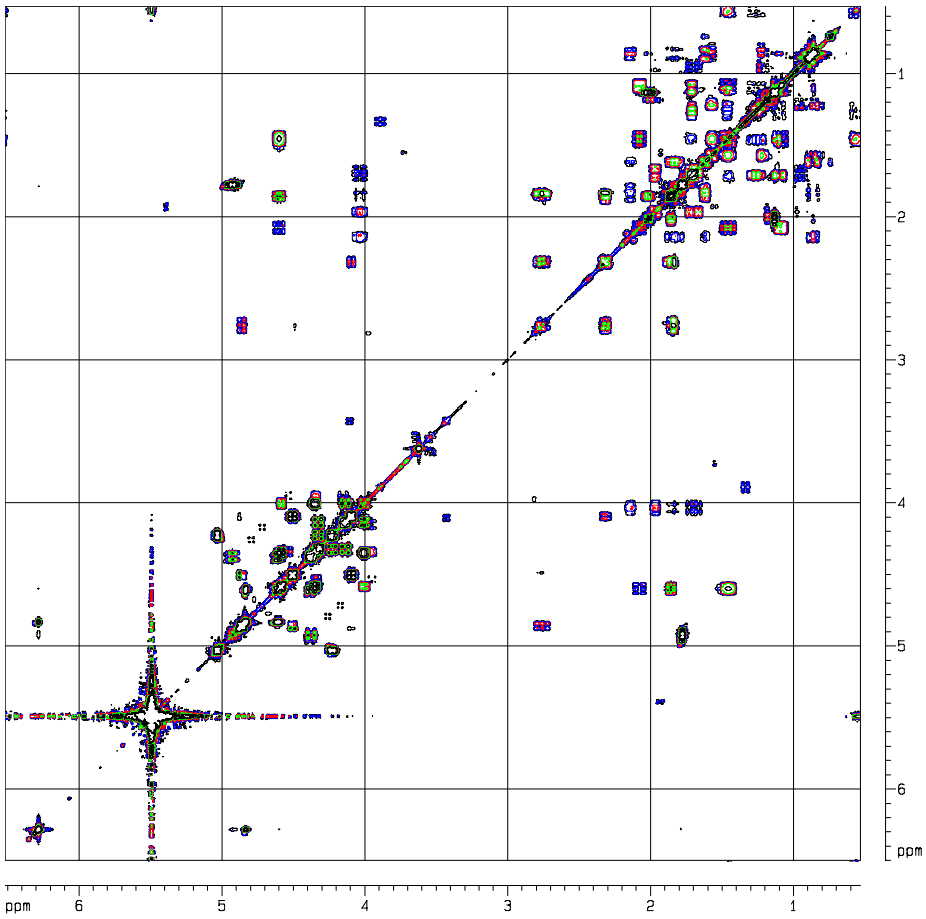


Figure 3.3 COSY spectrum of saponin **1**

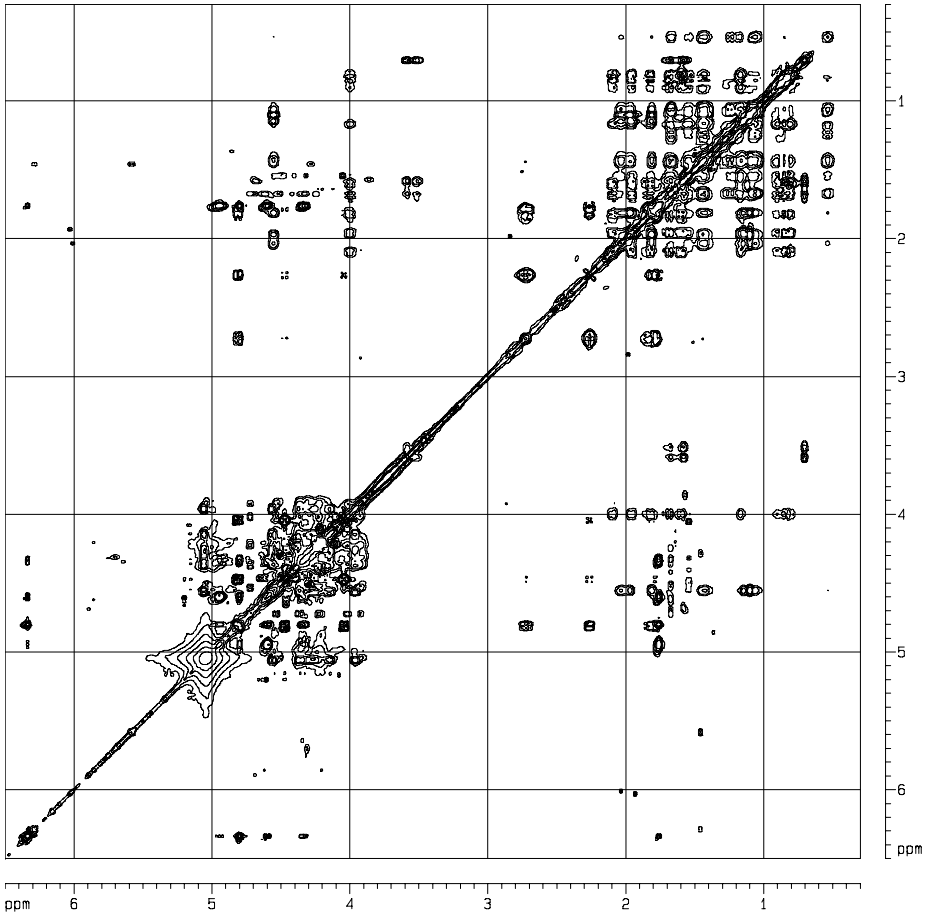


Figure 3.4 TOCSY spectrum of saponin **1**

Table 3.4 COSY connectivities (δ C_5D_5N) observed for glycosyl protons of saponin **1**

δ ^{1}H	correlated signal(s)	δ ^{1}H	correlated signal(s)
5.05 (H-1')	4.45 (H-2')	6.33 (H-1")	4.80 (H-2")
4.24 (H-2')	4.68 (H-1'), 4.07 (H-3')	4.80 (H-2")	6.33 (H-1"), 4.61 (H-3")
4.28 (H-3')	4.45 (H-2'), 3.88 (H-4')	4.61 (H-3")	4.80 (H-2"), 4.37 (H-4")
4.16 (H-4')	4.07 (H-3'), 3.66 (H-5')	4.37 (H-4")	4.61 (H-3"), 4.94 (H-5")
3.99 (H-5')	3.88 (H-4'), 1.49 (H-6')	4.94 (H-5")	4.37 (H-4"), 1.70 (H-6")
4.35 (H-6')	3.99 (H-5')	1.70 (H-6")	4.94 (H-5")

3.4.3.3 g-HSQC and g-HMBC Spectra

The g-HSQC spectrum correlated proton resonances with those of the corresponding carbons. The angular methyl group signals of saponin **1**, which occurred as a singlets at 0.83 and 0.87 ppm, and the secondary methyl group signal which occurred as a doublet at 1.12 ppm (d, J = 7.0 Hz) showed g-HSQC correlations at 16.4, 12.3 ppm and 14.8 ppm respectively.

The presence of a β-oriented hydroxyl group at C-3 was initially established by examination of the splitting pattern of the axial H-4 proton in a phase sensitive g-HSQC NMR experiment. A quartet-like signal, attributable to three couplings ($J_{4ax,4eq}$, $J_{4ax,5ax,}$ $J_{4ax,3ax}$) was observed. Notwithstanding the limited resolution of the 2D spectral data, it was apparent that all of these couplings were greater than or equal to *ca* 10 Hz. This indicated a *trans* diaxial relationship between H-4_{ax} and each of H-3 and H-5, and further defined the C-3 hydroxyl group to be equatorially oriented. NOESY and ROESY data (see Section **3.4.3.4**) subsequently verified this conclusion.

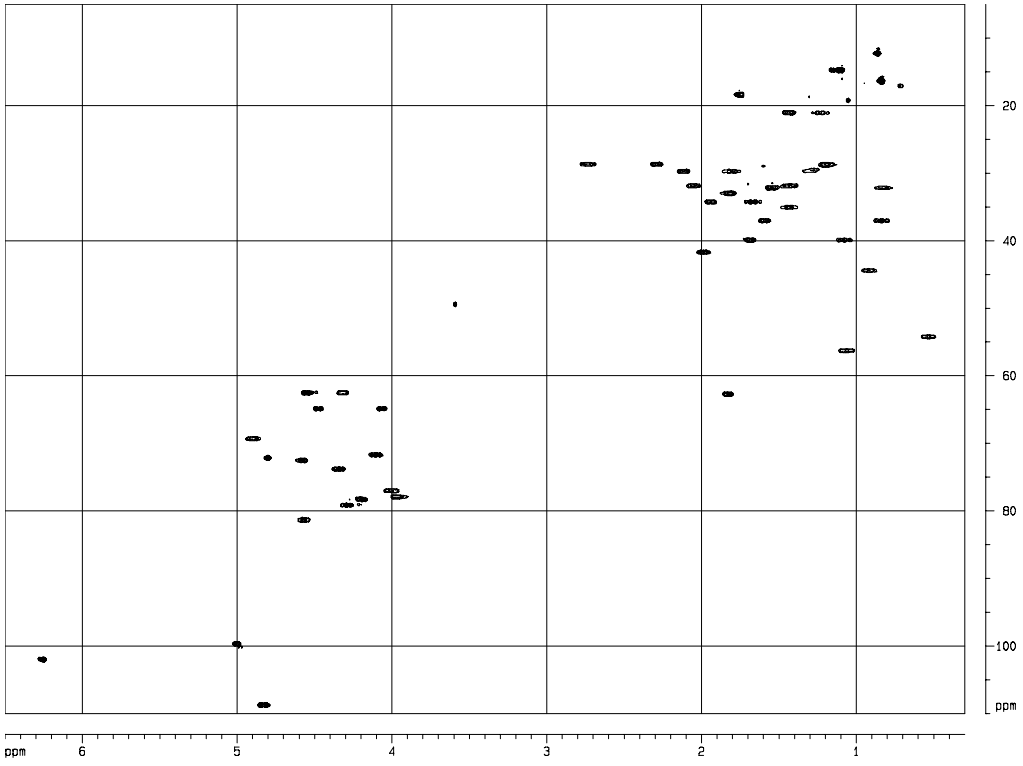


Figure 3.5 g-HSQC spectrum of the saponin **1**

The carbon signals which occurred at 99.6 and 102.1 ppm showed g-HSQC correlations with ^{1}H doublets which occurred at 5.05 ($J_{1',2'}$ = 7.6 Hz) and 6.33 ($J_{1'',2''}$ = 1.6 Hz) ppm respectively, consistent with their identification as the anomeric carbons of two glycosyl units.

g-HMBC spectral analyses gives valuable information for quaternary carbons assignments and confirmative analyses of neighbouring atoms in molecule. A g-HMBC correlation between the signal at 78.4 ppm (C-2') and the proton signal at 6.33 ppm (H-1'') indicated that the rhamnopyranosyl residue was attached at C-2' of the D-glucopyranosyl residue. A g-HMBC correlation between H-1' (5.05 ppm) and the C-3 genin signal at 77.1 ppm indicated that the glucopyranosyl residue was linked to the aglycone at C-3.

Structurally significant g-HMBC correlations observed for selected atoms of saponin **1** are listed in **Table 3.5**.

Table 3.5 g-HMBC correlations observed for selected atoms of saponin **1**

δ ^{1}H signal	correlated signal(s) (^{2}J or ^{3}J)
0.83 (H_3-18)	40.6 (C-13), 40.1 (C-12), 56.4 (C-14), 62.9 (C-17)
0.87 (H_3-19)	35.7 (C-10), 37.0 (C-1), 44.6 (C-5), 54.2 (C-9)
6.33 (H-1'')	78.4 (C-2')
5.05 (H-1')	77.1 (C-3)
4.46-4.03 (H_2-26)	108.6 (C-27)

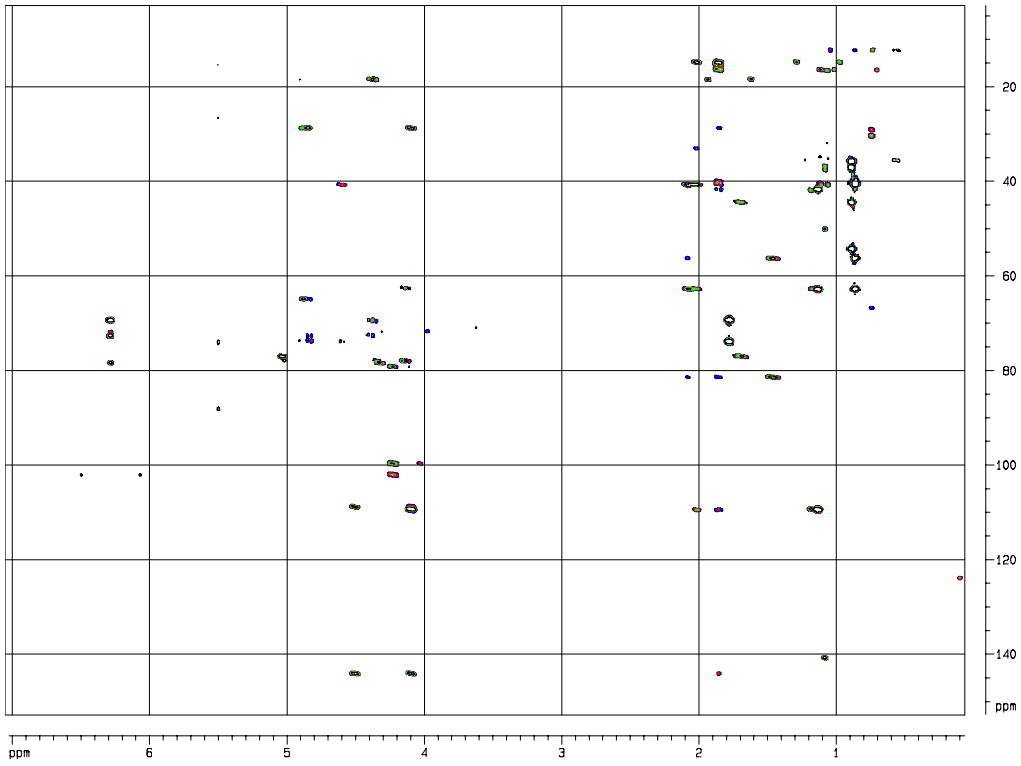


Figure 3.6 g-HMBC spectrum of saponin **1**

3.4.3.4 NOESY and ROESY Spectra

NOESY and ROESY data verified that C-3 glycosyl group was equatorial (β) oriented since H-3α (3.99 ppm), H-1α (0.81 ppm), H-2α (2.10 ppm), H-4α (1.96 ppm) and H-5α (0.90 ppm) exhibited mutual correlations in separate NOESY and ROESY experiments.

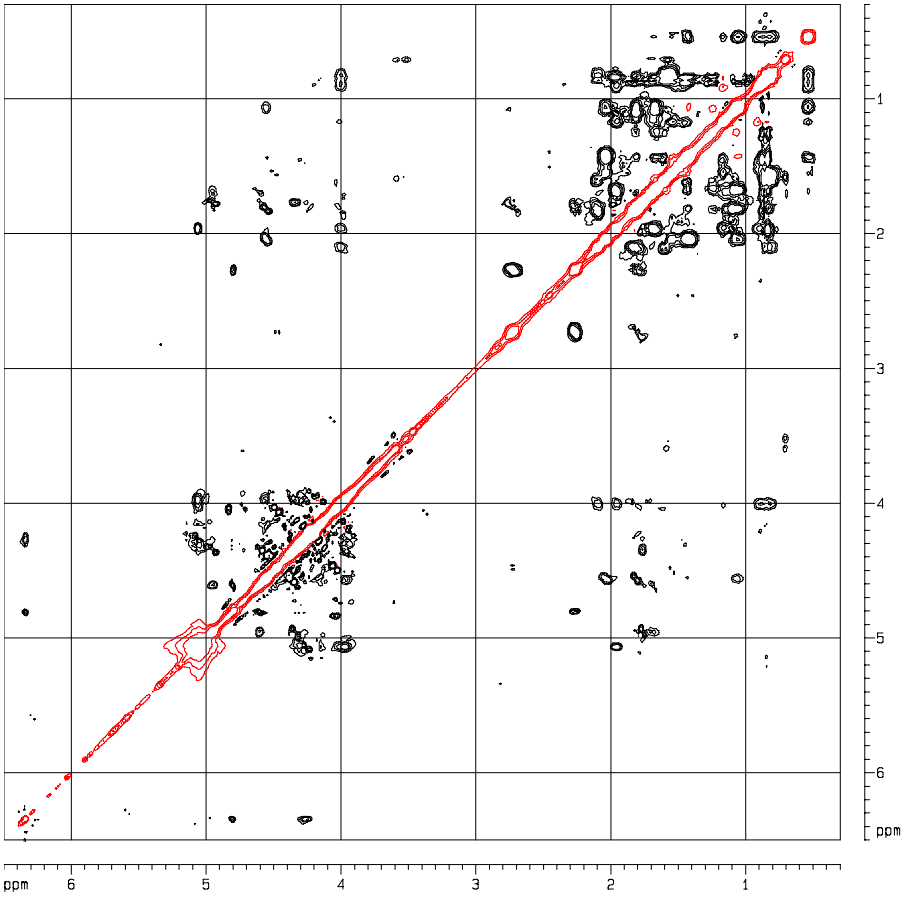


Figure 3.7 ROESY spectrum of the glycosyl region of saponin **1**

The rhamnosyl H-1" (6.33 ppm) resonance showed a strong correlation at 4.24 ppm (H-2') indicating that rhamnosyl sugar residue to be attached to the glucosyl C-2' atom.

The foregoing ESMS and NMR data showed that saponin 1 contained a 2'-*O*-α-L rhamnopyranosyl-β-D-glucopyranosyl moiety which was attached at *O*-3 of 5α-spirost-25(27)-en-3β-ol (or $\Delta^{25(27)}$-tigogenin). This genin, which has a limited occurrence in nature, was first reported from *Hosta kiyosumiensis* [24,125]. Later, it was isolated from *Tristagma uniflorum* and spectrally characterised [126]. Saponin **1**, for which the trivial name raukāukain* is proposed is the dominant steroidal saponin from the dry leaves of *C. australis*. This saponin demonstrated antifungal activity against *T. mentagrophytes* (see Chapter Four)**.**

*We thank Mr Waldo Houia of the School of Māori and Pacific Development, The University of Waikato, for suggesting raukāuka (= leaves of the Ti kōuka) for this saponin.

3.5 Structure Elucidation of Saponin 2

Saponin **2,** 5α-spirost-25(27)-ene-1β,3β-diol 1-{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranoside}, was isolated from the chloroform extract of fresh leaves of *C. australis*.

3.5.1 IR Spectrum

Strong IR absorptions at 3422 and 1044 cm^{-1} and IR absorption at 980 cm^{-1}, 920 cm^{-1}, 900 cm^{-1} and 865 cm^{-1} confirmed the glycosidic nature of saponin 2 and the presence of a spiroketal moiety in this saponin [60-62].

3.5.2 ESMS

The molecular formula of **2**, $C_{39}H_{62}O_{12}$ was confirmed by positive ion ESMS, which showed a M_2Na^+ ion at *m/z* 1467. Under MS-MS (MS^2) conditions this ion fragmented to afford a MNa^+ ion at *m/z* 745, which in turn afforded a *m/z* 599 ion (MS^3 ion), consistent with the loss of a rhamnopyranosyl unit from the MNa^+ ion (MS^2 ion)

3.5.3 NMR Spectra

The complete assignment of the ^1H and ^{13}C NMR signals of saponin **2** presented in **Table 3.6** were established from detailed analyses of one and two dimensional NMR data (see following Sections).

Table 3.6 ^{1}H and ^{13}C NMR signal assignments (δ C_5D_5N) established for saponin **2**

atom	**^{13}C**	**^{1}H**	**atom**	**^{13}C**	**^{1}H**
1	83.1	3.78	22	109.4	
2	37.6	2.18, 2.69	23	33.1	1.75 (2H)
3	73.2	3.92	24	28.8	2.22, 2.68
4	39.4	1.66, 1.71	25	144.2	
5	43.0	1.02	26	64.9	4.00, 4.43
6	28.7	1.24 (2H)	27	108.7	4.75, 4.79
7	32.4	0.81, 1.53			
8	36.5	1.51	1'	99.9	4.68, *d,* $J_{1',2'}$ = 7.8 Hz
9	55.2	0.98	2'	76.6	4.45, *dd,* $J_{2',3'}$ = 9.4 Hz
10	41.4		3'	76.8	4.07 *dd,* $J_{3',4'}$ = 3.5 Hz
11	23.5	1.38, 3.15	4'	67.9	3.88 *dd,* $J_{4',5'}$ = 1.1 Hz
12	40.6	1.19, 1.57	5'	71.1	3.66, *m*
13	40.3		6'	17.1	1.49, *d*, $J_{5'6'}$ = 6.5 Hz
14	56.9	1.10			
15	32.3	1.40, 2.01	1"	101.3	6.31, *d,* $J_{1",2"}$ = 1.6 Hz
16	81.4	4.57	2"	72.3	4.71, *dd,* $J_{2",3"}$ = 3.4 Hz
17	63.0	1.80	3"	72.1	4.62, *dd*, $J_{3",4"}$ = 9.5 Hz
18	16.8	0.81	4"	74.1	4.28, *dd,* $J_{4"5"}$ = 9.5 Hz
19	8.6	1.21	5"	69.1	4.81, *m*
20	41.9	1.92	6"	18.8	1.70, *d,* $J_{5",6"}$ = 6.4 Hz
21	14.8	1.02			

3.5.3.1 ^{1}H and ^{13}C NMR Spectra

The ^{1}H NMR spectrum of saponin **2** in pyridine-d_5 included three methyl group protons at 0.81 (s), 1.21 (s), 1.02 (d) ppm, two oxygenated methylene protons at 4.75 and 4.79 (s) ppm and two anomeric glycosidic protons at 4.68 (d, J = 7.8 Hz) and 6.31 (d, J = 1.6 Hz) ppm.

The genin portion of the ^{13}C NMR spectrum of **2** contained 27 signals including three methyl groups nine non-oxygenated and one oxygenated methylene groups, six non-oxygenated and three oxygenated methine groups and two non-oxygenated and one oxygenated quaternary carbons. In addition to the foregoing signals, a C=CH_2 signal was observed at 108.7 ppm and a quaternary C=CH_2 signal at 144.2 ppm indicating the presence of a 25(27)-olefinic unsaturation. A quaternary carbon (C-22) signal also appeared at 109.4 ppm. Thus a dihydroxy spirostane skeleton with an exocyclic double bond on the F ring was indicated.

The ^{13}C NMR chemical shifts of C-5, C-7, C-9 and C-19, (43.0, 32.4, 55.2 and 8.6 ppm respectively) were consistent with the presence of a 5α-spirostane steroidal skeleton (tigogenin type), rather than a 5β-spirostane skeleton [71,78]. The orientation of the C-1 and C-3 hydroxyl groups were established to be equatorial (β-) (see Section **3.5.3.4).**

The glycosyl portion of the ^{13}C NMR spectrum of saponin **2** contained twelve signals comprising ten oxygenated methine signals and two methyl groups. The anomeric region contained signals at 99.9 and 101.3 ppm (see Section **3.5.3.3**).

3.5.3.2 COSY and TOCSY Spectra

Starting with the signal at 4.68 ppm (H-1') the couplings were traced around the pyranosyl ring giving $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 9.4 Hz, $J_{3',4'}$ = 3.5 Hz and $J_{4',5'}$ = 1.1 Hz indicating a β-D-fucopyranosyl residue. Similarly, starting with the signal at 6.31 ppm (H-1") coupling constants were measured around the ring giving $J_{1",2"}$ = 1.6 Hz, $J_{2",3"}$ =

3.4 Hz, $J_{3'',4''}$ = 9.5 Hz and $J_{4'',5''}$ = 9.5 Hz. This pattern was consistent with the presence of an α-L-rhamnopyranosyl residue.

These and some other structurally significant COSY and TOCSY correlations arising from selected protons of saponin **2** are listed in **Table 3.7**

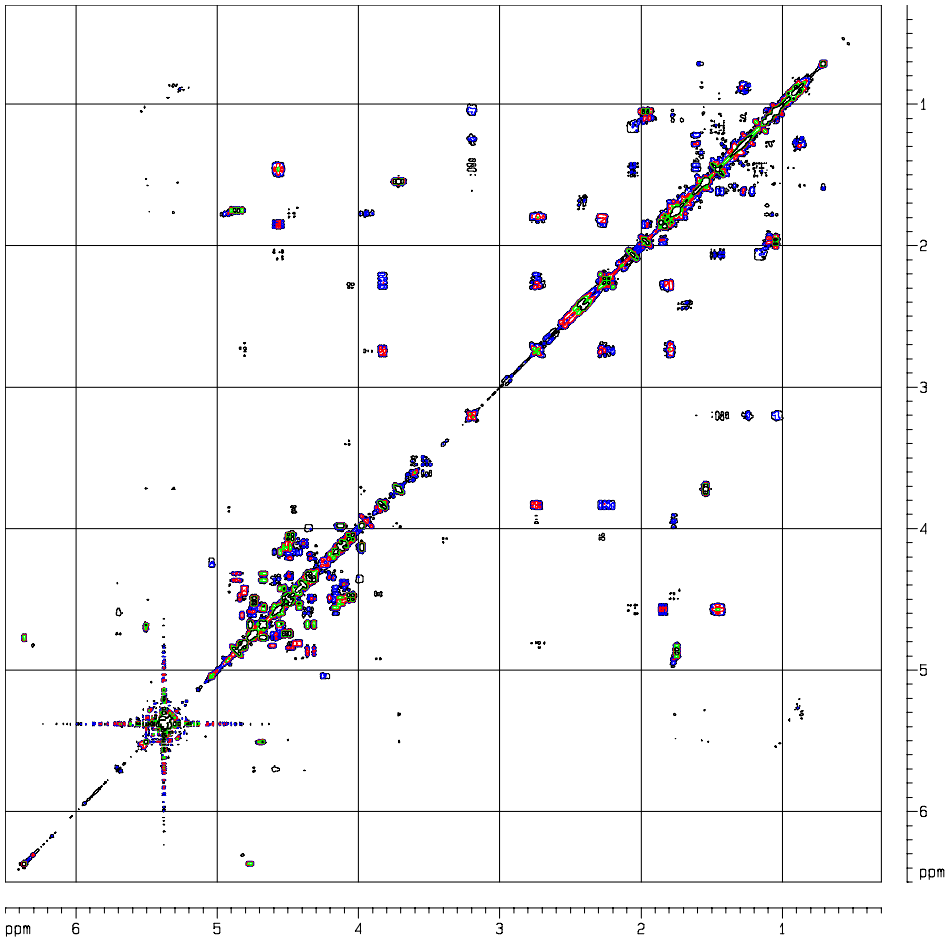


Figure 3.8 COSY spectrum of saponin **2**

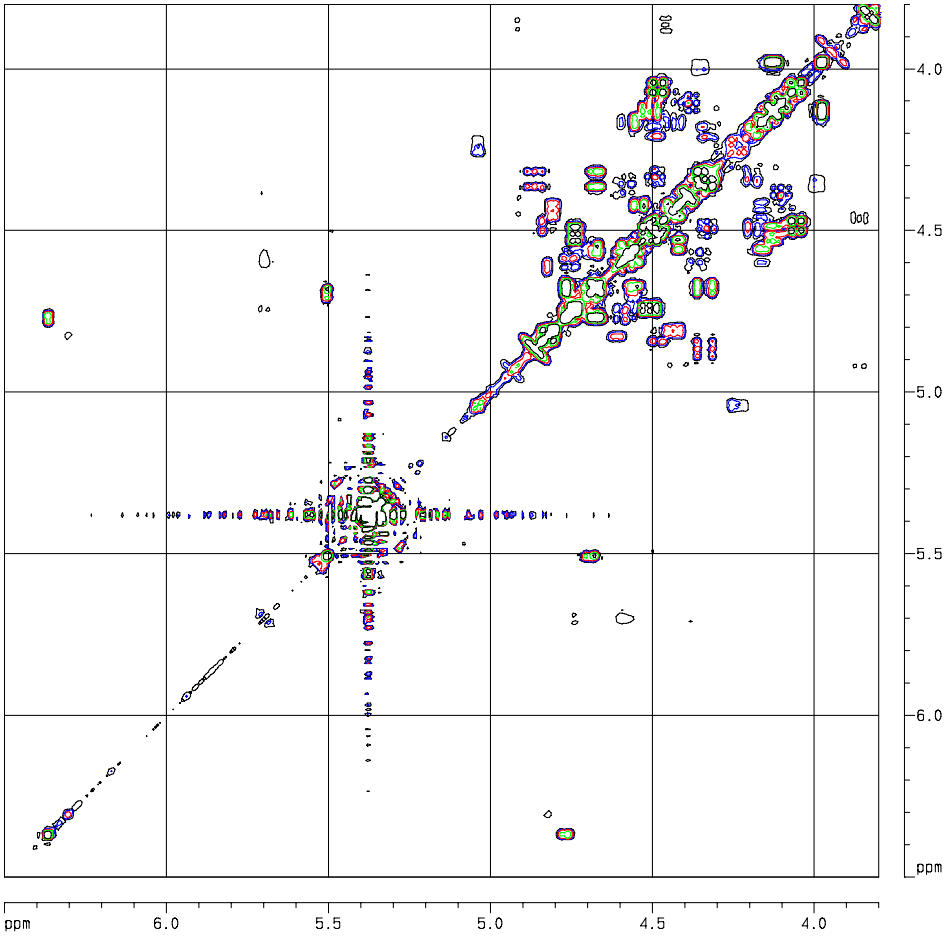


Figure 3.9 COSY spectrum of the glycosyl region of saponin **2**

Table 3.7 COSY connectivities (δ C_5D_5N) observed for glycosyl protons of saponin **2**

δ ^1H	correlated signal(s)	δ ^1H	correlated signal(s)
4.68 (H-1')	4.45 (H-2')	6.31 (H-1")	4.71 (H-2")
4.45 (H-2')	4.68 (H-1'), 4.07 (H-3')	4.71 (H-2")	6.31 (H-1"), 4.62 (H-3")
4.07 (H-3')	4.45 (H-2'), 3.88 (H-4')	4.62 (H-3")	4.71 (H-2"), 4.28 (H-4")
3.88 (H-4')	4.07 (H-3'), 3.66 (H-5')	4.28 (H-4")	4.62 (H-3"), 4.81 (H-5")
3.66 (H-5')	3.88 (H-4'), 1.49 (H-6')	4.81 (H-5")	4.28 (H-4"), 1.70 (H-6")
1.49 (H-6')	3.66 (H-5')	1.70 (H-6")	4.81 (H-5")

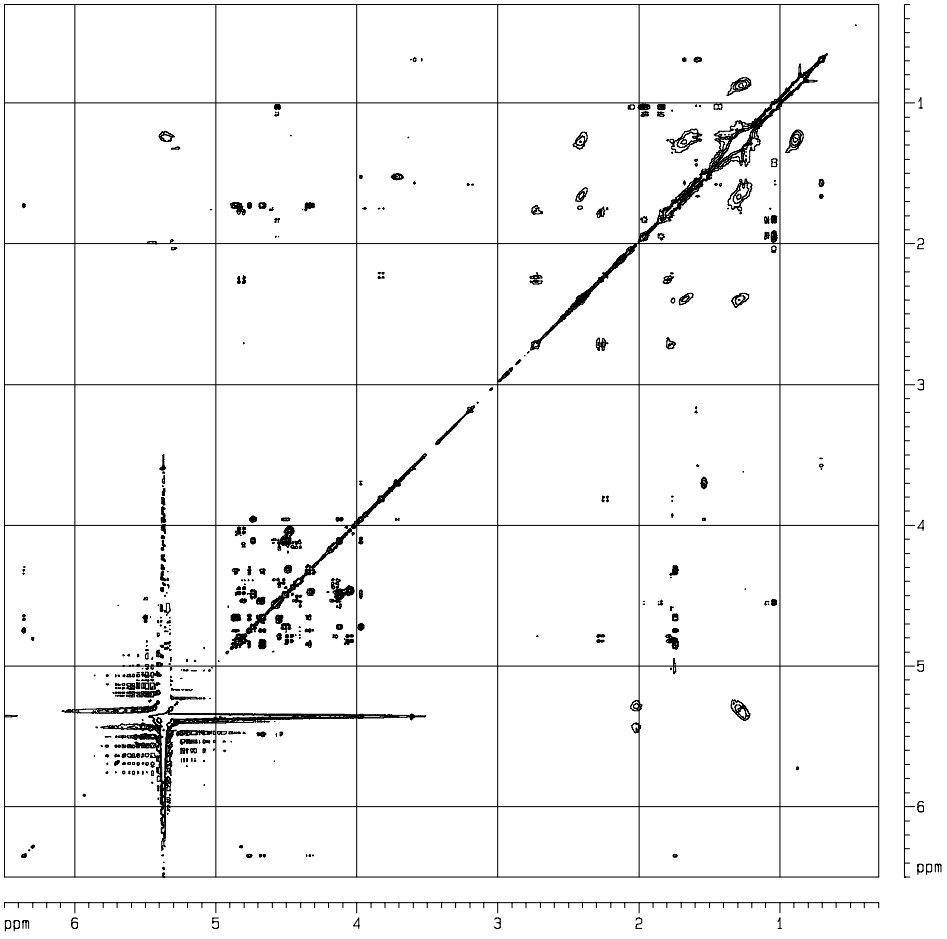


Figure 3.10 TOCSY spectrum of saponin **2**

The COSY and TOCSY spectra of saponin **2** also included correlations between H-11α (3.15 ppm) and H-12α (1.57 ppm), H-11β (1.38 ppm), H-12 β (1.19 ppm) and H-9α (0.98 ppm), also H-1α (3.78 ppm) and H-2α (2.69 ppm), H-2 β (2.18 ppm), H-4α (1.71 ppm) and H-4 β (1.66 ppm).

3.5.3.3 g-HSQC and g-HMBC Spectra

The anomeric region of glycosyl units contained signals at 99.9 and 101.3 ppm and these were coupled in the g-HSQC spectrum to H-1' and H-1", doublets at 4.68 ppm ($J_{1',2'}$ = 7.8 Hz) and 6.31 ppm ($J_{1'',2''}$ = 1.6 Hz) respectively. These coupling constants are consistent with the presence of a β-D-fucopyranosyl and an α-L-rhamnopyranosyl residue, respectively, in saponin **2**.

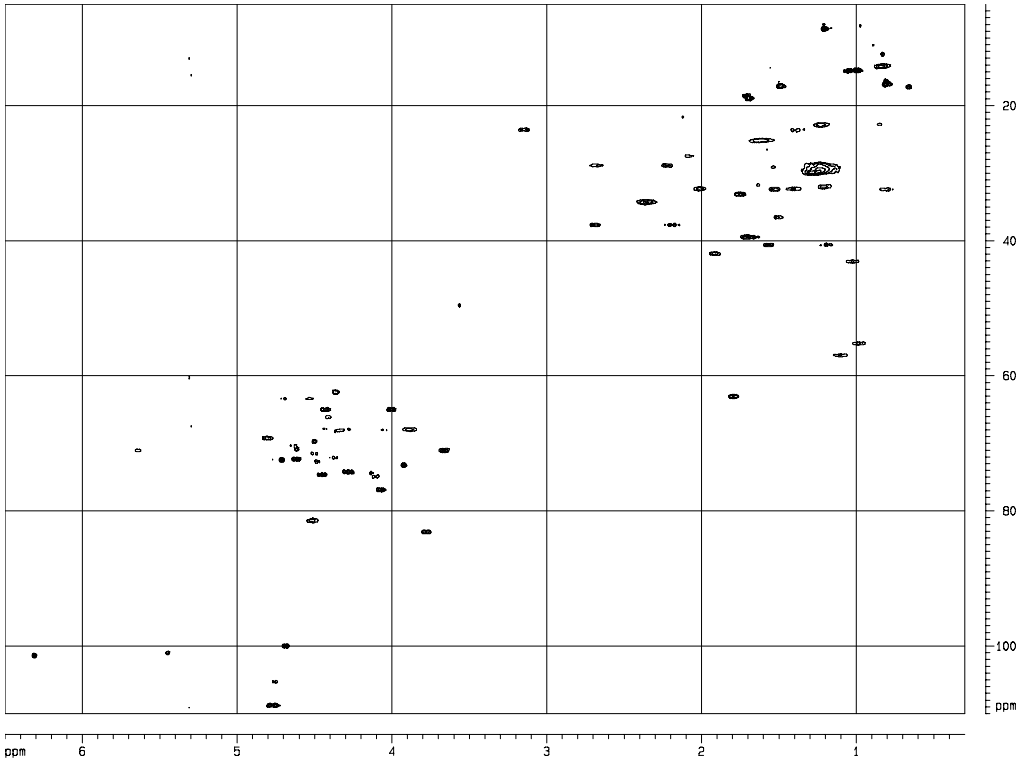


Figure 3.11 g-HSQC spectrum of saponin **2**

The location of the glycosyl linkages was elucidated from a combination of g-HMBC and ROESY spectral data (see **Figure 3.12**, **Table 3.8** and Section **3.5.3.4**)

A g-HMBC cross peak between the rhamnopyranosyl H-1" signal (6.31 ppm) and C-2' (76.6 ppm) indicated that the rhamnopyranosyl residue was attached at C-2' of the D-fucopyranosyl residue. Similarly a g-HMBC cross peak between fucopyranosyl H-1' signal (4.68 ppm) and C-1 of the genin (83.1 ppm) indicated that the fucopyranosyl residue was linked to the aglycone at C-1.

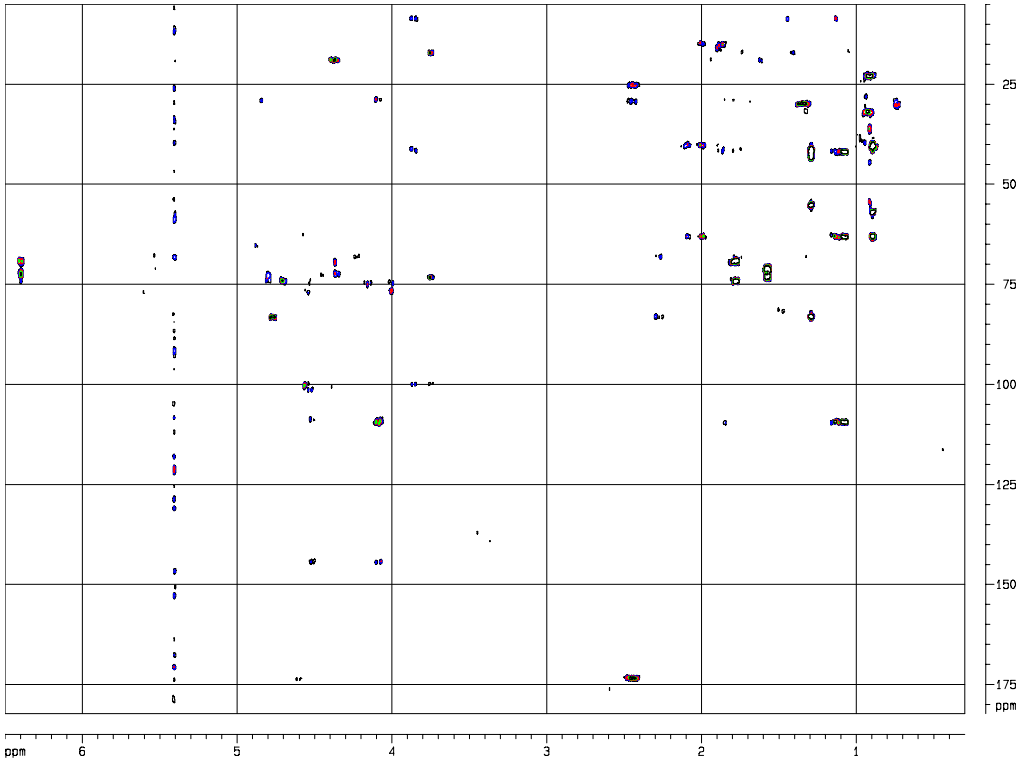


Figure 3.12 g-HMBC spectrum of saponin **2**

Table 3.8 g-HMBC correlations observed for selected atoms of saponin **2**

δ ^{1}H signal	correlated signal(s) (^{2}J or ^{3}J)
0.81 (H-18)	40.3 (C-13), 40.6 (C-12), 56.9 (C-14), 63.0 (C-17)
1.21 (H-19)	41.4 (C-10), 83.1 (C-1), 43.0 (C-5), 55.2 (C-9)
6.31 (H-1")	76.6 (C-2')
4.68 (H-1')	83.1 (C-1)

3.5.3.4 NOESY and ROESY Spectra

NOESY and ROESY data verified that the orientation of the C-1 and C-3 hydroxyl groups were equatorial (β-) since H-1α (3.78 ppm), H-3α (3.92 ppm) and H-5α (1.02 ppm) exhibited mutual correlations in separate NOESY and ROESY experiments.

Thus saponin **2** is 5α-spirost-25(27)-ene-1β,3β-diol 1-{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranoside}. Saponin **2** did not exhibit biological activity in the assays used.

3.6 Structure Elucidation of Saponin 3

The *n*-butanol extracts of senescent leaves of *C. australis* afforded a third new spirostanol glycoside that was identified as 5α-spirost-25(27)-ene-1β,3β-diol 1-*O*{β-D- fucopyranoside}, saponin **3.**

3.6.1 IR Spectrum

Strong IR absorptions at 3400 and 1044 cm^{-1} and IR absorption at 980 cm^{-1}, 920 cm^{-1}, 900 cm^{-1} and 865 cm^{-1} confirmed the spirostanol nature of saponin **3** [60-62].

3.6.2 ESMS

The molecular formula of **3**, $C_{33}H_{52}O_8$ was defined by ESMS which showed an $[M_2\text{-}H]^-$ ion at *m/z* 1151 in the negative ion ESMS spectrum, and an $[M_2Na]^+$ ion at *m/z* 1175 in the positive ion ESMS spectrum.

3.6.3 NMR Spectra

The complete assignment of the ^1H and ^{13}C NMR signal of saponin **3** presented in **Table 3.9** were established from detailed analyses of one and two dimensional NMR data (see following Sections).

Table 3.9 ^{1}H and ^{13}C NMR signal assignments (δ C_5D_5N) established for saponin **3**

atom	**^{13}C**	**^{1}H**	**atom**	**^{13}C**	**^{1}H**
1	81.2	3.95	22	109.3	
2	38.0	1.99, 2.88	23	33.0	1.78, 1.82
3	67.5	3.94	24	28.8	2.27, 2.74
4	39.5	1.65, 1.76	25	144.2	
5	42.8	1.13	26	64.8	4.03, 4.47
6	28.7	1.28 (2H)	27	108.3	4.78, 4.82
7	32.1	0.88, 1.59			
8	36.3	1.54	1'	101.2	4.81, *d,* $J_{1',2'}$ = 7.5 Hz
9	54.8	1.03	2'	72.1	4.33, *dd* $J_{2'3'}$ = 9.4 Hz
10	42.3		3'	75.1	4.08, *dd* $_{3'4'}$ = 3.5 Hz
11	23.5	1.40, 3.17	4'	72.3	4.02, *dd* $_{4'5'}$ = 1.2 Hz
12	40.5	1.23, 1.64	5'	71.0	3.78, *m,* $J_{5',6'}$ = 6.5 Hz
13	40.4		6'	17.1	1.59 *d*
14	56.6	1.15			
15	32.2	1.45, 2.06			
16	81.2	4.54			
17	63.0	1.83			
18	16.7	0.85			
19	8.2	1.03			
20	41.7	1.88			
21	14.7	1.05			

3.6.3.1 ^{1}H and ^{13} NMR Spectra

The ^{1}H NMR spectrum of saponin **3** in pyridine-d_5 included three methyl group protons at 0.85 (s), 1.03 (s), 1.05 (d) ppm, two oxygenated methylene protons at 4.78 and 4.82 (s) ppm and an anomeric glycosidic proton at 4.81 ppm (d, J = 7.5 Hz).

The genin portion of the ^{13}C NMR spectrum of **3** contained 27 signals including three methyl group carbon signals, nine non-oxygenated and one oxygenated methylene carbon signals, six non-oxygenated and three oxygenated methine group signals and two non-oxygenated and one oxygenated quaternary carbon signals. In addition to these 25 signals, a CH_2 signal was observed at 108.3ppm and a C signal at 144.2 ppm indicating the presence of a 25(27)-olefinic unsaturation. A quaternary carbon (C-22) signal appeared at 109.3 ppm. Thus a dihydroxyspirostane skeleton with an exocyclic double bond on F ring was indicated.

The A/B ring junction was established as *trans* by examination of the ^{13}C NMR chemical shifts of C-5, C-7, C-9 and C-19 (42.8, 32.1, 54.8 and 8.2 ppm respectively), which corresponded to those expected for a 5α-spirostane type steroidal skeleton [71,78]. The orientation of the C-1 and C-3 hydroxyl groups were established to be equatorial, (β-) (see Section **3.6.3.4).**

The glycosyl portion of the ^{13}C NMR spectrum contained six signals comprising five oxygenated methine signals and a methyl group signal. The anomeric methine signal occurred at 101.2 ppm (see Section **3.6.3.3).**

3.6.3.2 COSY and TOCSY Spectra

Starting with the signal at 4.81 ppm (H-1') the couplings were traced around the pyranosyl ring giving $J_{1',2'}$ = 7.5 Hz, $J_{2',3'}$ = 9.4 Hz, $J_{3',4'}$ = 3.5 Hz and $J_{4',5'}$ = 1.2 Hz $J_{5',6'}$ = 6.5 Hz indicating a β-D-fucopyranosyl residue.

These and some other structurally significant COSY and TOCSY correlations arising from selected protons of saponin **3** are listed in **Table 3.10.**

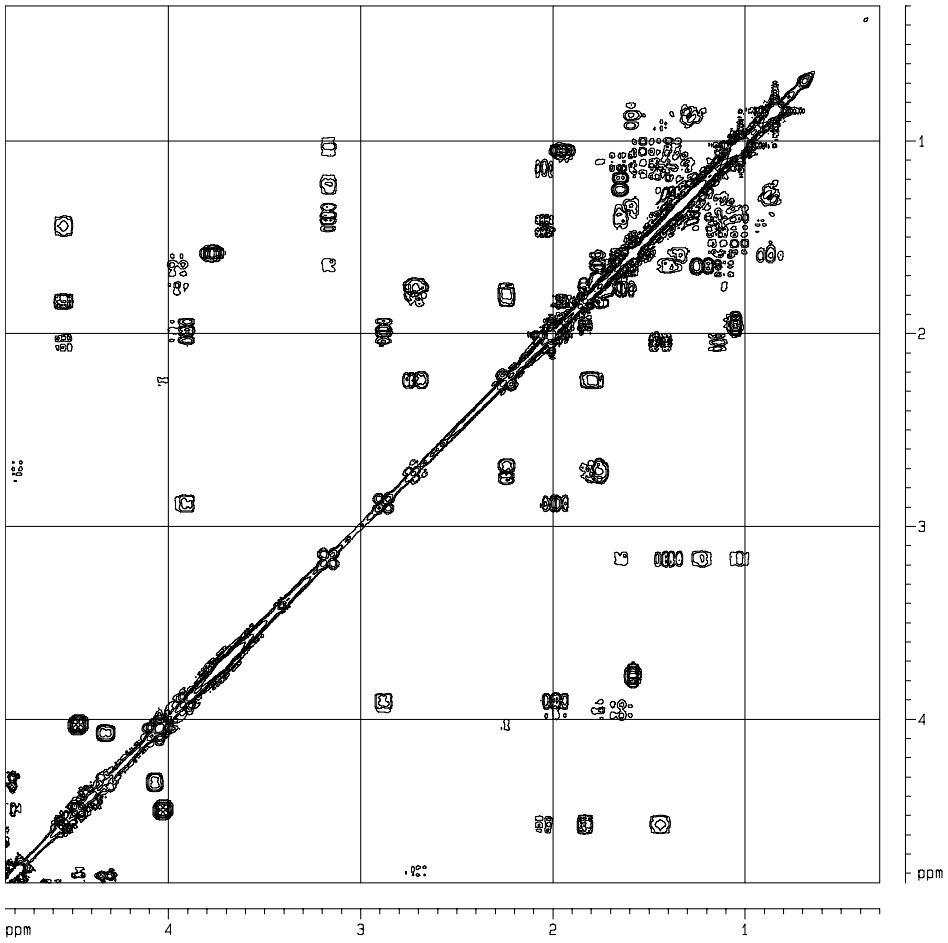


Figure 3.13 COSY spectrum of the fucosyl region of saponin **3**

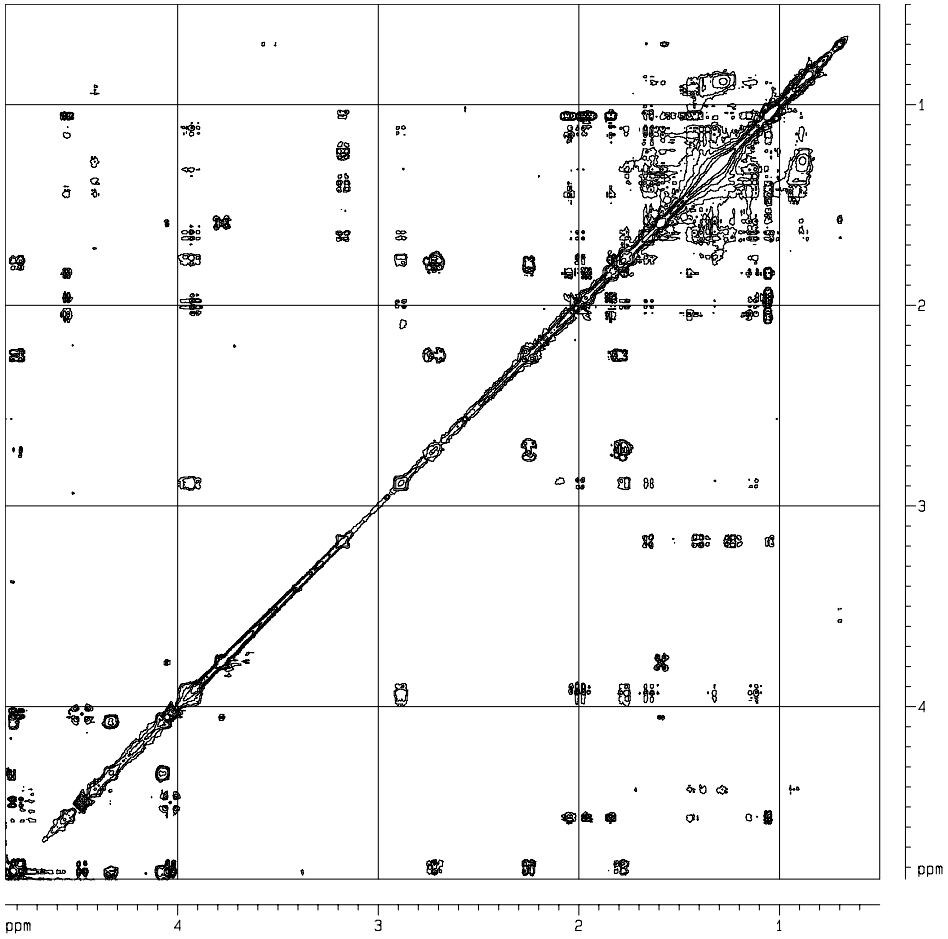


Figure 3.14 TOCSY spectrum of saponin **3**

Table 3.10 COSY and TOCSY connectivities (δ C_5D_5N) observed for selected protons of saponin **3**

δ ^{1}H	correlated signal(s)
4.81 (H-1')	4.33 (H-2')
4.33 (H-2')	4.81 (H-1'), 4.07 (H-3')
4.07 (H-3')[a]	4.33 (H-2'),
4.02 (H-4')[a]	3.78 (H-5')
3.78 (H-5')	3.88 (H-4'), 1.59 (H-6')
1.59 (H-6')	3.78 (H-5')
4.80 (H-27)	4.47 (H-26α), 2.74 (H-24α)
4.54 (H-16)	2.06 (H-15α), 1.83 (H-17), 1.45 (H-15β)
3.95 (H-1)	2.88 (H-2α), 1.99 (H-2β)

3.94 (H-3)	1.76 (H-4α), 1.65 (H-4β)
3.17 (H-11 α)	1.64 (H-12α), 1.40 (H-11β),1.26 (H-12β)
2.74 (H-24 α)	2.27 (H-24β), 1.78/1.82(H-23α/β)

[a] no cross peak H-3' and H-4' observed

3.6.3.3 g-HSQC and g-HMBC Spectra

The anomeric glycosyl proton (H-1') which resonated at 4.81 ppm (d, $J_{1',2'}$ = 7.5 Hz) showed a correlation in the g-HSQC spectrum to the carbon which occurred at 101.2 ppm. The g-HSQC spectrum of the glycosyl region of saponin **3** is shown in **Figure 3.15**.

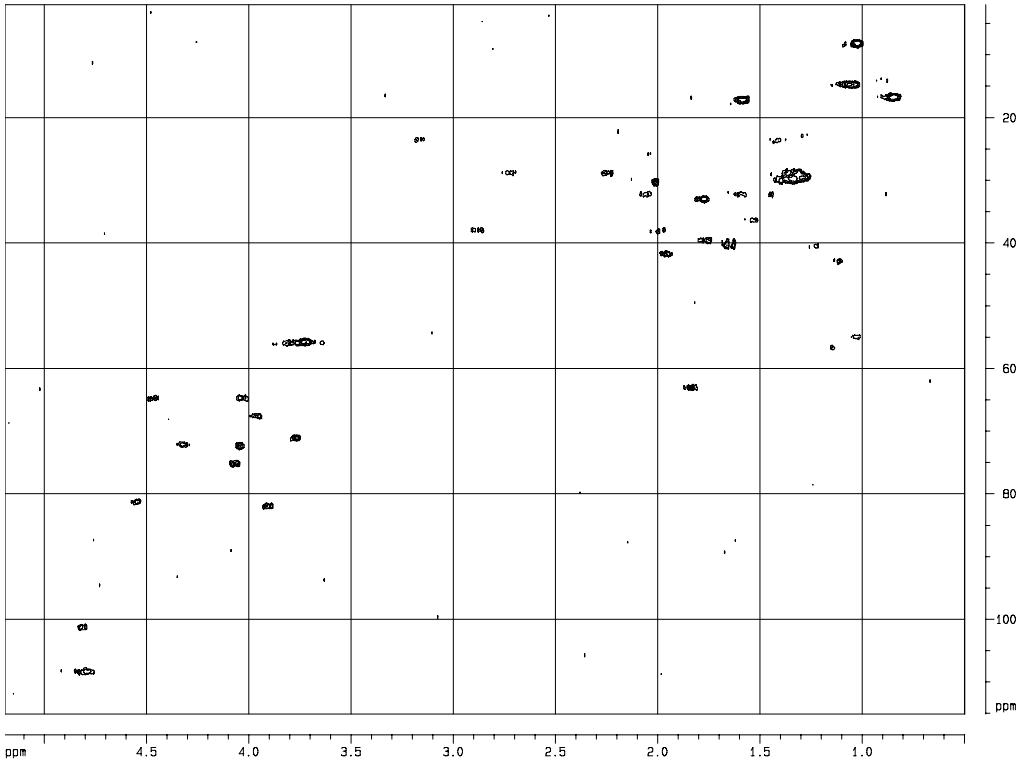


Figure 3.15 g-HSQC spectrum of saponin **3**

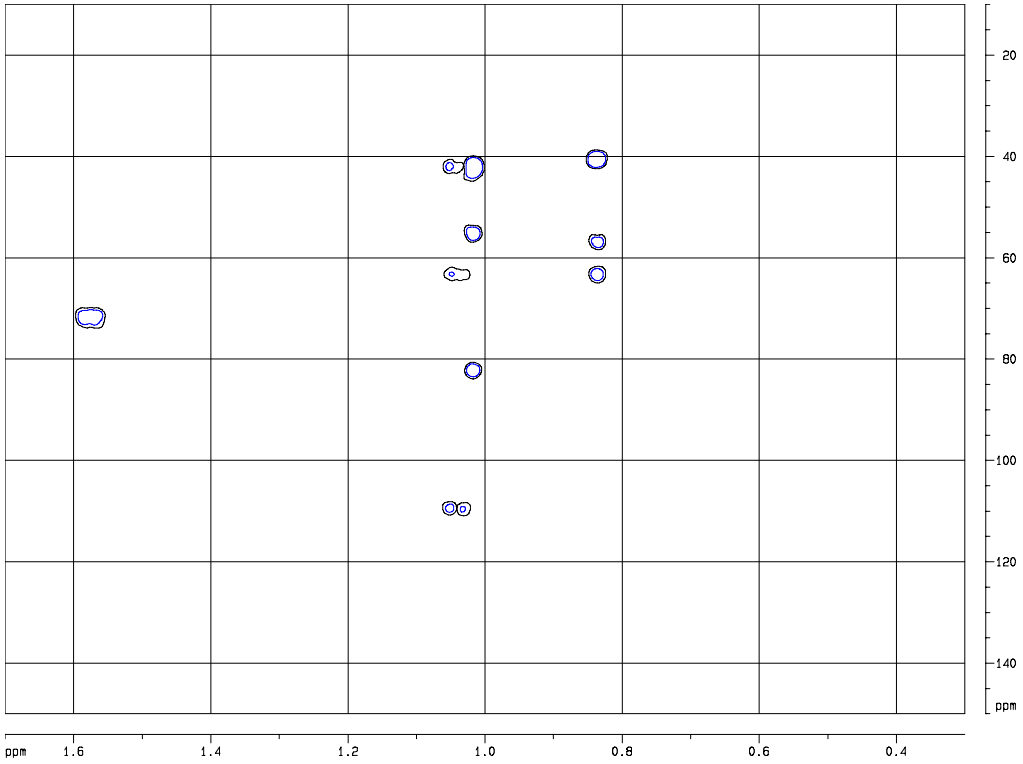


Figure 3.16 g-HMBC spectrum of the expanded region of saponin **3**

A g-HMBC correlation observed between H-18 (0.85 ppm) and C-13/C-12 (40.4/40.5 ppm), C-14 (56.6 ppm), C-17 (63.0 ppm), and between H-19 (1.03 ppm) and C-10 (42.3 ppm), C-9 (54.8 ppm) and C-1 (81.2 ppm). Also, a g-HMBC correlation between H-1' (4.81 ppm) and C-1 (81.2 ppm) indicated that the fucopyranosyl residue was linked to the aglycone at C-1.

3.6.3.4 NOESY and ROESY Spectra

NOESY and ROESY data verified that the orientation of the C-1 and C-3 hydroxyl groups were equatorial (β-) since mutual NOE's were exhibited by the proton signals which occurred at 3.95 (H-1α), 3.94 (H-3α) and 1.13 (H-5α) ppm.

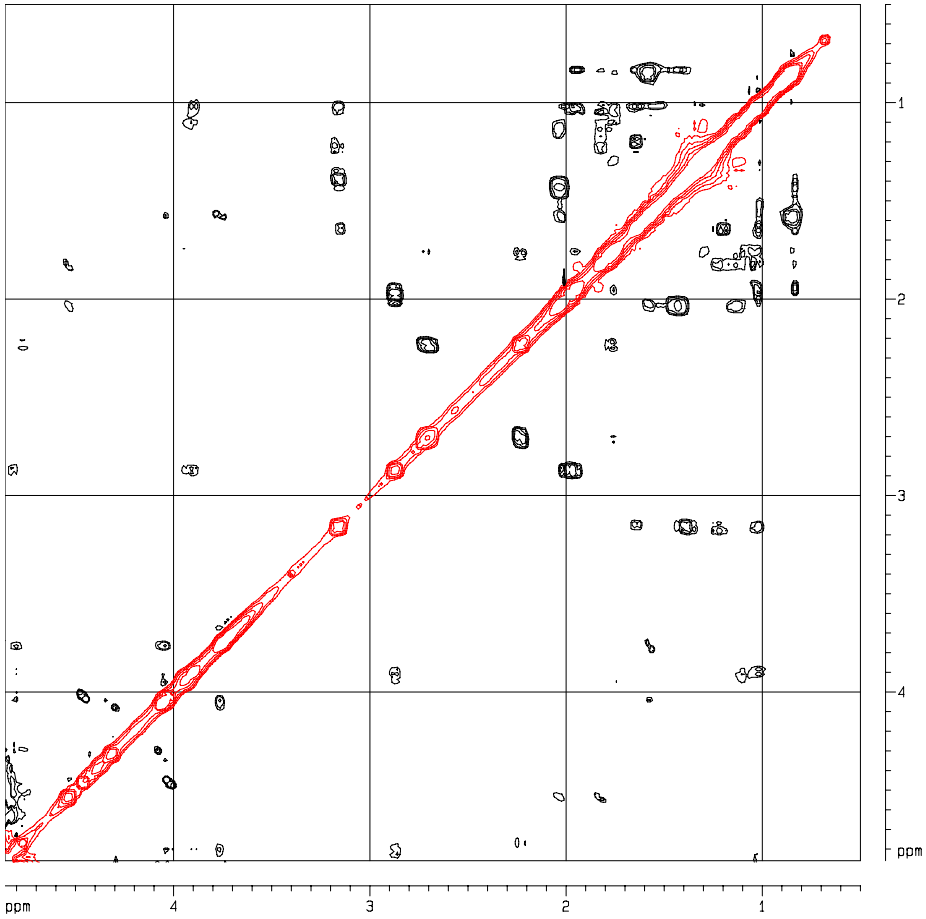


Figure 3.17 ROESY spectrum of the genin region of saponin **3**

Thus saponin **3** was identified as 5α-spirost-25(27)-ene-1β,3β-diol 1-*O*-β-D-fucopyranoside. Saponnin **3** did not exhibit biological activity in the assays used.

3.7 Structure Elucidation of Saponin 9

The *n*-butanol extract of dried stems of *C. australis* afforded (25*S*)-5α-spirostane-1β,3α-diol 1-*O*-β-D-glucopyranoside, saponin **9**.

3.7.1 IR Spectrum

Strong IR absorptions at 3400 and 1044 cm^{-1}, 980 cm^{-1}, 920 cm^{-1}, 900 cm^{-1} and 865 cm^{-1} confirmed the spirostanol nature of saponin **9** [60-62].

3.7.2 ESMS

The molecular formula $C_{33}H_{54}O_9$ for saponin **9** was defined by positive ion ESMS which showed $[M_2Na]^+$, $[MNa]^+$ and $[MH]^+$ ions at *m/z* 1211, 617 and 595 respectively.

3.7.3 NMR Spectra

The complete assignment of the ^1H and ^{13}C NMR signals of saponin **9** presented in **Table 3.11** was established from detailed analyses of one and two dimensional NMR data (see following Sections).

Table 3.11 ^1H and ^{13}C NMR signal assignments (δ C_5D_5N) established for saponin **9**

	^{13}C	^1H		^{13}C	^1H
1	78.7	4.60	22	109.7	
2	34.9	1.87, 2.64	23	26.2	1.45, 1.90
3	65.8	4.36	24	26.1	1.36, 2.13
4	37.0	1.58 (2H)	25	27.4	1.59
5	38.9	2.02	26	65.0	3.36, 4.08
6	28.6	1.28 (2H)	27	16.2	1.08 (3H d, *J*=?)
7	32.4	0.85, 1.58			
8	36.3	1.54	1'	100.5	5.00, *d*, $J_{1',2'}$ = 7.7 Hz
9	54.6	1.10	2'	75.5	4.02, *dd*, $J_{2',3'}$ = 8.7 Hz

10	42.4		3'	78.5	4.18, *dd,* $J_{3',4'}$ = 9.1 Hz
11	23.8	1.45, 3.09	4'	72.1	4.11, *dd*, $J_{4',5'}$ = 9.4 Hz
12	40.2	1.25, 1.70	5'	78.1	3.83, *t*
13	40.6		6'	63.3	4.52, 4.30, *d*
14	56.5	1.03			
15	32.3	1.43, 2.02			
16	81.1	4.50			
17	62.8	1.79			
18	16.8	0.86 (3H s)			
19	7.1	1.03 (3H s)			
20	42.3	1.90			
21	14.7	1.10 (3H d, *J* =7.5Hz)			

3.7.3.1 ^{1}H and ^{13}C NMR Spectra

The ^{1}H NMR spectrum of saponin **9** in pyridine-d_5 included four methyl protons at 0.86 (s), 1.03 (s), 1.10 (d) and 1.08 (d) ppm and an anomeric glycosidic proton at 5.00 ppm (d, J = 7.5 Hz).

The genin portion of the ^{13}C NMR spectrum of **9** contained 27 signals including four methyl groups, nine non-oxygenated and one oxygenated methylene, seven non-oxygenated and three oxygenated methine and two non-oxygenated and one oxygenated quaternary carbon signals. A quaternary carbon (C-22) signal appeared at 109.7 ppm. These data indicated a dihydroxyspirostane skeleton with an exocyclic equatorial secondary methyl group (25*S*) in ring F.

The H_3-27 resonance of **9** occurred at 1.08 ppm, *ca* 0.3 ppm downfield of the corresponding resonance of (25*R*)-spirostanes. All of the ^{13}C resonances of the ring F atoms of **9**, except C-22 (**Table 3.11**) occurred at higher field than for the (25*R*)-spirostanes[21]. The A/B ring junction was established as *trans* by examination of the ^{13}C NMR chemical shifts of C-5, C-7, C-9 and C-19 (38.9, 32.4, 54.6 and 7.1 ppm respectively) which corresponded to the 5α-spirostane type steroidal skeleton [71,78].

The orientation of the C-1 hydroxyl group was established to be equatorial (β-) and the orientation of the 3-OH group was shown to be axial (α-) (see Section **3.7.3.4).** Also, the orientation of the 3-OH group was shown to be axial by the lesser width of the H-3β multiplet ($W_{1/2}$ ~ 10 Hz, fully resolved) of **9** compared to that observed for the H-3α multiplet of **3** ($W_{1/2}$ ~ 15 Hz, partially resolved).

The glycosyl portion of the ^{13}C NMR spectrum contained six signals comprising five oxygenated methine signals and a one oxygenated methylene signals. The anomeric methine signal of saponin **9** occurred at 100.5 ppm.

3.7.3.2 COSY and TOCSY Spectra

Starting with the signal at 5.00 ppm (H-1') couplings were traced around the pyranosyl ring giving $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 8.7 Hz, $J_{3',4'}$ = 9.1 Hz and $J_{4',5'}$ = 9.4 Hz. This data indicated a β-D-glucopyranosyl residue. These and some other structurally significant COSY and TOCSY correlations arising from selected protons of saponin **9** are listed in **Table 3.12**.

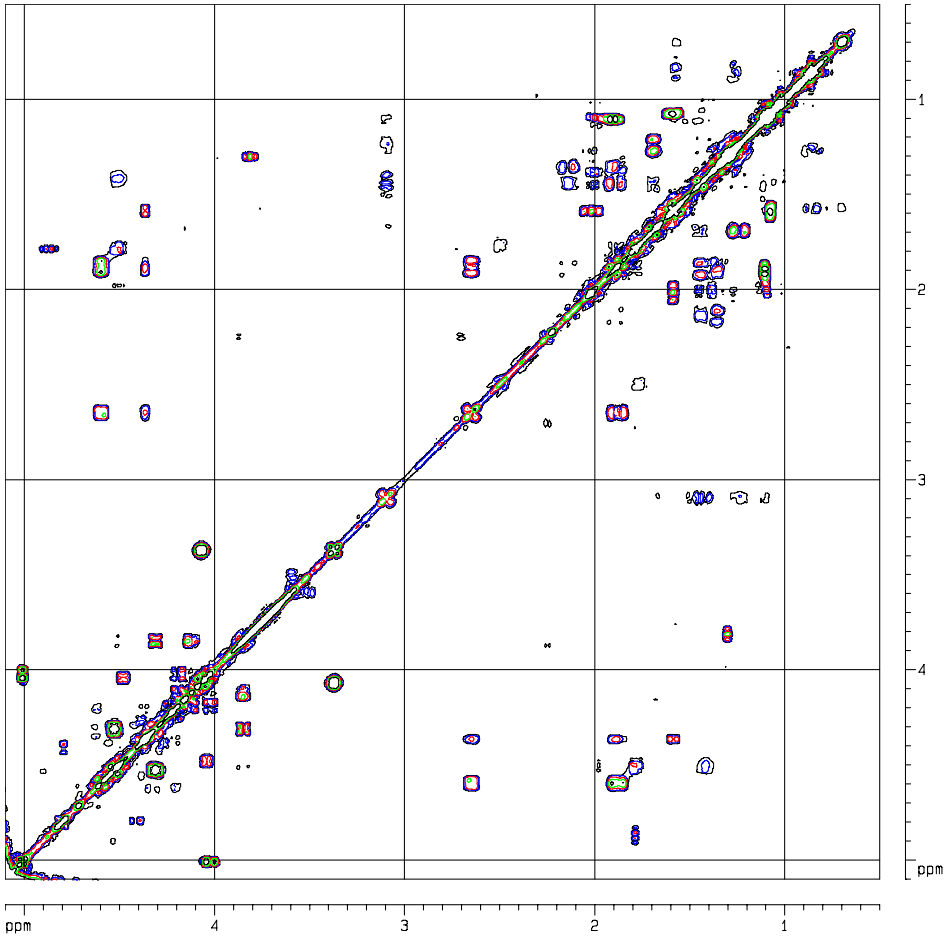


Figure 3.18 COSY spectrum of saponin **9**

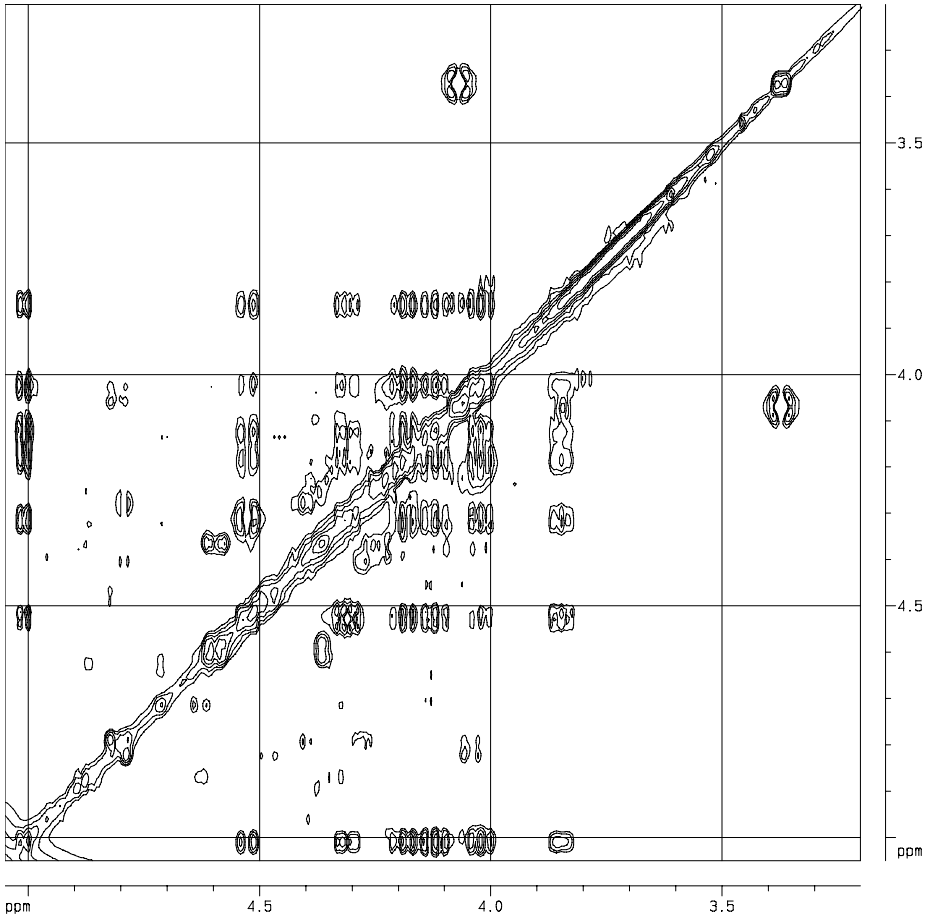


Figure 3.19 TOCSY spectrum of expanded region of saponin **9**

Table 3.12 TOCSY connectivity (δ C_5D_5N) observed for glucosyl proton of saponin **9**

δ ^1H	correlated signal(s)
5.00 (H-1')	4.52 (H-6'α), 4.30 (H-6'b), 4.18 (H-3'), 4.11 (H-4'), 4.02 (H-2'), 3.83 (H-5')
4.52 (H-6'α)	4.30 (H-6'b), 5.00 (H-1'), 4.18 (H-3'), 4.11 (H-4'), 4.02 (H-2'), 3.83 (H-5')

The COSY and TOCSY spectra of saponin **9** also included correlations between H-23α/23β (1.90/1.45 ppm), H-24α/H-24β (1.36 and 2.13 ppm), and H-25 proton (1.59 ppm).

3.7.3.3 g-HSQC and HMBC Spectra

The angular methyl group protons of saponin **9** which occurred as a singlets at 0.86 and 1.03 ppm, and as doublets at 1.10 and 1.08 ppm (J = 7.5 Hz and 7.2 Hz respectively), showed g-HSQC correlations to carbon signals which occurred at 16.8, 7.1 ppm and 14.7, 16.2 ppm respectively.

The g-HSQC spectrum of the glycosyl region of saponin **9** is shown in **Figure 3.20**. The carbon signal which occurred at 100.5 ppm showed a correlation to the anomeric H-1' glycosyl proton which resonated at 5.00 ppm (*d*, $J_{1',2'}$ = 7.7 Hz). The carbon signals which occurred at 78.5, 78.1, 75.5, 72.1 and 63.3 ppm showed correlations to H-3', H-5', H-2', H-4' and H-6' glucosyl protons which resonated at 4.18 ppm (*dd,* $J_{3',4'}$ = 9.1 Hz), 3.83 ppm (*t*), 4.02 ppm (*dd*, $J_{2',3'}$ = 8.7 Hz), 4.11 ppm (*dd*, $J_{4',5'}$ = 9.4 Hz) and 4.52 and 4.30 ppm (*d*) respectively. Also the C-26 carbon signal which occurred at 65.0 ppm showed correlation to the (H-26) protons at 4.08 and 3.36 ppm accordingly.

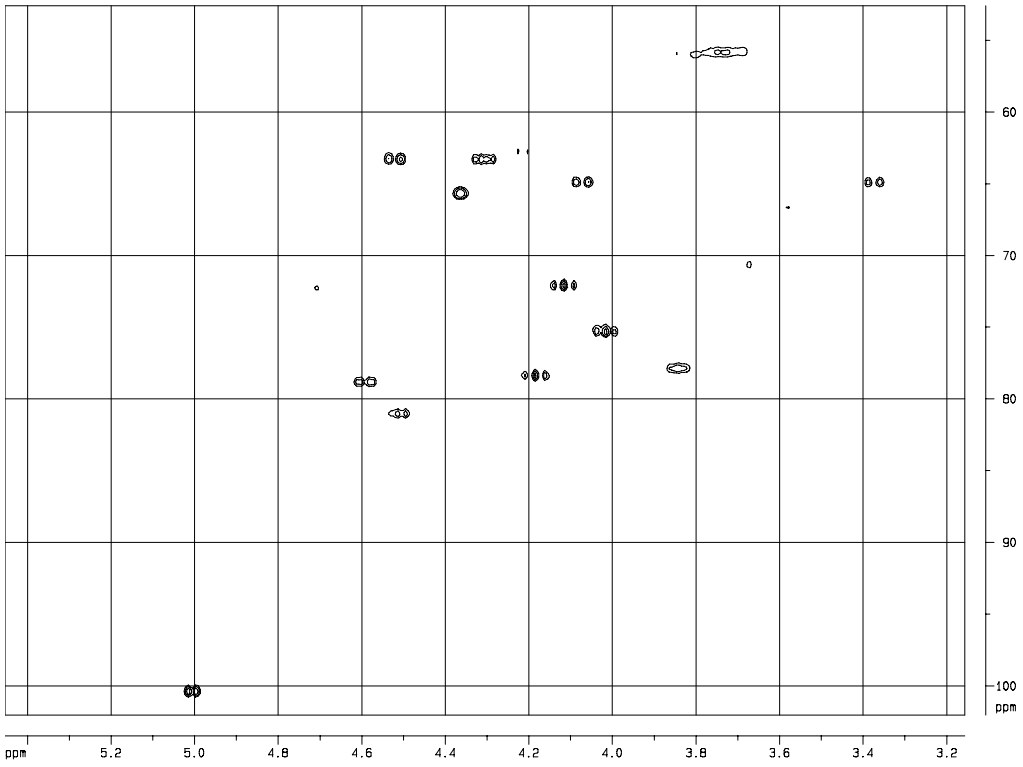


Figure 3.20 g-HSQC spectrum of the glucosyl region of **9**

A g-HMBC spectrum included a correlation between H-1' (5.00 ppm) and C-1 (78.7 ppm), which indicated that the glucopyranosyl residue was linked to the aglycone at C-1. Also, a g-HMBC spectrum showed H-18 (0.86 ppm) and H-19 (1.03 ppm), correlations (^{2}J, ^{3}J, and ^{4}J) to C-13 (40.6 ppm), C-12 (40.2 ppm), C-14 (56.5 ppm), C-17 (62.8 ppm) and C-11 (23.8 ppm), C-10 (42.4 ppm), C-1 (78.7 ppm), C-5 (38.9 ppm), C-9 (54.6 ppm), C-6 (28.6 ppm) accordingly.

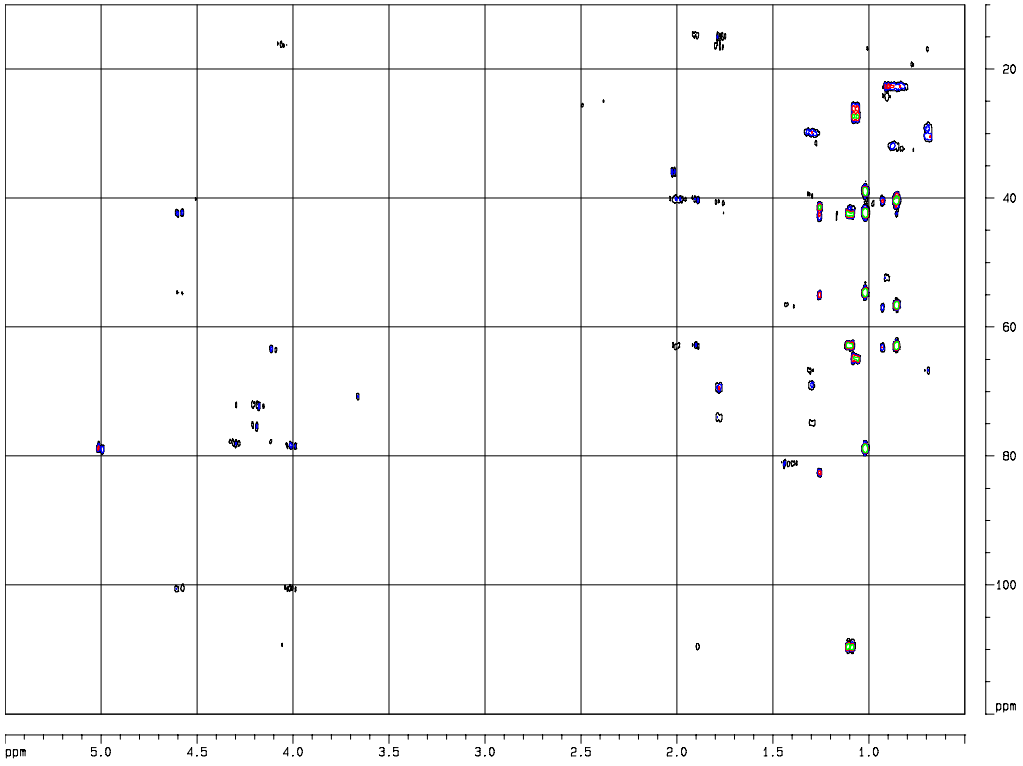


Figure 3.21 g-HMBC spectra of saponin **9**

3.7.3.4 NOESY and ROESY Spectra

The orientation of the C-1 hydroxyl group was established to be equatorial (β-) since the genin H-1α (4.60 ppm) exhibited strong correlations at H-2α (2.64 ppm) and H-5α (2.02 ppm) in separate NOESY and ROESY experiments. The absence of ROESY or NOESY correlations between the H-1 and H-3 signals were also consistent with the presence in saponin **9** of an axial 3α-OH group**.** Similarly, the glucosyl H-1' (5.00 ppm) resonance exhibited strong ROESY correlations at H-1α (4.60 ppm), H-3' (4.18 ppm) and H-5'(3.83 ppm), demonstrated that H-1' H-3' and H-

5' are mutually (α-) oriented and therefore the glucopyranosyl unit is (β-) linked to C-1 aglycone **(Figure 3.22).**

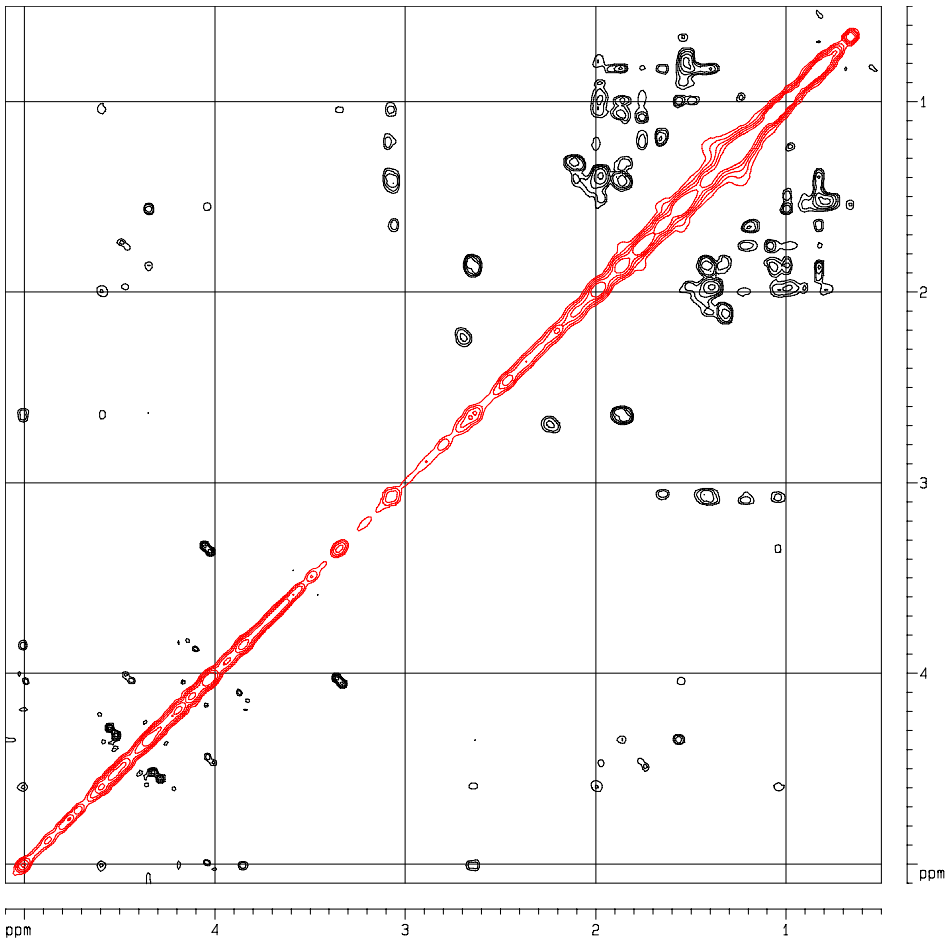


Figure 3.22 ROESY spectrum of the genin region of saponin **9**

Thus saponin **9** was identified as (25*S*)-5α-spirostane-1β,3α-diol 1-*O*-β-D-glucopyranoside. This saponin showed moderate cytotoxicity against *Herpes simplex type 1 virus* (ATCC VR733) and *Polio Virus Type 1* (Pfizer vaccine strain). See Chapter Four**.**

3.8 Structure Elucidation of Flavonoid Glycosides 4 and 5

A *ca* 47:53 mixture of two flavonoid glycosides which were identified as isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside} (**4**) (also

known as 3'-methylquercetin-3-*O*-rutinoside, isorhamnitin-3-rutinozide, or rhamnazin-3-O-rutinoside) [120,121,127,128] and isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside} (**5**) (also known as isorhamnetin-3-O-robinobioside) [120,127-129], were isolated from the *n*-butanol extract of dried leaves of *C. australis*. Efforts to separate the two flavonoid glycosides were not successful.

3.8.1 NMR Spectra

The complete assignment of the ^{1}H and ^{13}C NMR signals of flavonoid glycosides **4** and **5** presented in **Table 3.13** was established from detailed analyses of one and two-dimensional NMR data.

Table 3.13 ^{13}C and ^{1}H NMR chemical shifts (δ C_5D_5N) determined for mixture of **4** and **5**.

	4		**5**	
atom	^{13}C[b]	^{1}H	^{13}C	^{1}H
2	157.8		157.8	
3	136.1		136.1	
4	178.4		178.5	
5	157.9		157.9	
6	100.1	6.76, $J_{6,8}$ = 2.1 Hz	100.1	6.78, $J_{6,8}$ =2.1 Hz
7	166.9		166.9	
8	94.9	6.73	94.8	6.74
9	162.7		162.8	
10	104.9		104.9	
1'	123.7		123.7	
2'	114.4	8.38, $J_{2',6'}$ = 2.1 Hz	114.6	8.61, $J_{2',6'}$ = 2.1 Hz
3'	148.1		148.1	
4'	151.4		151.4	
5'	116.4	7.30, $J_{5',6'}$ = 8.4 Hz	116.2	7.27, $J_{5',6'}$ = 8.4 Hz
6'	123.5	7.91	123.0	7.82

OCH_3	56.1	3.93	56.5	4.03
Glu/Gal-1"	104.3[a]	6.16, $J_{1",2"}$ = 7.5 Hz	104.4[c]	6.22, $J_{1",2"}$ = 7.8 Hz
Glu/Gal-2"	76.2	4.30, $J_{2",3"}$ = 9.3 Hz	73.2	4.71, $J_{2",3"}$ = 9.3 Hz
Glu/Gal-3"	78.6	4.32, $J_{3",4"}$ = 9.8 Hz	75.1	4.27, $J_{3",4"}$ = 3.3 Hz
Glu/Gal-4"	71.6	4.05, $J_{4",5"}$ = 9.4 Hz	69.5	4.40, $J_{4",5"}$ = 1.8 Hz
Glu/Gla-5"	77.6	4.12, $J_{5",'6a"}$ = 5.9 Hz	75.1	4.16, $J_{5",6a"}$ = 5.6 Hz
Glu/Gal-6"	68.3	4.50, 4.02	66.6	4.42, $J_{5",6b"}$ = 4.2 Hz, 3.99 $J_{6a",6b"}$ = 11.4 Hz
Rha-1"'	102.6	5.25, $J_{1"',2"'}$ = 1.2 Hz	101.9	5.33, $J_{1"',2"'}$ = 1.3 Hz
Rha-2"'	72.5	4.12	72.1	4.18
Rha-3"'	69.5	4.38	69.7	4.35
Rha-4"'	73.8	4.28	73.8	4.18
Rha-5"'	72.1	4.40	69.7	4.40
Rha-6"'	18.4	1.48	18.5	1.52

a Refs 120,121,127,128, 130

b Refs 121,127

c Refs 120,121,127-130

3.8.1.1 ^{1}H and ^{13}C NMR Spectra

The ^{1}H NMR spectrum of the mixture of **4** and **5,** determined in pyridine-d_5 was comprised of a signals attributable to a *ca* 47:53 mixture of **4** and **5**.

The ^{1}H NMR spectrum of **4** included five aryl protons at 6.76 ppm (H-6), 6.73 ppm (H-8), 8.38 ppm (H-2') (*d*, $J_{2',6'}$ = 2.1 Hz), 7.30 (H-5') (*dd,* $J_{5',6'}$ = 8.4 Hz), and 7.91 ppm (H-6') (*dd,* $J_{6',5'}$ = 8.4 Hz), a methoxyl (-OCH_3) proton at 3.93 ppm (s) and two anomeric glycosidic protons at 6.16 (*d,* $J_{1",2"}$ = 7.5 Hz) and 5.25 ppm (d, $J_{1"',2"'}$ = 1.2 Hz). Other proton NMR signals are listed in **Table 3.13**.

The ^{1}H NMR spectrum of flavonoid glycoside **5** included five protons at 6.78 ppm (H-6), 6.74 ppm (H-8), 8.61 ppm (H-2') (*d*, $J_{2',6'}$ = 2.1 Hz), 7.27 ppm (H-5'), (*dd,* $J_{5',6'}$ = 8.4 Hz), and 7.82 ppm (H-6') (*dd,* $J_{6',5'}$ = 8.4 Hz), a methoxyl (-OCH_3) proton at 4.03 (s), and two anomeric glycosidic protons at 6.22 ppm (*d,* $J_{1",2"}$ = 7.8 Hz) and 5.33 ppm (*d*, $J_{1"',2"'}$ = 1.3 Hz). Other proton NMR signals are listed in **Table 3.13**.

The genin portion of the ^{13}C NMR spectrum of **4** and **5** contained total of 16 signals including methoxyl group signals at 56.1 and 56.6 ppm (C-3' of **4** and **5** respectively), and conjugated keto signals at 178.4 and 178.5 ppm (C-4 of **4** and **5** respectively).

The glycosyl portion of the ^{13}C NMR spectrum of **4** and **5** contained twelve signals comprising ten oxygenated methine signals, one oxygenated methylene and a methyl group respectively.

3.8.1.2 COSY and TOCSY Spectra

Correlations observed in COSY and TOCSY spectra, along with coupling constant data were consistent with the presence of glucopyranosyl and rhamnopyranosyl units in **4** and galactopyranosyl and rhamnopyranosyl units in **5**, respectively. Starting with the signals at 6.16 ppm ($J_{1",2"}$ = 7.5 Hz, H-1"), **4** and 6.22 ppm ($J_{1",2"}$ = 7.8 Hz, H-1"), **5** the couplings were traced around the pyranosyl ring giving $J_{2",3"}$ = 9.3 Hz, $J_{3",4"}$ = 9.8 Hz and $J_{4",5"}$ = 9.4 Hz indicating a β-D-glucopyranosyl residue for **4** and $J_{2",3"}$ = 9.3 Hz, $J_{3",4"}$ = 3.3 Hz and $J_{4",5"}$ = 1.8 Hz indicating a β-D-galactopyranosyl residue for **5.** Similarly, starting with the signal at 5.25 ppm ($J_{1"',2"'}$ = 1.2 Hz, H-1"'), **4** and 5.33 ppm ($J_{1"',2"'}$ = 1.3 Hz, H-1"'), **5**, coupling constants were measured around the ring giving $J_{1"',2"'}$ = 1.2 Hz and 1.3 Hz for **4** and **5** accordingly. This pattern defined the presence of α-rhamnopyranosyl unit, which was assumed to be an α-L-rhamnopyranosyl unit, as commonly found in with other natural products.

The COSY and TOCSY spectra of **4** and **5** also included correlations between H-5' (7.30 and 7.27 ppm in **4** and **5** respectively) and H-6' (7.91 and 7.82 ppm in **4** and **5**

respectively), H-2' (8.38 and 8.61 ppm in **4** and **5** respectively) and H-6'(7.91 and 7.82 ppm in **4** and **5** respectively) and H-6 (6.76 and 6.78 ppm in **4** and **5** respectively) and H-8 (6.73 and 6.74 ppm in **4** and **5** respectively) (see **Figures 3. 23** and **3.24**).

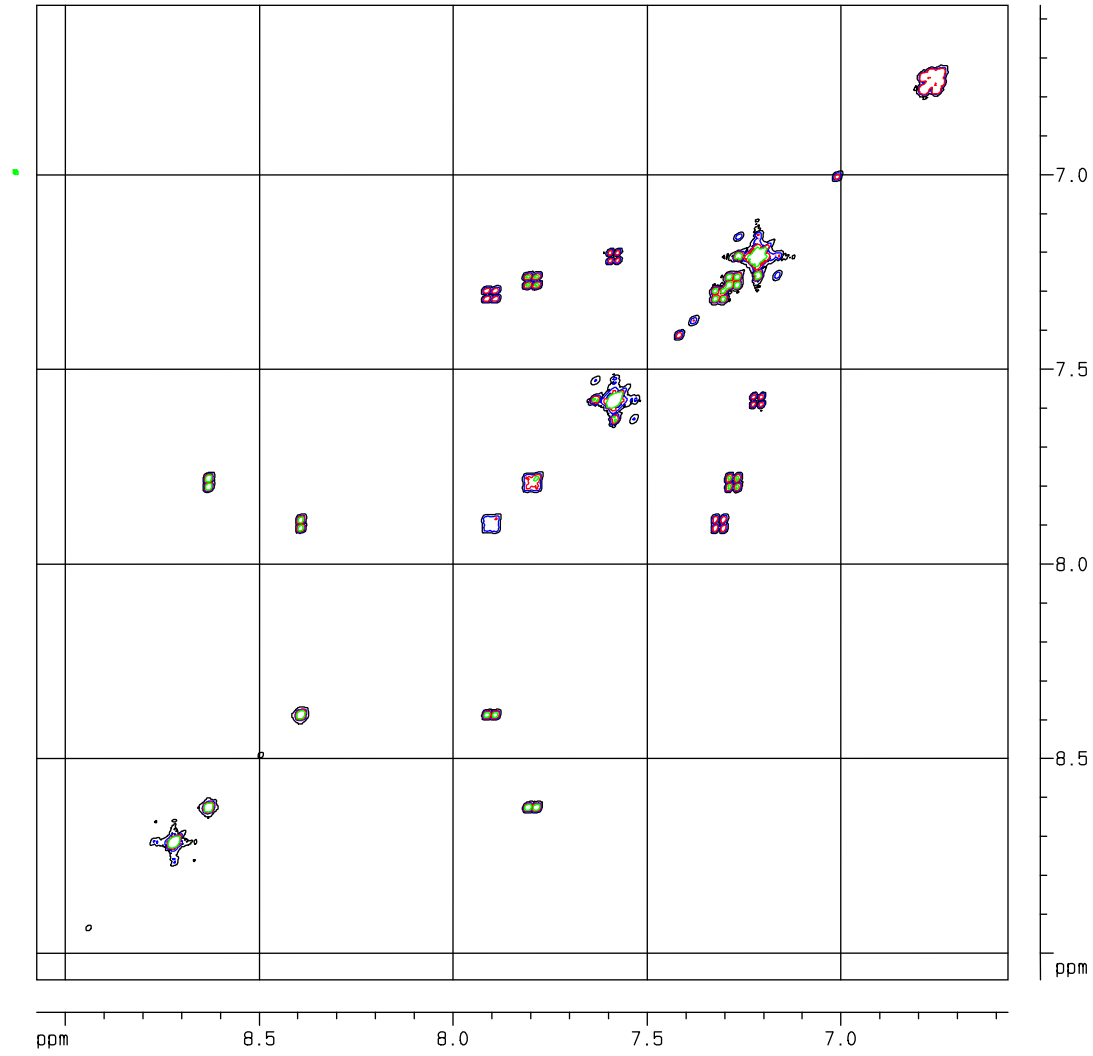


Figure 3.23 COSY spectrum of the aromatic region of **4** and **5**

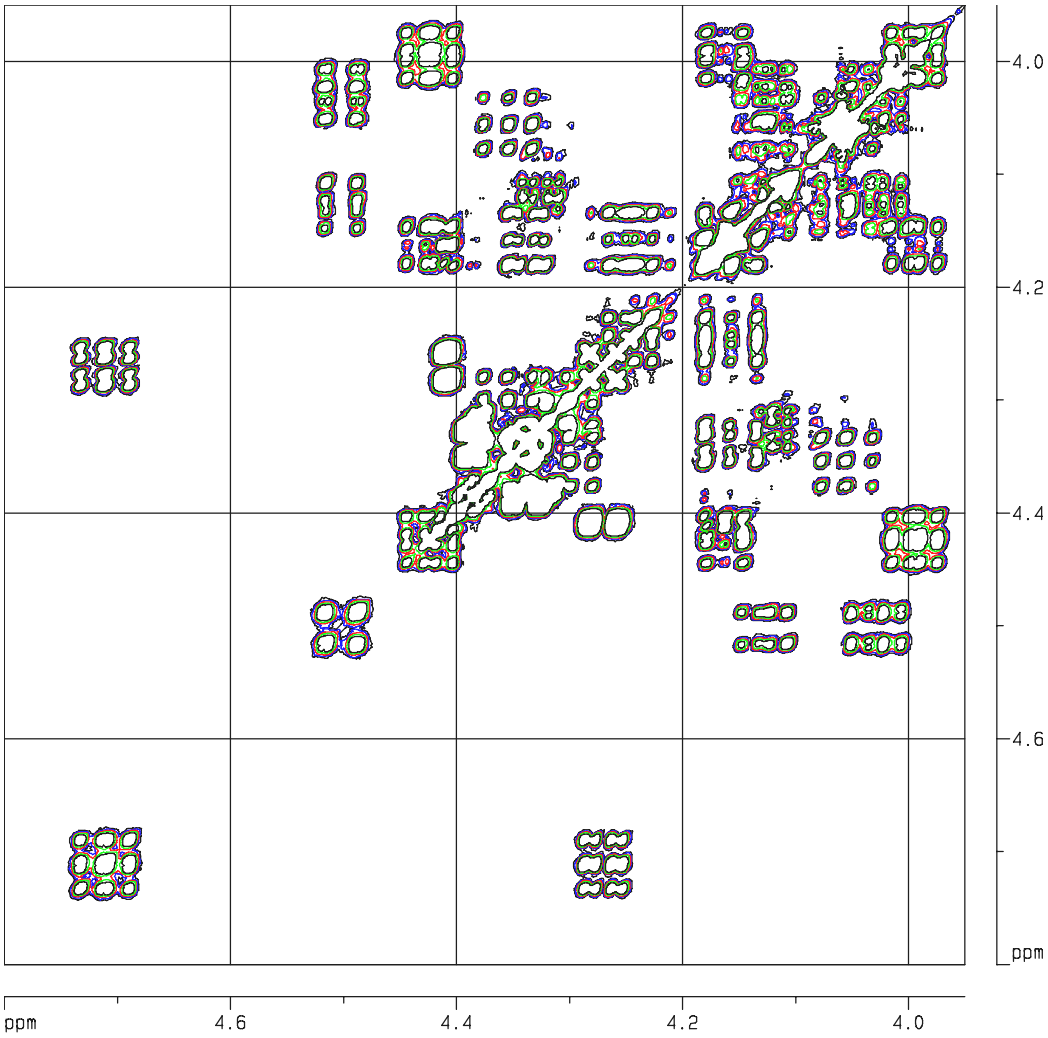


Figure 3.24 COSY spectrum of the glycosyl region of **4** and **5**

The TOCSY spectrum of the glycosidic region of **4** and **5** traced out the connectivity for the glycopyranosyl residues of **4** of **5**, and in the case of glucosyl residue included correlations between 6.16 ppm (H-1") to 4.50 ppm (H-6"), 4.30 ppm (H-2") 4.32 ppm (H-3"), 4.12 ppm (H-5") and 4.05 ppm (H-4") for **4** and in the case of galactosyl residue included correlations between 6.22 ppm (H-1") to 4.71 (H-2"), 4.40 ppm (H-4") and (H-3") for **5** (see **Figure 3.25).**

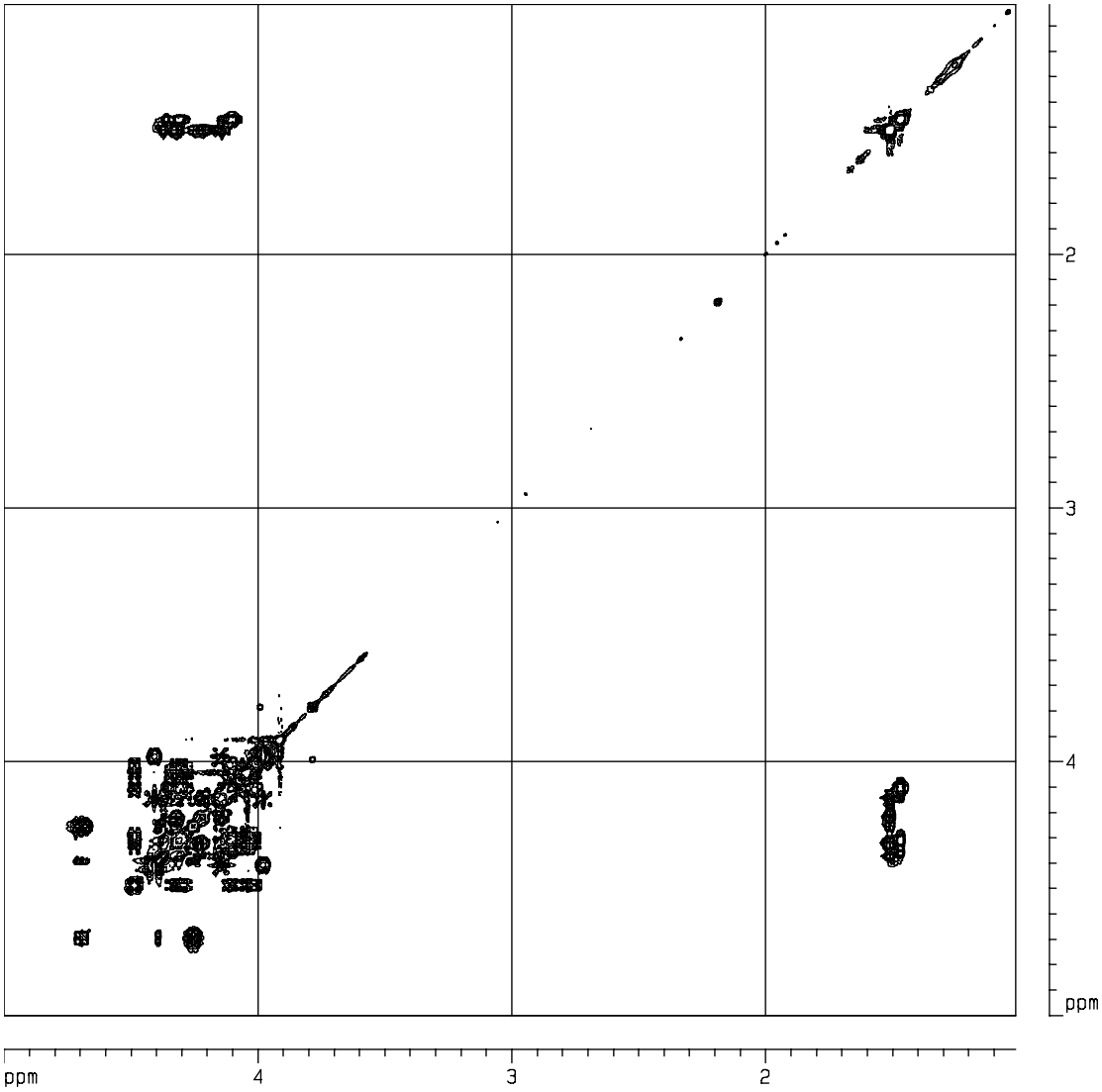


Figure 3.25 TOCSY spectrum of expanded region of **4** and **5**

3.8.1.3 g-HSQC and g-HMBC Spectra

The g-HSQC spectrum of the mixture of the two flavanoid glycosides showed correlations which linked all of the proton resonances with those of the corresponding carbons. For example, the carbon signals for **4** and **5**, which occurred at 104.3 and 104.4 ppm, (C-1" of **4** and **5** respectively), and 102.6 and 101.9 ppm (C-1"' of **4** and **5** respectively), showed g-HSQC correlations with ^{1}H doublets which occurred at 6.16 or 6.22 ppm ($J_{1",2"}$ = 7.5 Hz or 7.8 Hz of **4** and **5** respectively) and 5.25 or 5.33 ppm

($J_{1''',2'''}$ = 1.2 Hz or 1.3 Hz of **4** and **5** respectively), consistent with their identification as the anomeric carbons of two glycosyl units.

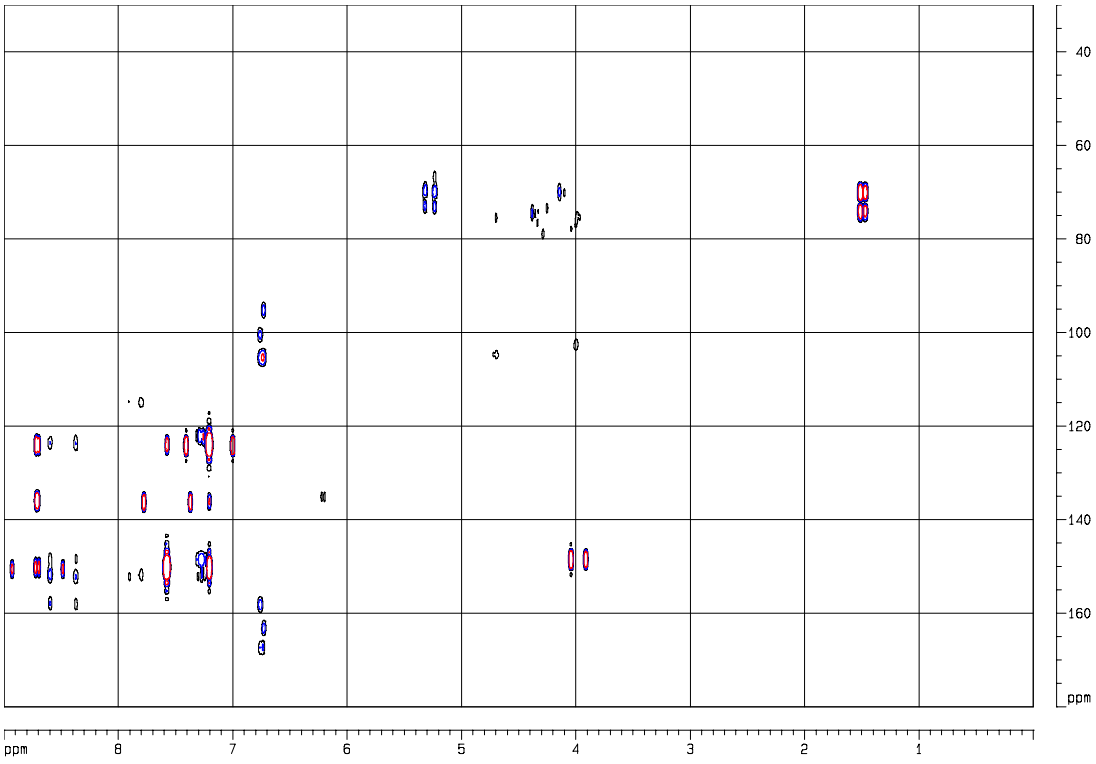


Figure 3.26 g-HMBC spectrum of the glycosyl region of the mixture of **4** and **5**

The aromatic proton signals which occurred a at 6.76 or 6.78 ppm (H-6 of **4** and **5** respectively) and 6.73 or 6.74 ppm (H-8 of **4** and **5** respectively), showed g HSQC correlations at 100.1 ppm (C-6 of **4** and **5**) and 94.9 or 94.8 ppm (C-8 of **4** and **5** respectively). Similarly the aromatic proton signals which occurred at 8.38 or 8.61 ppm (H-2' of **4** and **5** respectively) correlated to carbon signals which occurred at 114.4 or 114.6 ppm (C-2' of **4** and **5** respectively), the aromatic proton signals which occurred at 7.30 or 7.27 ppm (H-5' of **4** and **5** respectively) correlated to carbon signals which occurred at 116.4 or 116.2 ppm (C-5' of **4** and **5** respectively), the aromatic proton signals which occurred at 3.93 or 4.03 ppm (-OCH_3 of **4** and **5** respectively) correlated to carbon signals which occurred at 56.1 and 56.5 ppm (C-3' **4** and **5** respectively).

A g-HMBC correlation between H-1" signals of **4** and **5** (6.16 or 6.22 ppm of **4** and **5** respectively) and the C-3 flavonoid signals at 136.1 ppm in each of **4** and **5** indicated that the glucopyranosyl and galactopyranosyl residues were linked to the flavonoid skeleton at C-3.

Importantly, a g-HMBC correlation between the proton signals at 5.25 or 5.33 ppm (H-1'" of **4** and **5** respectively) and the ^{13}C signals at 68.3 or 66.6 ppm (C-6" of **4** and **5** respectively) indicated the presence of rhamnopyranosyl residues linked to C-6" in each of **4** and **5**.

These and other structurally significant HMBC correlations observed for **4** and **5** in **Table 3.14** and depicted graphically in **Figure 3.26.**

Table 3.14 g-HMBC correlations observed for selected atoms of **4** and **5**

δ ^{1}H signal	correlated signal(s) (^{2}J or ^{3}J)
8.38 or 8.61 ppm (H-2')	157.8 (C-2), 151.4 ppm (C-4'), 148.1 ppm (C-3'), 123.7 (C-1')
3.93 or 4.03 ppm (-OCH_3)	148.1ppm (C-3'),
6.73 and 6.74 ppm (H-8)	166.9 ppm (C-7), 162.7 or 162.3 ppm (C-9), 157.9 ppm (C-5), 104.9 ppm (C-10), 100.1 ppm (C-6)
6.76 or 6.78 ppm (H-6)	166.9 ppm (C-7), 157.9 ppm (C-5), 104.9 ppm (C-10), 94.9 or 94.8 ppm (C-8)

3.8.1.4 NOESY and ROESY Spectra

The location of the interglycosyl linkages of **4** and **5** were established using ROESY and NOESY experiments. The rhamnosyl H-1''' (5.25 or 5.33 ppm of **4** and **5** respectively) resonances showed strong correlations at 4.50 or 4.42 ppm (H-6'' of **4** and **5** respectively) indicating the rhamnosyl sugar residues of **4** and **5** to be attached to the glucosyl or galactosyl C-6'' atoms respectively.

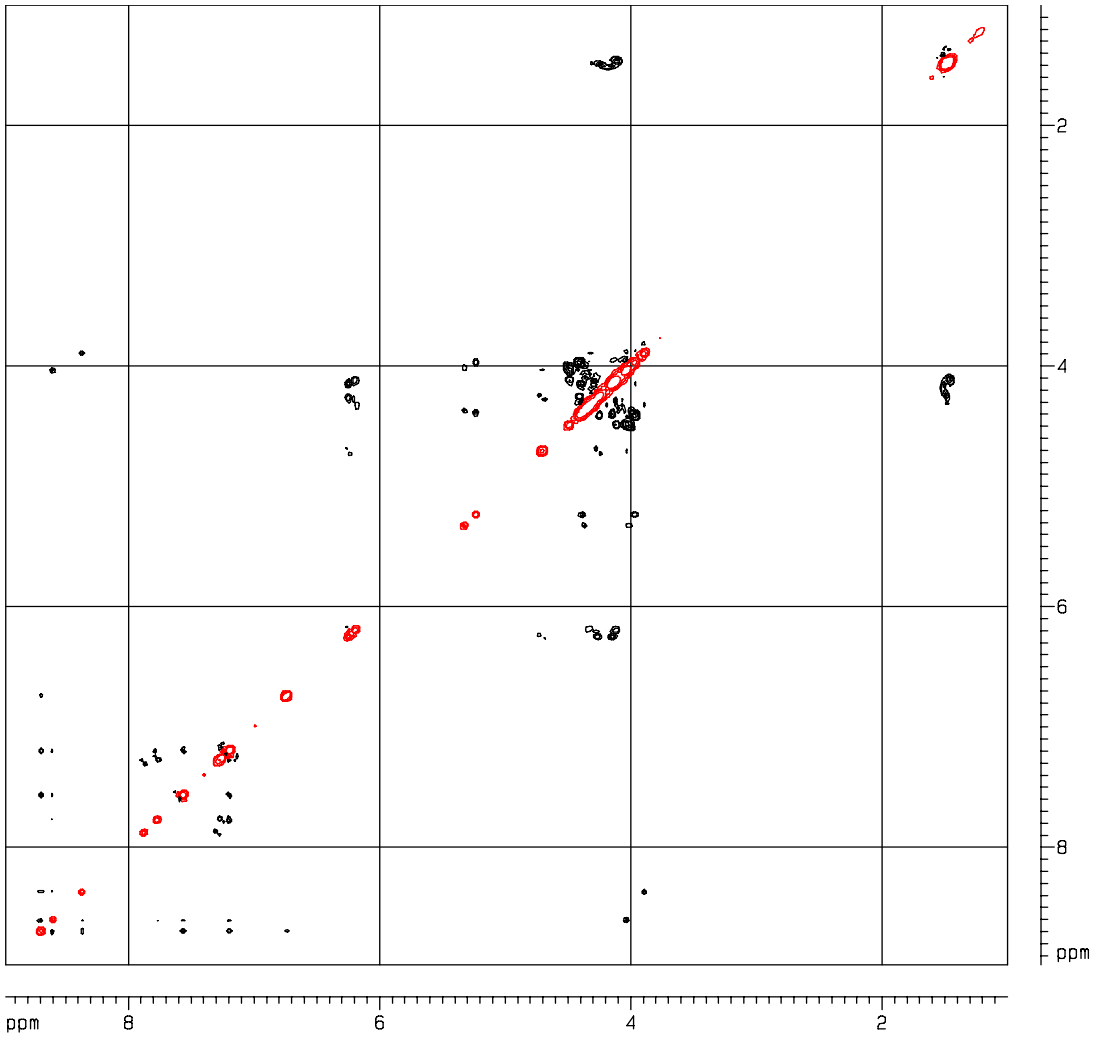


Figure 3.27 ROESY spectrum of **4** and **5**

Thus glycosides **4** and **5** were identified as isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside} [120,121,127,128], and isorhamnetin-3-*O*-{*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside} [120,127-129] respectively. Flavonoid glycosides **4** and **5** did not exhibit biological activity in the assays reported in Chapter Four.

3.9 Structure Elucidation of Compound 6

Compound **6** was isolated from the *n*-butanol extract of senescent leaves of *C. australis* and identified as β-sitosterol glucoside (**6**) (22,23-dihydrostigmast-5-en-3-*O*-β-D-glucoside).

3.9.1 NMR Spectra

The complete assignment of the ^{1}H and ^{13}C NMR signals of compound **6** presented in **Table 3.15** was established from detailed analyses of one and two dimensional NMR data (see following Sections).

Table 3.15 ^{1}H and ^{13}C NMR signal assignments (δ C_5D_5N) established for compound **6**

atom	^{13}C	^{1}H	atom	^{13}C	^{1}H
1	37.5	1.74, 1.00	22	34.2	
2	30.3	2.15, 1.75	23	26.4	1.26
3	78.6	3.92	24	46.0	1.00
4	39.4	2.76, 2.49	25	29.4	0.88
5	140.9		26	19.2	0.87, (*d*, *J* = 6.6.Hz)
6	122.0	5.40	27	20.0	0.89, (*d*, *J* = 7.0 Hz)
7	32.2	1.92, 1.55	28	23.4	1.32
8	32.1	1.39	29	12.2	0.91, (*d*, *J* = 7.5 Hz)
9	50.3	0.91			
10	36.9		1'	102.6	5.02 *d*, $J_{1',2'}$ = 7.8 Hz

11	21.3	1.48	2'	75.4	4.09 *dd,* $J_{2',3'}$ = 8.5 Hz
12	40.0	1.98, 1.12	3'	78.7	4.32 *t,* $J_{3',4'}$ = 9.2 Hz
13	42.5		4'	71.7	4.32 *t,* $J_{4',5'}$ = 9.1 Hz
14	56.8	0.96	5'	78.1	4.01 *t,*
15	24.5	0.92	6'	62.8	4.59, 4.45 *dd*
16	28.6	1.09			
17	56.2	1.12			
18	12.0	0.67, (*s*)			
19	19.4	0.95 (*s*)			
20	36.4				
21	19.0	1.00 (*d*, *J* = 6.5 Hz)			

3.9.1.1 ^{1}H and ^{13}C NMR Spectra

The ^{1}H NMR spectrum of compound **6** in C_5D_5N included six methyl group protons at 0.67 (C-18) (s), 0.95 (C-19) (s) 1.00, (C-21) (d, *J* = 6.5 Hz), 0.87 (C-26) (d, *J* = 6.6 Hz), 0.89 (C-27) (d, *J* = 7.0 Hz) and 0.91 ppm (C-29) (d, *J* = 7.5 Hz) and an anomeric glycosidic proton at 5.02 ppm (d, *J* = 7.8 Hz).

The genin portion of the ^{13}C NMR spectrum of **6** contained 29 signals including six methyl groups, eleven methylene groups, eight non oxygenated, one oxygenated methine group and three non oxygenated quaternary carbons. These data indicated the genin to be monohydroxylated.

The ^{13}C NMR chemical shifts of C-5, C-6, C-7, C-9 and C-19, (140.9, 122.0, 32.2, 50.3 and 19.4 ppm respectively) were consistent with the presence of a Δ5-steroidal Skeleton [71,78], while the chemical shift of the glycosylated carbon (C-3, 78.6 ppm) corresponded closely to that reported by Faizi *et al* [122] for the C-3 carbon of β-sitosterol glucoside (78.34 ppm).

The glycosyl portion of the ^{13}C NMR spectrum of compound **6** consisted of six signals and was comprised of four oxygenated methine signals in the region 70-80

ppm, an oxygenated methylene signal at 62.8 ppm and an anomeric methine signal at 102.6 ppm. Coupling constant and COSY and TOCSY spectral data (**Figure 3.28** and **Figure 3.29**) were consistent with the presence of a glucosyl unit.

3.9.1.2 COSY and TOCSY Spectra

Starting with the signal at 5.02 ppm (H-1') couplings were traced around the pyranosyl ring giving $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 8.5 Hz, $J_{3',4'}$ = 9.2 Hz and $J_{4',5'}$ = 9.1 Hz. This data indicated a β-D-glucopyranosyl residue [122,130].

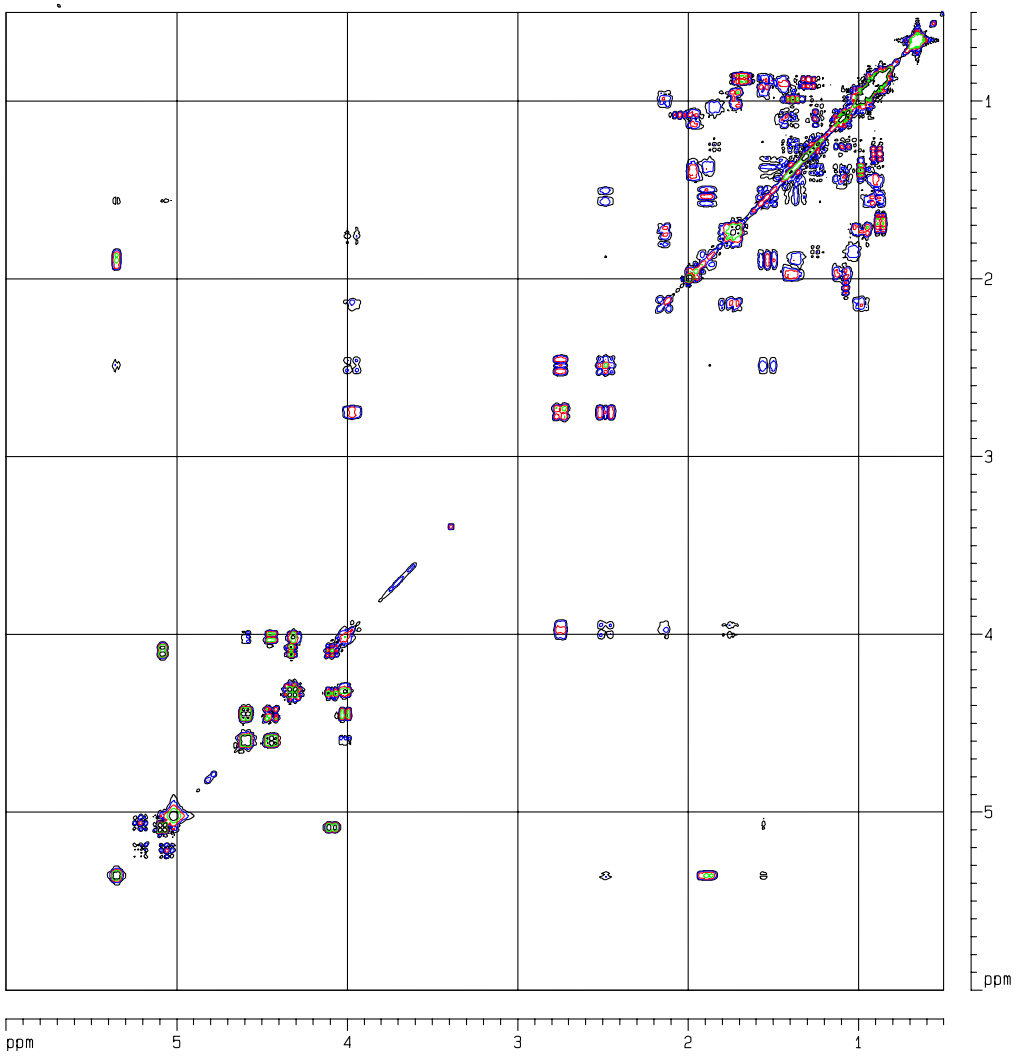


Figure 3.28 COSY spectrum of compound **6**

TOCSY correlations observed for glucosyl protons of compound **6** demonstrated connectivity between 5.02 ppm (H-1') and 4.59 (H-6'β), 4.45 (H-6'α), 4.32 (H-3', H-4'), 4.09 (H-2'), and 4.01 ppm (H-5).

The TOCSY spectra of compound **6** also showed correlations between the genin proton at 3.92 ppm (H-3) and protons which occurred at 2.76 (H-4β), 2.49 (H-4α), 2.15 (H-2β) 1.75 (H-2α), 1.74 (H-1β) and 1.00 (H-1α). Correlations were also observed between 5.40 ppm (H-6) and 2.76 (H-4β), 2.49 (H-4α), 1.92 (H-7β), 1.55 (H-7α), 1.39 (H-8), 1.12 (H-12α), 0.96 (H-14) and 0.91 (H-9) respectively.

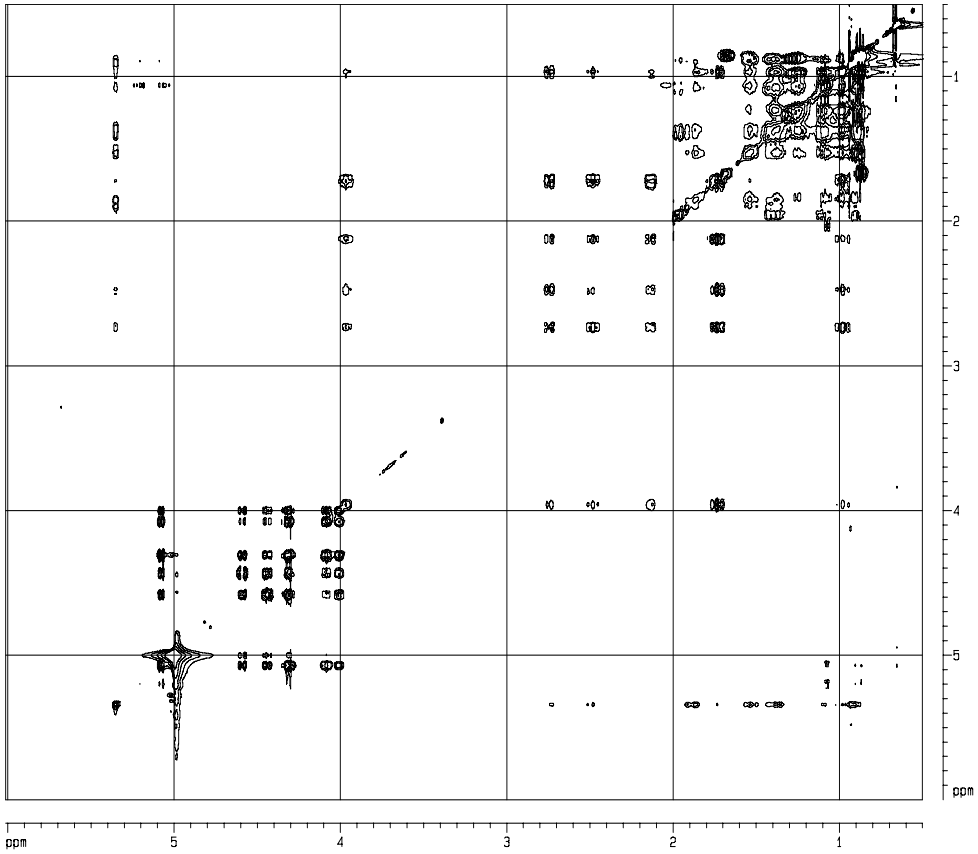


Figure 3.29 TOCSY spectrum of expanded region of compound **6**

3.9.1.3 g-HSQC and g-HMBC Spectra

The six angular methyl group protons of compound **6** which occurred as a singletsat 0.67 and 0.95 ppm, or as doublets at 1.00, 0.87, 0.89 and 0.91 ppm (J = 6.5 Hz , J = 6.6 Hz, J = 7.0 and J = 7.5 Hz respectively), showed g-HSQC correlations to carbon signals which occurred at 12.0, 19.4 ppm and 19.0, 19.2, 20.0 and 12.2 ppm respectively. These and other HSQC correlations observed for compound **6** are shown in **Figure 3.30**.

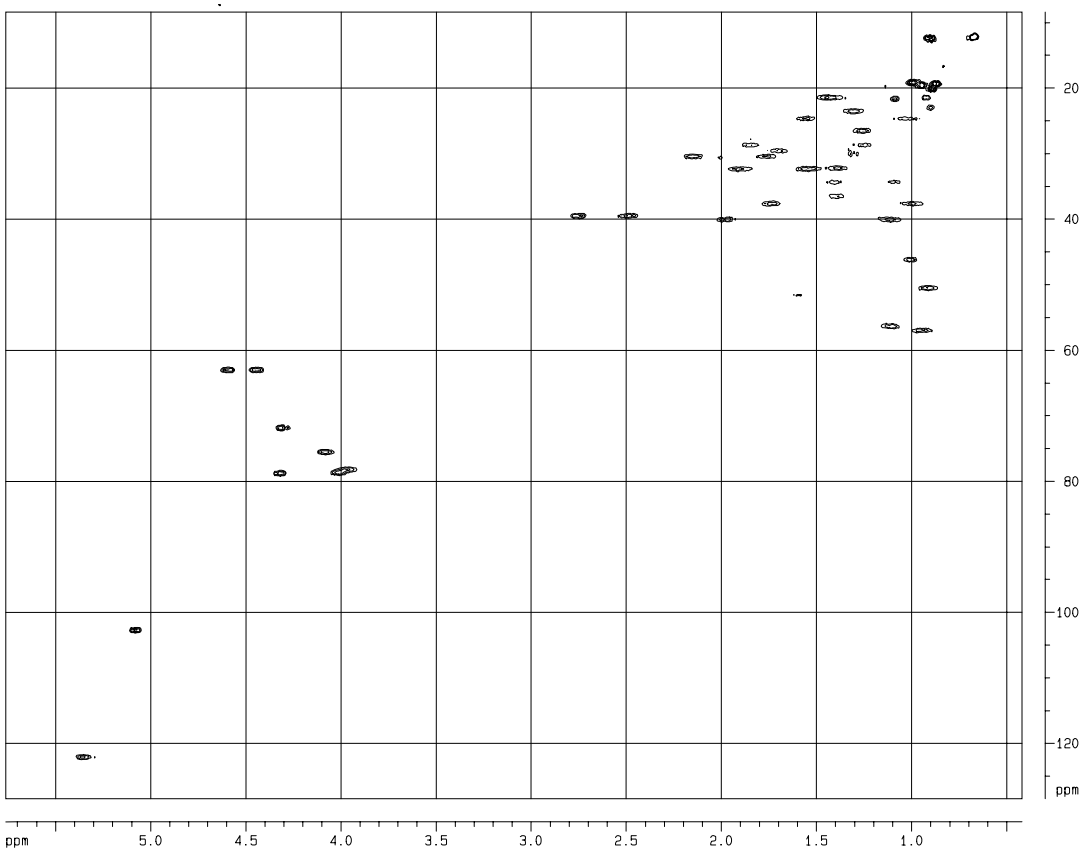


Figure 3.30 g-HSQC spectrum of compound **6**

The g-HMBC spectrum of **6** included a correlation between H-1' (5.02 ppm) and C-3 (78.6 ppm), which indicated that the glucopyranosyl residue was linked to the aglycone at C-3. Some other structurally significant HMBC correlations are listed in **3.16** and depicted graphically in **Figure 3.31.**

Table 3.16 g-HMBC correlations observed for selected atoms of compound **6**

δ ^{1}H signal	correlated signal(s) (^{2}J or ^{3}J)
0.67 (H-18)	42.5 (C-13), 40.0 (C-12), 56.2 (C-17)
0.95 (H-19)	36.9 (C-10), 37.5 (C-1), 140.9 (C-5), 50.3 (C-9).
0.87 (H-26), 0.89 (H-27)	29.4 (C25)
0.91 (H-29)	23.4 (C-28), 46.0 (C-24)

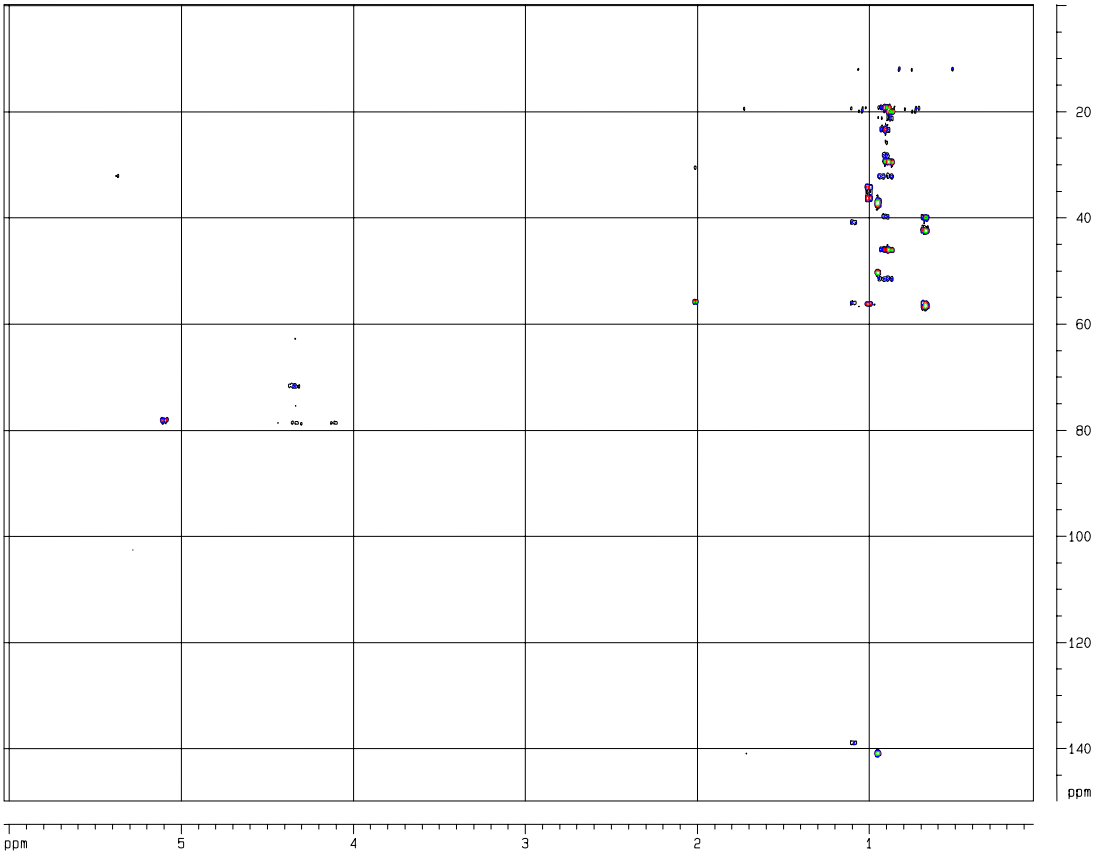


Figure 3.31 g-HMBC spectra of compound **6**

3.9.1.4 NOESY and ROESY Spectra

NOESY and ROESY data verified that the C-3 glucosyl group was equatorial (β-) oriented since H-3α (3.92 ppm), H-1α (1.00 ppm), H-2α (1.75 ppm) and H-4α (2.49 ppm) exhibited mutual correlations in separate NOESY and ROESY experiments.

The glucosyl H-1' (5.02 ppm) signal showed a strong NOESY and ROESY correlation at 3.92 ppm (H-3) indicating that glucopyranosyl residue to be attached to the genin at C-3 atom.

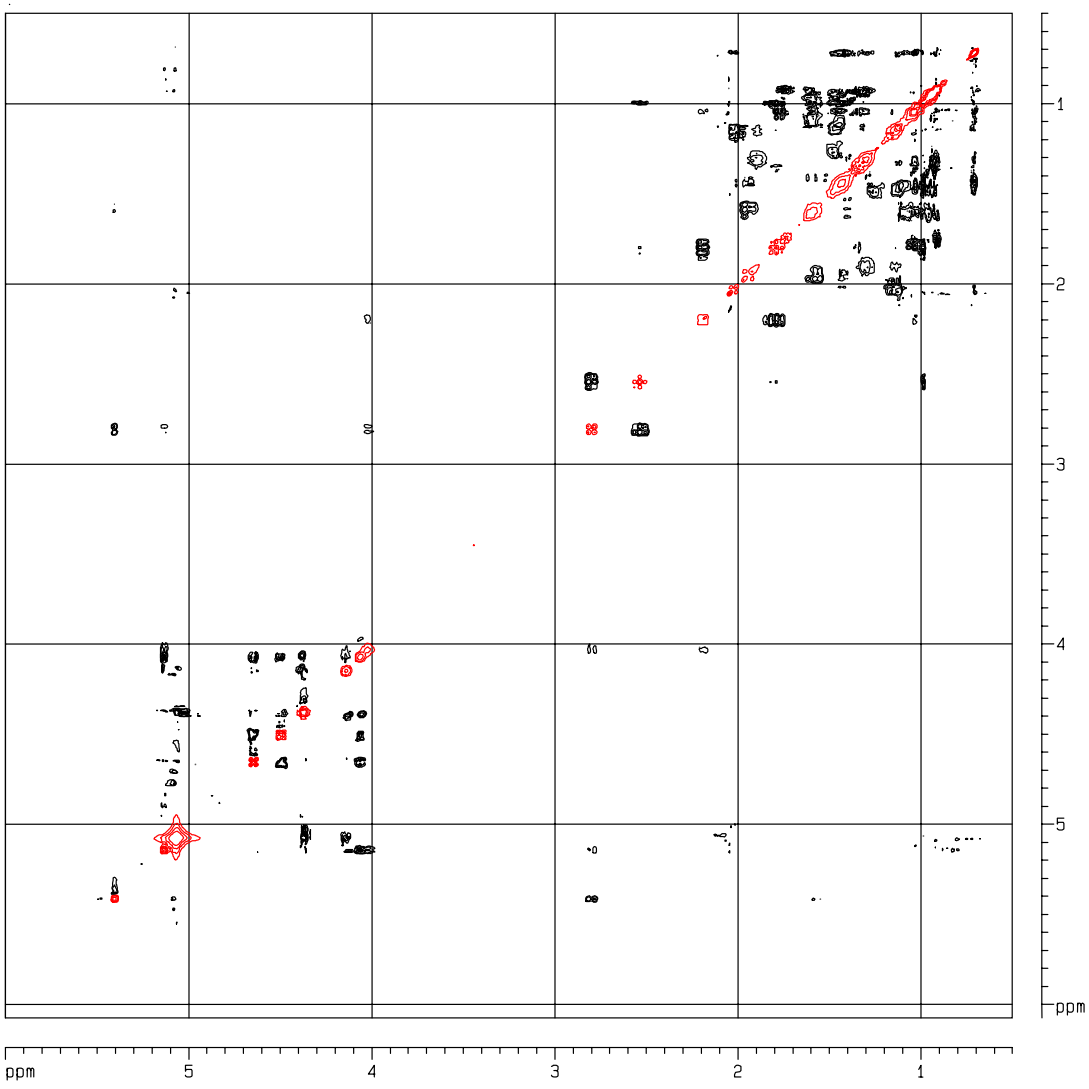


Figure 3.32 ROESY spectrum of the glucosyl region of compound **6**

Thus compound **6** was identified as a β-sitosterol glucoside or 22,23-dihydroxystigmast-5-en-3-*O*-β-D-glucopyranoside. The NMR assignments presented in **Table 3.15** for **6** correspond closely to those reported by Faizi *et al* [122] for this compound which they incorrectly named as stigma-5-en-3-*O*-β-D-glucopyranoside.

3.10 Other Saponins

Two diosgenin saponins were also detected in the methanol extract of senescent leaves of *C. australis*.

3.10.1 Prosaponin A of Dioscin

One of the diosgenin saponins was detected as a minor component of some of the fractions (see Section **2.9**) which afforded saponin **1**. The NMR spectra of these fractions were consistent with their being *ca* 3:1 mixtures of saponin **1** and diosgenin 3-*O*{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glycopyranoside}, also known in the literature as prosaponin A of dioscin [123].

The ^{13}C NMR spectrum of fractions which contained prosapogenin A of dioscin included signals at 140.7, 12l.6, 109.4 and 100.1 ppm attributable to the Δ5-sapogenin structure and the anomeric glucosyl and rhamonosyl carbons respectively. These signals corresponded closely to those reported by for this compound [71,78].

3.10.2 Desrhamnoprosapogenin A of Dioscin (Trillin)

Some fractions from the methanol extraction of senescent leaves of *C. australis* (see Section **2.9**) afforded a saponin which was tentatively identified as desrhamnoprosapogenin A dioscin, or diosgenin 3-*O*-β-D-glucopyranoside, also known in the literature as trillin [71,78,123].

CHAPTER FOUR

Biological Activity of Saponins and Extracts from C. australis

4.1 Biological Activity of Steroidal and Flavonoid Glycosides

Anticancer, antitumour, antiinflamatory, antiviral, antifungal and molluscicidal activities have been variously reported for steroidal [3,15,17-22,25-27,29,30,32,38,40-44,47,48,50,51,53,123,131-137] and flavonoid [120,138] glycosides derived from a variety of plants other than *Cordyline spp*.

4.1.1 Biological Activity of Steroidal Glycosides of Genus *Cordyline*

Traditional medicine often provides clues to biological activity in native plants [14]. The Maori used an infusion of leaves of *Cordyline spp* as a remedy against dysentery, diarrhea and cuts [86,87,100]. Softened leaves were applied as an ointment onto cuts, cracks in the skin and sores [86,87,100]. The young inner shoot and top of the stem were boiled and eaten by nursing mothers and were also given to children for colic [86,87,103]. Malays treated dysentery with a decoction of *C. fruticosa* and the fern *Ligodium spp* [87]. *C. fruticosa* has been used in New Caledonia for a variety of ailments of the mouth and stomach [87].

Nursing mothers have used *C. terminalis* in Tubuai and Tahiti for diarrhea, in Tahiti for abscesses, in Samoa for skin disease and in Hawaii for headaches, in the Philippines for diarrhea, dysentery and for heart ailment [87,98,100,139]. Extracts of *C. stricta* leaves have been used for their haemostatic property in traditional Chinese medicine [98].

C. dracenoides (*Kunth*) is used in traditional medicine in the South of Brazil as an anti-inflammatory preparation for the treatment of rheumatoid and related diseases [140]. Results obtained from pharmacology experiments carried out in Brazil suggested that a crude extract from *C. dracenoides* exhibits a potent and long-lasting antioedematogenic effect and has central nervous system depressant effects. The authors pointed out that this effect may be related to the presence of steroidal saponins in this plant [140] .

A nonpolar compound from *C. terminalis* (Ti or Ki), which is a traditional Polynesian medicinal plant, showed antibacterial activity against certain American Type Culture Collection (*ATCC*) bacteria using the Kirby-Bauer method [141].

4.2 Biological Activity of Crude Saponin Extracts and Isolated Substances from *C. australis*

4.2.1 Antimicrobial, Antivirus, Antitumour and Cytotoxicity Assay of Extracts from *C. australis*

Nine samples of crude plant extract were tested for antimicrobial, antiviral, cytotoxicity and antitumour activity. Samples included the butanol-soluble fraction of a methanol extract of dry leaves, dry stems, fresh leaves, dry roots and senescent leaves (Extract 1, Extract 2, Extract 3 Extract 4, Extract 5) and the water-soluble fraction remaining after butanol extraction of the methanol extract of dry leaves, dry stems, fresh leaves, dry roots (Extract 1', Extract 2', Extract 3' Extract 4'), eleven samples of isolated substances, saponins **1** (7 samples), **2, 3, 9** and flavonoid glycosides **4** and **5** (as a mixture). Of these only Extracts 1-5 and saponin **1**, **3**, and **9** showed biological activity. Extracts 1-5, saponin **1** and **3** showed antifungal activity against *T. mentagrophytes*, the most sensitive organism, **Table 4.1.** Saponin **9** showed moderate cytotoxicity against *Herpes simplex type 1 virus* (ATCC VR7330) and *Polio Virus Type 1* (Pfizer vaccine strain).

Table 4.1 Antifungal activity against *T. mentagrophytes* and saponin content for bioactive samples from *C. australis*

Sample	Dose (μg)	Zone of inhibition (mm)	Compound isolated	GCMS results for conj.sapogenin types
Extract 1	600	5	**1**	Tigogenin (*R*, *S*, $\Delta^{25(27)}$), diosgenin
Extract 2	600	7	**9**	Not studied
Extract 3	600	8	-	Tigogenin (*R*, *S*, $\Delta^{25(27)}$)
Extract 4	600	5	-	Tigogenin (*R*, *S*, $\Delta^{25(27)}$), diosgenin
Extract 5	200	3	**1, 3, 6**	Tigogenin (R, *S*, $\Delta^{25(27)}$)
1	2.3*	2	**1**	
3	200	1	**3**	

*Minimum inhibitory doze

The results presented in **Table 4.1** showed that Extracts 1-5 have antifungal activity and Extracts 2 and 3 showed the strongest activity against *T. mentagrophytes*. While GC MS showed significant levels of conjugated sapogenins in the methanol extract of fresh, dried and senescent leaves, only saponin **1** has been isolated from the dry leaves and saponins **1** and **3** from the senescent leaves of the butanol-soluble fractions (Extract 1 and Extract 5) of the methanol extraction of *C. australis*. The results in **Table 4.1** indicate that saponin **1,** with the minimum inhibitory dose 2.3 μg is probably responsible for the activities presented for Extracts 1 and 5. Saponin **9** with the moderate cytotoxicity activity did not show any antifungal activity. Unfortunately, time did not permit further study of Extracts 1-5 for isolation and characterisation of saponins. Due to time constraint, only Extracts 1, 2 and 5 were investigated more closely.

4.2.2 Anti-Protozoal and Cytotoxicity Screening of some Extracts from *C. australis*

One sample of crude saponin extract, the butanol-soluble fraction of a methanol extract of senescent leaves of *C. australis* (Extract 5), and two isolated substances, saponin **1** and flavonoid glycosides **4** and **5** (as a mixture) were tested for antiprotozoal and cytotoxicity activity at the Tibotec Pharmaceutical Research and Development Company in Geneva Switzerland, **Table 4.2**.

The results outlined in **Table 4.2** are given in micromolar concentrations producing 50% inhibition (IC_{50}) in the assays used.

Table 4.2 Anti-protozoal and cytotoxicity screening assay results of some samples from *C. australis* (Tibotec)

Code	Results of activity	IC_{50} (μM)			
		P. falciparum[a]	*T. brucei*[b]	*T. cruzi*[c]	*MRC5* cell
Extract 5	weak	12	23.479	>32	16.241
1	aspecific	10	5.012	3.896	3.283
4 and **5**	ND	>32	>32	>32	>32

Results from the same assays for standard compounds used:

[a] Chloroquine:0.018

[b] Suramin:0.14

[c] Nifurtimox:0.66

The results presented in **Table 4.2** showed that saponin **1** has aspecific activity, that is it affected the protozoa, but this is likely to be a result of its cytotoxicity. The cytotoxicity shown here against *MRC5* cell was not duplicated against BSC-1 cells (African Green Monkey kidney),infected with *Herpes simplex type 1 virus (ATCC VR 733)* or *Polio virus type 1* (Pfiser vaccine strain) or *P388* (Murine Leukemia) as in the other assay (see Section **2.1.8)**. Extract 5 was very weakly antiprotozoal also weakly cytotoxic and the mixture of **4** and **5** was inactive.

The results show that saponin **1** has strong antifungal activity against *T. mentagrophytes* and also has some aspecific activity and cytotoxicity against *MRC5* cell.

The results indicate saponin **1** would be worth further investigation and development. Saponin **9** also warrants further investigation and studies as do the extracts of fresh leaves, dry stems and dry root.

References

1. Hostettmann, K.; Hostettmann, M.; Marston, A. Saponins. *Methods in Plant Biochemistry* **1991**, *7*, 435-471.

2. Hostettmann, K.; Marston, A. *Saponins*; Chemistry and pharmacology of natural products; Cambridge University Press: Cambridge, **1995**.

3. Hostettmann, K.; Wolfender, J.-L. The search for biologically active secondary metabolites. *Pesticide Science* **1997**, *51*, 471-482.

4. Kofler, L. *Die saponine*; Julius Springer Verlag: Vienna, 1972.

5. Oakenfull, D.; Sidhu, S. Saponins. In *Toxicants of plant origin*; P. R. Cheebo, Ed.; CRC Press: Boca Ratton, Florida, **1986**; pp 98-143.

6. Vasilyeva, I. S.; Paseshnichenko, V. A. Steroid glycosides of the plnats and the cell cultures of *Dioscorea* species: Metabolism and biological activity. *Uspekhi Biologicheskoi Khimii* **2000**, *40*, 153-204.

7. Wall, M. E.; Davis, K. H. Chemistry, pharmacology, and toxicology of steroidal glycosides. In *Toxins Anim.Plant Origin: Proc. 2nd Int. Symp. Anim. Plant Toxins,* ; A. De Vries and E. Kochva, Eds.; Gordon and Breach: New York, **1972**; pp 597-624.

8. Kamernitskii, A. V.; Abubakirov, N. K.; Gorovits, M. B.; Vollerner, Y. E.; Voishvillo, N. E.; Reshetova, I. G.; Paseshnichenko, V. A. *Chemistry of Spirostanols*; Nauka: Moscow, USSR, **1986**.

9. Hostettmann, K.; Marston, A. Twenty years of research into medicinal plants: Results and perspectives. *Phytochemistry Reviews* **2002**, *1*, 275-285.

10. Kasai, R.; Yamasaki, K.; Tanaka, O. Saponins. In *Naturally occuring glycosides*; R. Ikan, Ed.; John Wiley: Chichester, New York, **1999**; pp 295-310.

11. Kren, V.; Martinkova, L. Glycosides in medicine: "The role of glycosidic residue in biological activity". *Current Medicinal Chemistry* **2001**, *8*, 1303-1328.

12. Wagle, A.; Kelkar, G. D.; Heble, M. R. Production of steroids and saponins. In *Biotechnology: Secondary metabolites*; K. G. Ramawat and J. M. Merillon, Eds.; Science Publishers: Enfield, N.H., **1999**; pp 219-239.

13. Gupta, S. K.; Zafar, R.; Pathak, D. Review on phytochemical and pharmacological aspects of *Tribulus terrestris Linn*. *Indian Drugs* **1997**, *34*, 422-426.

14. Hostettmann, K.; Marston, A.; Maillard, M.; Hamburger, M. *Phytochemistry of plants used in traditional medicine*; Clarendon Press: Oxford, **1995**.

15. Ikeda, T.; Tsumagari, H.; Honbu, T.; Nohara, T. Cytotoxic activity of steroidal glycosides from solanum plants. *Biological & Pharmaceutical Bulletin* **2003**, *26*, 1198-1201.

16. Kijima, T.; Takasaki, M.; Tokuta, H.; Nishino, H.; Duc, N. M.; Kasai, R.; Yamazaki, K. Anticancer agents containing triterpenoid saponin of *Panax vietnamensis*. Japanese patent 2001039998, **2001**.

17. Kintia, P. K.; Lazurevskii, G. V.; Balashova, N. N.; Baleshova, I. T.; Surushiu, A. L.; Lyakh, V. A. *The structure and biological activity of steroid glycosides of the spirostane and furostane series*; Shtiintsa: Kishinev, USSR, **1987**.

18. Lacaille-Dubois, M. A.; Wagner, H. A review of the biological and pharmacological activities of saponins. *Phytomedicine* **1996**, *2*, 363-386.

19. Lacaille-Dubois, M. A.; Wagner, H. Bioactive saponins from plants: an update. *Studies in Natural Products Chemistry* **2000**, *21*, 633-687.

20. Lazurevskii, G. V.; Kintya, P. K.; Kovalchuk, L. P.; Burtseva, S. A.; Mashchenko, N. E.; Bobeiko, V. A. Antimicrobial activity of steroid glycosides in connection with their chemical structure. *Doklady Akademii Nauk SSSR* **1980**, *254*, 1479-1482

21. Mimaki, Y. Structure and physiological activity of saponin. The strongest antitumor substance from Liliaceae. *Farumashia* **1996**, *32*, 1208-1213.

22. Mimaki, Y.; Yokosuka, A.; Kuroda, M.; Sashida, Y. Cytotoxic activities and structure-cytotoxic relationships of steroidal saponins. *Biological & Pharmaceutical Bulletin* **2001**, *24*, 1286-1289.

23. Okanishi, T.; Akahori, A.; Yasuda, F.; Takeuchi, Y.; Iwao, T. Steroidal sapogenins of sixteen Liliaceae plants. *Chemical &Pharmaceutical Buletin* **1975**, *23*575-579.

24. Patel, A. V.; Blunden, G.; Crabb, T. A.; Sauvaire, Y.; Baccou, Y. C. A review of naturally-occurring steroidal sapogenins. *Fitoterapia* **1987**, *58*, 67-107.

25. Spinu, K. I.; Kintia, P. K.; Grushko, T. P.; Vorozhbit, B. U.; Bologa, V. F.; Skofertsa, P. G.; Vutkarev, V. P. Antiviral activity of steroidal glycosides. *Book of Abstracts, 210th ACS National Meeting, Chicago, IL, August 20-24* **1995**, AGFD-149.

26. Dimoglo, A. S.; Choban, I. N.; Bersuker, I. B.; Kintya, P. K.; Balashova, N. N. Structure-activity correlations for the antioxidant and antifungal properties of steroid glycosides. *Bioorganicheskaya Khimiya* **1985**, *11*, 408-413.

27. Liu, M.; Yu, B.; Guo, Z.; Li, C.; Hui, Y. Syntheses and antitumor activities of diosgenyl glycosides. *Carbohydrate Letters* **1997**, *2*, 423-428.

28. Malinow, M. R. Tigogenin glycosides for treatment of hypercholesterolemia. Patent number US 4602003, **1986**.

29. Nakamura, T.; Komori, C.; Lee, Y.; Hashimoto, F.; Yahara, S.; Nohara, T.; Ejima, A. Cytotoxic activities of *Solanum* steroidal glycosides. *Biological & Pharmaceutical Bulletin* **1996**, *19*, 564-566.

30. Sof'ina, Z. P.; Pukhal'skaya, E. C.; Romanova, I. N. New directions in the search for antitumor drugs. Steroidal glycosides. *Tselenapravlennyi Poisk Nov. Protivorak. Protivovirusn. Prep.* **1978**, 79-89.

31. Uniyal, G. C.; Agrawal, P. K.; Sati, O. P.; Thakur, R. S. Studies on Indian medicinal plants. Part 34. Agaveside C, a steroidal glycoside from *Agave cantala*. *Phytochemistry* **1991**, *30*, 1336-1339.

32. Yao, X.; Hu, K.; Dong, A. Steroid glycoside compounds for treating cancer and preparation method thereof. Patent number CN 1243129, **2000**.

33. Marker, R. E.; Wagner, R. B.; Ulshafer, P. R.; Wittbecker, E. L.; Goldsmith, D. P. J.; Ruof, C. H. Sterols. CLVII. Sapogenins. 69. Isolation and structures of thirteen new steroidal sapogenins. New sources for known sapogenins. *Journal of the American Chemical Society* **1943**, *65*, 1199-1209.

34. Wall, M. E.; Eddy, C. R.; Willaman, J. J.; Correll, D. S.; Schubert, B. G.; Gentry, H. S. Steroidal sapogenins. XII. Survey of plants for steroidal sapogenins and other constituents. *Journal of the American Pharmaceutical Association* **1954**, *43*, 503-505.

35. Madaeva, O. S. Steroid sapogenins as raw materials for hormone synthesis. *Meditsinskaya Promyshlennost SSSR* **1961**, *15*, 35-38.

36. Wall, M. E. Steroid sapogenins and derived steroid hormones. *American Perfumer and Aromatics* **1960**, *75*, 63-73.

37. Itabashi, M.; Segawa, K.; Ikeda, Y.; Kondo, S.; Naganawa, H.; Koyano, T.; Umezawa, K. A new bioactive steroidal saponin, furcreastatin, from the plant *Furcraea foetida*. *Carbohydrate Research* **2000**, *323*, 57-62.

38. Mimaki, Y.; Kuroda, M.; Fukasawa, T.; Sashida, Y. Steroidal saponins from the bulbs of *Allium karataviense*. *Chemical & Pharmaceutical Bulletin* **1999**, *47*, 738-743.

39. Mimaki, Y.; Kuroda, M.; Kameyama, A.; Yokosuka, A.; Sashida, Y. Aculeoside B, a new bisdesmosidic spirostanol saponin from the underground parts of *Ruscus aculeatus*. *Journal of Natural Products* **1998**, *61*, 1279-1282.

40. Mimaki, Y.; Kuroda, M.; Kameyama, A.; Yokosuka, A.; Sashida, Y. New steroidal constituents of the underground parts of *Ruscus aculeatus* and their cytostatic activity on HL-60 cells. *Chemical & Pharmaceutical Bulletin* **1998**, *46*, 298-303.

41. Mimaki, Y.; Kuroda, M.; Obata, Y.; Sashida, Y.; Kitahara, M.; Yasuda, A.; Naoi, N.; Xu, Z. W.; Li, M. R.; Lao, A. N. Steroidal saponins from the rhizomes of *Paris polyphylla* var. *chinensis* and their cytotoxic activity on HL-60 cells. *Natural Product Letters* **2000**, *14*, 357-364.

42. Mimaki, Y.; Nakamura, O.; Sashida, Y.; Nikaido, T.; Ohmoto, T. Steroidal saponins from the bulbs of *Triteleia lactea* and their inhibitory activity on cyclic AMP phosphodiesterase. *Phytochemistry* **1995**, *38*, 1279-1286.

43. Mimaki, Y.; Nikaido, T.; Matsumoto, K.; Sashida, Y.; Ohmoto, T. New steroidal saponins from the bulbs of *Allium giganteum* exhibiting potent inhibition of cAMP phosphodiesterase activity. *Chemical & Pharmaceutical Bulletin* **1994**, *42*, 710-714.

44. Bersuker, I. B.; Dimoglo, A. S.; Choban, I. N.; Lazur'evskii, G. V.; Kintya, P. K. Structure-antitumor activity relations in a series of steroidal glycosides. *Khimiko-Farmatsevticheskii Zhurnal* **1983**, *17*, 1467-1471.

45. Tran, Q. L.; Tezuka, Y.; Banskota, A. H.; Tran, Q. K.; Saiki, I.; Kadota, S. New spirostanol steroids and steroidal saponins from roots and rhizomes of *Dracaena angustifolia* and their antiproliferative activity. *Journal of Natural Products* **2001**, *64*, 1127-1132.

46. Kemertelidze, E. P.; Korkashvili, T. S.; Pkheidze , T. A.; Abuladze, G. V. A method for obtaining a sum of steroid glycosides possessing antiinflamatory action. Author's Certificate N1320928, **1987**, March 1.

47. Mulkijanyan, K.; Abuladze, G. Anti-exudative action of steroid glycosides' preparation ruscoponin from *Ruscus ponticus*. *Bulletin of the Georgian Academy of Sciences* **2000**, *161*, 254-256.

48. Peana, A. T.; Moretti, M. D. L.; Manconi, V.; Desole, G.; Pippia, P. Anti-inflammatory activity of aqueous extracts and steroidal sapogenins of *Agave americana*. *Planta Medica* **1997**, *63*, 199-202.

49. Miyakoshi, M.; Tamura, Y.; Masuda, H.; Mizutani, K.; Tanaka, O.; Ikeda, T.; Ohtani, K.; Kasai, R.; Yamasaki, K. Antiyeast steroidal saponins from *Yucca schidigera* (Mohave Yucca), a new anti-food-deteriorating agent. *Journal of Natural Products* **2000**, *63*, 332-338.

50. Ribeiro, A.; Zani, C. L.; de Almeida Alves, T. M.; Mendes, N. M.; Hamburger, M.; Hostettmann, K. Molluscicidal saponins from the pericarp of *Sapindus saponaria*. *International Journal of Pharmacognosy* **1995**, *33*, 177-180.

51. El-Sayed, M. M. Saponins of *Yucca aloifolia* as molluscicidal agents. *Egyptian Journal of Pharmaceutical Sciences* **1994**, *34*, 555-563.

52. Sauvaire, Y.; Ribes, G.; Baccou, J. C.; Loubatieres-Marian, M. M. Implication of steroid saponins and sapogenins in the hypocholesterolemic effect of fenugreek. *Lipids* **1991**, *26*, 191-197.

53. Kintya, P. K.; Vasilenko, Y. K.; Goryanu, G. M.; Bobeiko, V. A.; Suetina, I. V.; Mashchenko, N. E. Search for hypocholesteremic agents among a group of steroid glycosides. *Khimiko-Farmatsevticheskii Zhurnal* **1981**, *15*, 55-60.

54. Sangeeta, D.; Sidhu, H.; Thind, S. K. Urinary inhibitory activity towards calcium oxalate crystal growth in rats fed sodium glycolate and an aqueous extract of *Tribulus terrestris*. *Medical Science Research* **1994**, *22*, 627-628.

55. Umikashvili, R. S. Histochemical characteristics of the liver in experimental hypercholesterolemia under the action of *Tribulus terrestris* saponins. *Soobshcheniya Akademii Nauk Gruzinskoi SSR/Bulletin of the Georgian Academy of Science of the Georgian SSR* **1972**, *67*, 729-731.

56. Kemertelidze, E.; Benidze, M. Steroidal glycosides of *Yucca gloriosa* L. and their influence on plant growth. *Bulletin of the Georgian Academy of Sciences* **2001**, *164*, 91-93.

57. Tschesche, R.; Wulf, G. Saponins of the spirostanol series. VIII. Common occurrence of 25α - 25β - sapogenins in the saponins from *Digitalis purpurea* and *D. lanata*. *Tetrahedron* **1962**, *18*, 559-567.

58. Tschesche, R.; Wulf, G. Chemie und biologie der saponine. Berlin, 1972; pp 462-606.

59. Marker, R. E.; Lopez, J. Steroid sapogenins. N163. The biogenezis of steroidal sapogenins in plants. *Journal of the American Chemical Sociaty* **1947**, *69*, 2383-2385.

60. Jones, R. N.; Katzenellenbogen, E.; Dobriner, K. Steroid metabolism. XVII. The infrared absorption spectra of the steroid sapogenins. *Journal of the American Chemical Society* **1953**, *75*, 158-166.

61. Wall, M. E.; Eddy, C. R.; McClennan, M. L.; Klumpp, M. E. Detection and estimation of steroidal sapogenins in plant tissue. *Analytical Chemistry* **1952**, *24*, 1337-1341.

62. Eddy, C. R.; Wall, M. E.; Scott, M. K. Catalog of infrared absorption spectra of steroidal sapogenin acetates. *Analytical Chemistry* **1953**, *25*, 266-271.

63. Ganzera, M.; Bedir, E.; Khan, I. A. Determination of steroidal saponins in *Tribulus terrestris* by reversed-phase high-performance liquid chromatography and evaporative light scattering detection. *Journal of Pharmaceutical Sciences* **2001**, *90*, 1752-1758.

64. Lin, J. T.; Xu, C. J. High-performance liquid chromatography of steroidal sapogenins. *Journal of Chromatography* **1984**, *287*, 105-112.

65. Combarieu, E.; Falzonim, M.; Fuzzati, N.; Gattesco, F.; Giori, A.; Lovati, M.; Pace, R. Identification of *Ruscus* steroidal saponins by HPLC-MS analysis. *Fitoterapia* **2003**, *74*, 423.

66. Dinan, L.; Harmatha, J.; Lafont, R. Chromatographic procedures for the isolation of plant steroids. *Journal of Chromatography, A* **2001**, *935*, 105-123.

67. Faul, W. H.; Djerassi, C. Mass spectrometry in structural and stereochemical problems. CXCIV. Mass spectrometric fragmentations of steroidal sapogenins. *Organic Mass Spectrometry* **1970**, *3*, 1187-1213.

68. Blunden, G.; Jaffer, J. A.; Jewers, K.; Robinson, J. M. Configurational assignments to spirostan-3-ols by mass spectrometry. *Steroids* **1980**, *36*, 299-303.

69. Budzikiewicz, H.; Djerassi, C.; Williams, D. H. *Structural elucidation of natural products by mass spectrometry. II: Steroids, terpenoids, sugars and miscellaneous classes.*; Holden-Day, INC.: san Francisco, London, Amsterdam, **1964**.

70. Wilkins, A. L.; Miles, C. O.; Smith, B. L.; Meagher, L. P.; Ede, R. M. GC-MS method for analysis of steroidal sapogenins in plant and animal samples associated with ovine photosensitization. In *Plant-Associated Toxins: Agricultural, Phytochemical and Ecological Aspects, Proceedings of the 4th International Symposium on Poisonous Plants, Fremantle, Australia, Sept. 26-Oct. 1, 1993*, **1994**; pp 263-268.

71. Agrawal, P. K.; Jain, D. C.; Gupta, R. K.; Thakur, R. S. Carbon-13 NMR spectroscopy of steroidal sapogenins and steroidal saponins. *Phytochemistry* **1985**, *24*, 2479-2496.

72. Evans, F. J. Thin-layer chromatography-mass spectrometry for the identification of sapogenins from *Digitalis* species. *Biomedical Mass Spectrometry* **1974**, *1*, 166-168.

73. Takeda, K.; Hara, S.; Wada, A.; Matsumoton, N. A systematic, simultaneous analysis of steroid sapogenins by thin-layer chromatography. *Journnal of Chromatography* **1963**, 562-564.

74. Eggert, H.; Djerassi, C. Carbon 13-NMR spectra of sapogenins. *Tetrahedron Letters* **1975**, *42*, 3635-3638.

75. Agrawal, P. K. Two dimensional NMR spectroscopy in the structural elucidation of natural products. *Journal of Scientific and Industrial Research* **1994**, *53*, 329-339.

76. Agrawal, P. K. *A systematic NMR approach for the determination of the molecular structure of steroidal saponins*; Plenum Press: New York, 1996.

77. Agrawal, P. K. NMR spectral analysis of some spirostanoids. *Indian Journal of Chemistry* **1998**, *37B*, 601-603.

78. Agrawal, P. K.; Jain, D. C.; Pathak, A. K. NMR spectroscopy of steroidal sapogenins and steroidal saponins: An update. *Magnetic Resonance in Chemistry* **1995**, *33*, 923-953.

79. Agrawal, P. K.; Mahmood, U.; Thakur, S. Torvonin-B, a spirostane saponin from *Solanum torvum*. *Heterocycles* **1989**, *29*, 1895-1899.

80. Agrawal, P. K.; Pathak, A. K. Nuclear magnetic resonance spectroscopic approaches for the determination of interglycosidic linkage and sequence in oligosaccharides. *Phytochemical Analysis* **1996**, *7*, 113-130.

81. Agrawal, P. K.; Bunsawansong, P.; Morris, G. A. NMR spectral investigations. Part 46. Dependence of the 1H NMR chemical shifts of ring F resonances on the orientation of the 27-methyl group of spirostane-type steroidal sapogenins. *Phytochemistry* **1997**, *47*, 255-257.

82. Agrawal, P. K.; Bunsawansong, P.; Morris, G. A. Complete assignment of the 1H and ^{13}C NMR spectra of steroidal sapogenins: Smilagenin and sarsasapogenin. *Magnetic Resonance in Chemistry* **1997**, *35*, 441-446.

83. Griffin, W. J.; Maunwongyathi, P. A comparison of four species of *Cordyline*. *Planta Medica* **1969**, *4*, 346-360.

84. Salmon, J. T. *The native trees of New Zealand*; Reed: Wellington, N.Z., **1980**.

85. Healy, A. J.; Edgar, E. *Flora of New Zealand. Volume III. Adventive cyperaceous, petalous & spathaceous monocotyledons*; Govt. Printer: Wellington, N.Z., 1980.

86. Simpson, P. *Dancing leaves: The story of New Zealand's cabbage tree, ti kōuka* Canterbury University Press: Christchurch, N.Z., **2000**.

87. Brooker, S. G.; Cambie, R. C.; Cooper, R. C. *New Zealand medicinal plants*; Rev. ed.; Reed: Auckland, N.Z., **1987**.

88. Crowe, A. *A field guide to the native edible plants of New Zealand*; Rev. ed.; Godwit Publishing: Auckland, New Zealand, **1997**.

89. Jewers, K.; Burbage, M. B.; Blunden, G.; Griffin, W. J. Brisbagenin and brisbenone, two new spirostanes from *Cordyline* species. *Steroids* **1974**, *24*, 203-208.

90. Jewers, K.; Manchanda, A. H.; Dougan, J.; Nagler, M. J.; Blunden, G.; Griffin, W. J. Cordylagenin, a new steroidal sapogenin diol from *Cordyline cannifolia* and *C. stricta*. *Tetrahedron Letters* **1974**, *15*, 1475-1478.

91. Blunden, G.; Jaffer, A. J.; Jewers, J. K.; Griffin, W. J. Epi-neotigogenin and epi-tigogenin, two new steroidal sapogenins from leaves of *Cordyline cannifolia* leaves. *Journal of Natural Products* **1979**, *42*, 478-482.

92. Blunden, G.; Jaffer, A. J.; Jewers, J. K.; Griffin, W. J. Steroidal sapogenins from leaves of *Cordyline* species. *Journal of Natural Products* **1981**, *44*, 441-447.

93. Blunden, G.; Jaffer, J. A.; Jewers, K.; Griffin, W. J. Crabbogenin, 1[beta]-hydroxycrabbogenin, strictagenin and pompeygenin, four new steroidal sapogenins from *Cordyline stricta* leaves. *Tetrahedron* **1981**, *37*, 2911-2915.

94. Blunden, G.; Sitton, D.; Beach, S. J.; Turner, C. H. Australigenin, a new steroidal sapogenin from *Cordyline australis* fruits. *Journal of Natural Products* **1984**, *47*, 266-269.

95. Griffin, W. J.; Blunden, G.; Jewers, K.; Burbage, M. B.; Nagler, M. J. Steroidal sapogenins from *Cordyline cannifolia* leaves. *Phytochemistry* **1976**, *15*, 1271-1274.

96. Cain, B. F.; Roche, S.; Cambie, R. C. A New Zealand phytochemical survey. Part 5. The monocotyledons. *New Zealand Journal of Science* **1962**, *12*, 537-555.

97. Mistry, P. "Characterization of the saponin and/or sapogenin compounds in Cabbage trees using GC/MS and NMR methods," The Waikato University NZ, **1998**.

98. Mimaki, Y.; Takaashi, Y.; Kuroda, M.; Sashida, Y. Steroidal glucosides from leaves of *Cordyline stricta*. *Phytochemistry* **1997**, *45*, 1229-1234.

99. Mimaki, Y.; Kuroda, M.; Takaashi, Y.; Sashida, Y. Steroidal saponins from the leaves of *Cordyline stricta*. *Phytochemistry* **1998**, *47*, 79-85.

100. Riley, M.; Enting, B. *Māori healing and herbal : New Zealand ethnobotanical sourcebook*; Viking Sevenseas N.Z.: Paraparaumu, N.Z., **1994**.

101. Brooker, S. G.; Cambie, R. C.; Cooper, R. C. *Economic native plants of New Zealand*; Botany Division DSIR: Christchurch, N.Z., **1988**.

102. Brooker, S. G.; Cambie, R. C.; Cooper, R. C. Economic native plants of New Zealand. *Economic Botany* **1989**, *43*.

103. Macdonald, C. *Medicines of the Maori: From their trees, shrubs, and other plants, together with foods from the same sources*; Collins: Auckland, N.Z., **1973**.

104. Fankhauser, B. Archaeometric studies of cordyline (TI) based on ethnobotanical and archaeological research. PhD Thesis, University of Otago, Dunedin, N.Z., **1986**.

105. Fankhauser, B. L.; Brasch, D. J. Preparation of high-fructose syrup from the New Zealand Cabbage Tree, *Cordyline australis*. *New Zealand Journal of Technology* **1985**, *1*, 27-31.

106. Brasch, D. J.; Fankhauser, B. L.; McDonald, A. G. A study of the glucofructofuranan from the New Zealand cabbage tree *Cordyline australis*. *Carbohydrate Research* **1988**, *180*, 315-324.

107. Morice, I. M. Seed fats of the New Zealand Agavaceae. *Journal of the Science of Food and Agriculture* **1962**, *13*, 666-669.

108. Morice, I. M. Two potential sources of linoleic acid in New Zealand. *New Zealand Journal of Science* **1965**, *8*, 446-449.

109. Morice, I. M. Seed fats of some New Zealand and Australian monocotyledons. *Phytochemistry* **1970**, *9*, 1829-1833.

110. Itoh, T.; Tamura, T.; Matsumoto, T. Pollinastanol in the seeds of *Cordyline indivisa*. *Phytochemistry* **1977**, *16*, 1081-1081.

111. Itoh, T.; Tamura, T.; Mitsuhashi, T.; Matsumoto, T. Sterols of Liliaceae. *Phytochemistry* **1977**, *16*, 140-141.

112. Laracine, C.; Lebreton, P.; Berthet, P. Biochemical contributions to the definition of the Agavaceae. *Lettres Botaniques* **1985**, 307-318.

113. Kameoka, H.; Wang, C.-P. The constituents of the steam volatile oil from the flower of *Cordyline australis* Hook. fil. *Nippon Nogei Kagaku Kaishi* **1977**, *51*, 189-194.

114. Blunden, G.; Carpenter, B. G.; Adrina-Romero, M.; Yang, M.-H.; Tyihak, E. Formaldehyde in the plant kingdom. *Acta Biologica Hungarica* **1988**, *49*, 239-246.

115. Matthews, J. S. Steroids. CCXXIII. Color reagent for steroids in thin-layer chromatography. *Biochimica et Biophysica Acta* **1963**, *69*, 163-165.

116. Stahl, E. *Thin-layer chromatography: A laboratory handbook*; 2nd ed.; Springer: Berlin, **1969**.

117. Sannie, C.; Heitz, S.; Lapin, H. Paper partition chromatography of sterolic sapogenins. *Comptes rendus hebdomadaires des seances de l'Academie des sciences* **1951**, *233*, 1670-1672.

118. Kiyosawa, S.; Hutoh, M.; Komori, T.; Nohara, T.; Hosokawa, I.; Kawasaki, T. Detection of proto-type compounds in diosgenin- and other spirostanol glycosides. *Chemical & Pharmaceutical Bulletin* **1968**, *16*, 1162-1164.

119. Konishi, T.; Kiyosawa, S.; Shoji, J. Studies on the coloration mechanism of furostanol derivatives with Ehrlich reagent. I. On the reaction of 3,25-dimethoxyfurosta-5,20-diene with Ehrlich reagent. *Chemical & Pharmaceutical Bulletin* **1984**, *32*, 2111-2116.

120. Harborne, J. B.; Mabry, T. J., Eds. *The flavonoids: Advances in research*; Chapman and Hall: London, **1982**.

121. Markham, K. R.; Ternai, B.; Stanley, R.; Geiger, H.; Mabry, T. J. Carbon-13 NMR studies of flavonoids-III: Naturally occurring flavonoid glycosides and their acylated derivatives. *Tetrahedron* **1978**, *34*, 1389-1397.

122. Faizi, S.; Ali, M.; Saleem, R.; Irfanullah, S. B. Spectral assignments and reference data: Complete 1H and 13C NMR assignments of stigma-5-en-3-O-glucoside and its acetyl derivative. *Magnetic Resonance in Chemistry* **2001**, *39*, 399-405.

123. Watanabe, Y.; Sanada, S.; Ida, Y.; Shoji, J. Comparative studies on the constituents of *Ophiopogonis* tuber and its congeners. II. Studies on the constituents of the subterranean part of *Ophiopogon planiscapus* Nakai. (1). *Chemical & Pharmaceutical Bulletin* **1983**, *31*, 3486-3495.

124. Espejo, O.; Llavot, J. C.; Jung, H.; Giral, F. Spirostanic diosgenin precursors from *Dioscorea composita* tubers. *Phytochemistry* **1982**, *21*, 413-416.

125. Takeda, K. i.; Okanisih, T.; Minato, H.; Shimaoka, A. Studies on the steroidal components of domestic plants-XLVI: Constituents of hosta species (3)[1] Δ25(27)-sapogenins. *Tetrahedron* **1965**, *21*, 2089-2093.

126. Brunengo, M. C.; Tombesi, O. L.; Doller, D.; Gros, E. G. Steroid sapogenins from *Nothoscordum gramineum* var. *philippianum* and *Tristagma uniflorum*. *Phytochemistry* **1988**, *27*, 2943-2945.

127. Agrawal, P. K. *Carbon-13 NMR of flavonoids*; Studies in Organic Chemistry 39; Elsevier: Amsterdam-Oxford-New York-Tokyo, **1989**.

128. Markham, K. R.; Geiger, H. *1H Nuclear magnetic resonance spectroscopy of flavonoids and their glycosides in hexadeuterodimethlsulfoxide*; Chapman and Hall: London-Glasgow-NewYork-Tokyo-Melborne-Madras, **1994**; 441-473.

129. Buschi, C. A.; Pomilio, A. B. Isorhamnetin 3-O-robinobioside from *Gomphera martiana*. *Journal of Natural Products* **1982**, *45*, 557-559.

130. Bock, K.; Thogersen, H. Nuclear magnetic resonance spectroscopy in the study of mono- and oligosaccharides. *Annual reports on NMR spectroscopy* **1982**, *13*, 1-57.

131. Mimaki, Y.; Kuroda, M.; Fukasawa, T.; Sashida, Y. Steroidal glycosides from the bulbs of *Allium jesdianum*. *Journal of Natural Products* **1999**, *62*, 194-197.

132. Shvets, S. A.; Gutsu, O. N.; Kintia, P. K. Steroidal glycosides from *Nicotiana tabacum L*. seeds and their biological activity. In *Saponins used in food and agriculture*; G. R. Waller and K. Yamasaki, Eds.; Plenum Press: New York, **1996**.

133. Shvets, S. A.; Kintia, P. K.; Naibi, M. A. Steroidal glycosides from *Petunia hybrida L*. seeds and their biological activity. In *Saponins used in traditional and modern medicine*; G. R. Waller and K. Yamasaki, Eds.; Plenum Press: New York, **1996**.

134. Bloor, S.; Qi, L. Cytotoxic saponins from New Zealand *Myrsine* species. *Journal of Natural Products* **1994**, *57*, 1354-1360.

135. Kanmoto, T.; Mimaki, Y.; Sashida, Y.; Mikaido, T.; Koike, K.; Ohmoto, T. Steroidal constituents from the underground parts of *Renieckea carnea* and their inhibitory activity on cAMP phosphodiesterase. *Chemical & Pharmaceutical Bulletin* **1994**, *42*, 926-931.

136. Mimaki, Y.; Nakamura, O.; Sashida, Y.; Koike, K.; Nikaido, T.; Ohmoto, T.; Nishino, A.; Satomi, Y.; Nishino, H. Structures of steroidal saponins from the tubers of *Brodiaea californica* and their inhibitory activity on tumor promoter-induced phospholipid metabolism. *Chemical & Pharmaceutical Bulletin* **1995**, *43*, 971-976.

137. Yan, W.; Ohtani, K.; Kasai, R.; Yamasaki, K. Steroidal saponins from fruits of *Tribulus terrestris*. *Phytochemistry* **1996**, *42*, 1417-1422.

138. Middleton, E.; Kandaswami, C. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer In *The*

flavonoids: Advances in research since 1986; J. B. Harborne, Ed.; Chapman and Hall: London, **1994**; pp 619-652.

139. Castillo, A. V. Physico-chemical investigation of *Cordyline terminalis* kunth (Family Liliaceae). *NRCP Research Bulletin* **1982**, *37*, 426-432.

140. Calixto, J. B.; De Lima, T. C. M.; Morato, G. S.; Nicolau, M.; Takahashi, R. N.; Valle, R. R.; Schmidt, C. C.; Yunest, R. A. Chemical and pharmacological analysis of the crude aqueous/alcoholic extract from *Cordyline dracaenoides*. *Phytochemistry Research* **1990**, *4*, 167-171.

141. Eggington, J.; Frederick, G. The isolation, antibacterial testing and structural analysis of a nonpolar compound obtained from *Cordyline terminalis*. Presented at 212th ACS National Meeting, CHED-144, Orlando, Fl., August 25-29, **1996**.