EXTRACTION, ISOLATION AND STRUCTURAL DETERMINATION OF ORGANIC COMPOUNDS FROM *SCAEVOLA SPINESCENS* R.Br.

A thesis submitted towards a

Doctor of Philosophy

by

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July, 2001

CONTENTS

ABSTRACT

The aim of this project was to extract, isolate and determine the structure of organic compounds from *Scaevola spinescens*, which is found widely in the arid to semi-arid regions of South Australia and through into most other states of Australia.

Scaevola spinescens is an Australian native plant that the Aboriginal people of Hawker in South Australia have traditionally used as a natural medicine to treat a variety of symptoms. There is much anecdotal evidence that indicates that *Scaevola spinescens* is a useful plant in traditional medicine.

Seventeen organic compounds were isolated and their structures elucidated by spectroscopic methods. Four further compounds were isolated but have not been completely identified.

The compounds isolated include triterpenes, iridoids, coumarins, flavonoids and other compounds.

Four novel compounds were isolated. They were:

- judarrylol, a triterpene
- emmarin, a coumarin
- alilydijosioside, an iridoid, which may be an artefact of the extraction process
- katecateroside, an iridoid

Of the seventeen organic compounds isolated, four were novel, fifteen were new to *Scaevola spinescens* and thirteen had not been reported from any plant from any species of *Scaevola*.

A table of the compounds (**Table A.1**) isolated and their structures are illustrated below.

All crude extracts were first tested with Meyers reagent (a test for alkaloids) with no positive results recorded.

The structures were deduced from MS data as well as routine and 2D NMR experiments, apart from (**95**) which was derived solely from GC/MS data.

Table A.1 : Compounds isolated from *Scaevola spinescens*

Compound	Novel	New to *Scaevola spinescens*	New to *Scaevola* genera
Hexadecanoic acid, methyl ester (**95**)		*	*
Taraxerol acetate (**92**)			
Taraxerol (**73**)			
Judarrylol (**96**)	*	*	*
Ursolic acid (**97**)		*	*
Emmarin (**98**)	*	*	*
Vanillic acid(**99**)		*	*
Daucesterol (**100**)		*	*
Alidyjosioside (**108**)	*	*	*
Scaevoloside (**6**)		*	
Katecateroside (**109**)	*	*	*
Loganin (**5**)		*	
Luteolin-7-*O*-glucuronide methyl ester (**110**)		*	*
2-C-(Hydroxymethyl)-D-ribonic acid-γ-lactone (**111**)		*	*
L-*threo*-Guaiacyl glycerol (112)		*	*
Luteolin-7-*O*- glycoside (**113**)		*	*
2-Deoxy-D-*chiro*-inositol(**114**)		*	*

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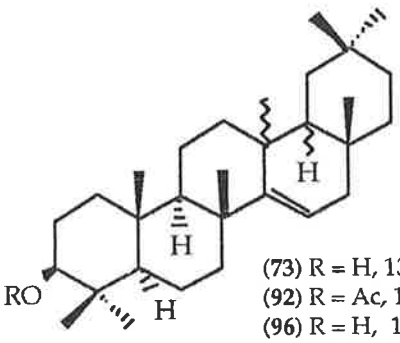
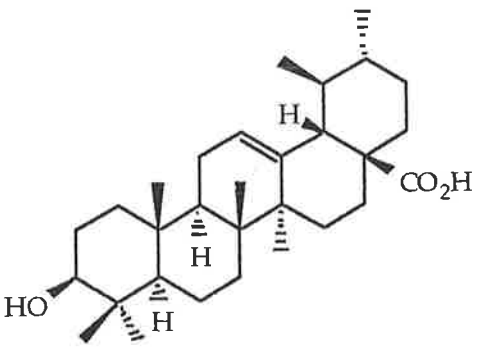


(**73**) R = H, 13α-CH_3, 18β-H
(**92**) R = Ac, 13α-CH_3, 18β-H
(**96**) R = H, 13β-CH_3, 18α-H



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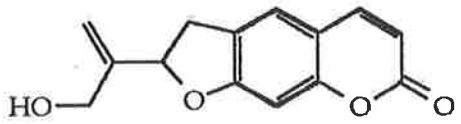

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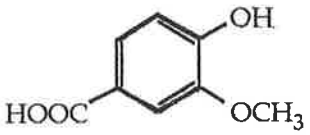

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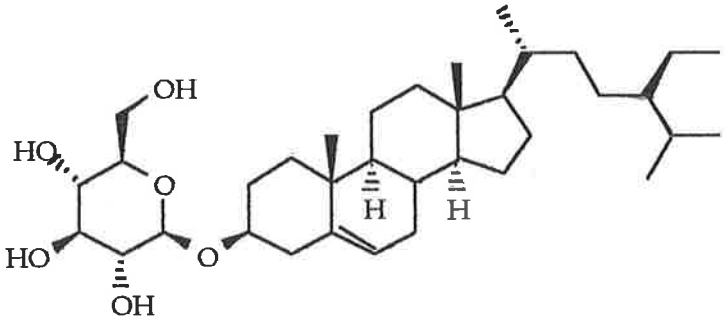

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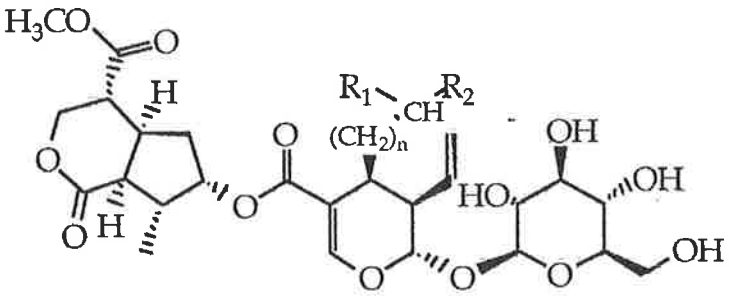
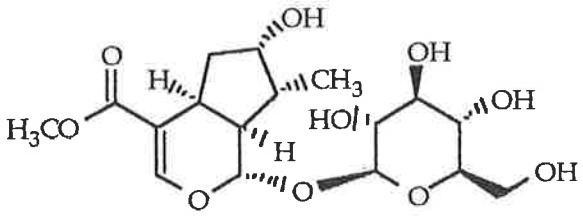


(6) R_1, R_2 = O, n = 1
(108) $R_1 = R_2 = OCH_3$
(109) R_1, R_2 = O, n = 2



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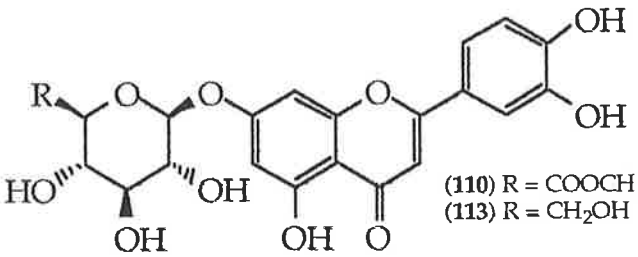
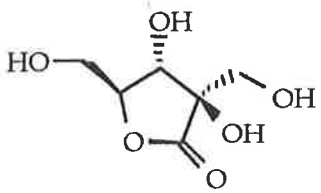


(110) R = $COOCH_3$
(113) R = CH_2OH



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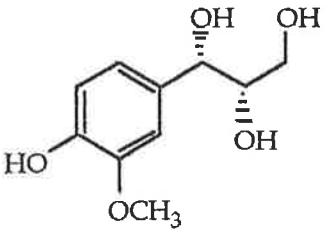

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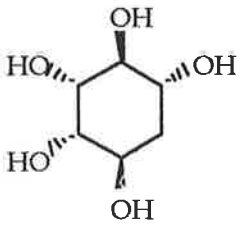

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A range of biological tests were carried out on both crude extracts, semi - purified fractions and certain isolated and purified compounds, as outlined in Chapter 11.

Crude Fractions 4, 8 and 9 showed moderate anti bacterial activity.

One novel coumarin - emmarin (**98**) - and vanillic acid (**99**) were isolated from Fraction 4.

The four iridoids - loganin (**5**), scaevoloside (**6**) alidyjosioside (**108**) and katecateroside (**109**) - were isolated from Fraction 8.

Luteolin-7-*O*-glucuronic acid methyl ester (**110**), 2-C-(hydroxymethyl)-D-ribonic acid-γ-lactone (**111**) and L-*threo*-guaiacyl glycerol (**112**) were isolated from Fraction 9.

These compounds need to be tested to determine whether they have anti bacterial activity.

The initial cytotoxicity test used - Procedure A - indicated a massive stimulatory effect at high concentrations of the more polar extracts on three cancer cell lines, while at lower concentrations there was some inhibition of growth. However these results could not be reproduced. Other cytotoxicity tests were inconclusive.

The anti viral tests showed no activity.

STATEMENT

This thesis contains no material previously submitted for a degree at any University, and, to my knowledge and belief, contains no material previously published or written by another person except where due reference is made in the text.

I give my consent for this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Sally Nobbs

ACKNOWLEDGMENTS

There are many people who have contributed to the completion of this thesis and whose help and inspiration have been invaluable to me.

Firstly, I am truly indebted to the care and motivation of Dr. Simon Pyke. Dr Pyke has been unfailing in his positive and professional approach to my work. His enthusiasm, care for detail, kindness and unfailing optimism have been of immense help. I truly could not have started, let alone completed this thesis without Dr Pyke's support.

There are some professional colleagues whom I must thank. I am indebted to Phil Clement, whose skill and patience in performing numerous NMR experiments for me was invaluable. Dr Bruce May was always very supportive and I really appreciated his help and that he actually read my draft! To all the fellow travellers in Lab 7, thankyou for the support and the fun.

My family has been very supportive of my efforts. My parents, Margaret and Jack Nobbs have been inspirations to me. They have been positive and enthusiastic at all times and I could not have completed this thesis without their help. My brothers John and Tony, sisters-in-law Jacqie and Sylvia and brother-in-law Ben have always encouraged me and supported me in any way they could. My sister Jackie has also been a major inspiration to me and I can now empathise more fully with her own experiences.

I could not have completed this thesis without the support of my place of employment, Wilderness School. To the Principal, Mrs Carolyn Grantskalns and to my Head of Department, Judy Cox, goes my eternal gratitude for their support and the interest they have always shown. To my many colleagues from school - both staff and students - thankyou. Thanks especially to Marian Bader for her tireless efforts in helping type the drafts for each chapter and to Jan Lyon who also read my first draft.

Thanks to all my friends who have helped me through the last six years of my life - Wendy and Greg, Kirstie, Jane Langrehr, Jane Keen, Libby, Fred and Siddi, Sandy and all the other friends that have helped.

Lastly to my "children" - Benjamin, Andrew, Jeremy and Little Jack - my beautiful nephews - and Carly Joanna and Emma-Louise Elsie Keech - those beautiful girls - all of whom I adore. They are the inspirations in my life and their presence in my life has kept me sane.

CHAPTER 1 INTRODUCTION

1.1 General historical use of natural products as medicines

Plants are a major source of biologically active organic compounds and have been used as natural medicines by most indigenous communities, including Aboriginal clans from South Australia.

Humans have used native plant material as medicinal and therapeutic agents throughout history. The earliest written prescriptions for medicines have been found on Egyptian papyrus dated around 3,500 years BC. In this collection, 800 medicinal prescriptions were found, many of them of plant origin that are now known to contain bioactive organic compounds.[1]

By 300 BC the Hippocratic collection of scientific medical documents had been established, while Dioscorides (surgeon to the Emperor Nero) carried on the botanical work of Theophrastus (370 - 286 BC) and wrote the "Materia Medica"- a book outlining the preparation and uses of more than 600 plants and plant products.[2] Galen (200 AD) outlined the preparation of herbs that provided the basis of medical treatments through until modern times.[1]

It has been suggested that over 75% of the world's population today receives their health care through the traditional use of natural medicines and many of the drugs common throughout the developed world are based on organic compounds isolated from plants.[1] The use of salicylic acid (aspirin), morphine and atropine are just three examples of the many medicines derived from plant sources.

More than 500 of the plant species growing wild in Australia have been and are still being used as medicines to treat a variety of ailments.[1]

The Australian Aborigines did not have a written language, but there is clear evidence in their oral history of the use of plants as medicines to treat a wide variety of symptoms. Steaming-oven stones for the steaming of aromatic plants have been found in hearths that are at least 4000 years before the present[3], and there is much anecdotal evidence for the use of medicinal plants that has been passed on to anthropologists.

The medicines were prepared and are still being prepared in a number of ways:[1,3]

- by using stones to bruise the plant material by pounding it, then moistening the material and placing it on the skin as a poultice.
- by burning aromatic leaves and twigs and breathing in the resultant fumes.
- the leaves and twigs were heated and then doused with water to form steam that was either breathed in or used as a steam bath.
- by extracting plant material with water and drinking the infusion. Before the arrival of Europeans, who provided drinking utensils, the Aborigines used to put the material in water in hollowed wooden containers and then place heated rocks into the infusion to increase the water temperature. They then drank the infusion. In this way, the water temperature did not usually increase above 50°C.

A number of ceremonial and medicinal drugs were widely traded among Aboriginal clans throughout mainland Australia, with only certain clans having the knowledge concerning the correct procedures to ensure the production of a 'safe' product. This knowledge was a very closely guarded secret, passed on from generation to generation.[4]

Duboisia hopwoodii is a plant that the Aborigines called pituri. It has a high nicotine content and was used both as a recreational drug and in certain ceremonies. It produced a general sense of well being, including hallucinatory effects and was widely traded along the numerous trade routes across Australia.[4]

Chemically, it is interesting to note that the preparation of pituri involved its treatment with alkali ash, which was produced by burning a particular wirra bush, *Acacia salicina*. The effect of this alkali ash was to increase greatly the power of the drug, by freeing the nicotine and allowing it to pass readily through mucous membranes.[4]

There appear to be various classes of organic materials that have a high therapeutic value. These were isolated from plant sources by Aborigines.[1]

These classes of organic materials include:

Alkaloids — Alkaloids are secondary metabolites, which are usually bitter and possess a definite and usually specific physiological effect and are by far the most well known plant medicines. They include caffeine, morphine, quinine, nicotine, atropine and a host of others. Fifteen per cent of all higher plants contain alkaloids.

Tannins	Tannins are used as astringents, drawing tissues together, and therefore used for treating surfaces such as inflamed mucous membranes. They were used to treat colds and coughs and to bath wounds.
Essential oils	Essential oils are widely used for a variety of treatments, in particular respiratory ailments. Eucalypts and the mint genera possess oil glands. The oil vapours can be inhaled to treat colds and upper respiratory infections and have also been known to possess anti-microbial properties.
Mucilage	Mucilage is the slimy drip from broken stems. Mucilage of some species has been used for the treatment of inflamed mucous membranes.
Latex	Latex is a fluid containing enzymes that digest protein and therefore can be used to remove small skin eruptions and clean the surfaces of ulcers and wounds.

Europeans have documented the use of plants by Australian Aborigines from as early as 1861. There are a large number of books and papers listing the diseases and symptoms for which Australian plants have been used.

These include "Wild Medicines in Australia" by Cribb and Cribb,[1] "Mankind" by Webb,[5,6] "The records of Western Australian Plants used by Aboriginals as Medicinal Agents" by Reid and Betts,[7] "Australian Medicinal Plants" by Lassack and McCartney,[8] articles by Cleland and Johnson in the Transactions of the Royal Society of South Australia[9] and, most recently, "Traditional Aboriginal Medicines in the Northern Territory of Australia" by Barr et al.[10]

Chemically and pharmacologically, the plants of Australia began to be investigated in the early 1940's in a CSIRO project known as the Australian Phytochemical Survey. The alkaloids were the major chemical group of interest as these were considered to be the most likely compounds to possess therapeutic activity.[11]

The phytochemical survey was augmented by the pharmacological testing of the alkaloids and then investigation of specific anti tumour activity, using both crude plant extracts and then pure substances.[11] However, there are many species of Australian plants that have not been investigated, including plants from the Goodeniaceae family and species from the *Scaevola* genus.

1.2 Family Goodeniaceae, *Scaevola* genus, *Scaevola spinescens*

1.2.1 Botanical Description

The family Goodeniaceae consists of eleven genera with approximately 400 species almost all of which are confined to the Southern Hemisphere.[12]

The *Scaevola* genus contains 96 species that occur throughout the tropical areas of the Indo-Pacific region as well as throughout Australia. Seventy of the seventy-one *Scaevola* species that occur in Australia are endemic, one of which is *Scaevola spinescens*. *Scaevola taccada* is the only species that is not unique to Australia and it occurs in many other areas of the world.[12]

Scaevola, according to one reference,[12] means "little hand", while a second reference[13] states that it derives from the Latin 'scaevus', meaning left, and that *Scaevola* was named after the Roman, Gaius Mucuis.

> According to legend, Gaius Mucius attempted to kill Lars Porsena, King of Clusium. However, he accidentally killed the King's secretary. On being arrested, and to show that he was not afraid of death, he thrust his right hand into a fire where it was consumed. The King set Mucius free (!) and gave him some land, and from that time on Mucius was known as Scaevola - left hand. [13]

Both 'little hand' and 'left hand' refer to the type of flower that all *Scaevola* species have. It is a flower that is shaped like an outspread hand.[13]

The full botanical description of *Scaevola spinescens* is attached in **Appendix A**.

The common names for *Scaevola spinescens* are prickly fan flower, fan bush, currant bush, poontoo[14] or maroon bush,[10,15] while the Aboriginal people who live near Hawker in South Australia - the Adnyamathanha - give it the name "yudli".[16]

It is a rigid, often tangled woody shrub, 50 cm to more than 1 m high with woody stems. Its leaves are very small (5-20 mm long, 2-5 mm broad), are shaped like a lance or a spear and are thick and fleshy. The leaves often cluster, and are accompanied by a rigid, sometimes forked spine. The flowers are cream-to-yellow, 10-16 mm long, born singly on slender stalks with 5 equal spreading petals on one side of an obvious fan-shaped arrangement.[14]

A photograph taken of a stand of *Scaevola spinescens* is shown in *Figure 1.2.1.1*.

Figure 1.2.1.1: Scaevola spinescens (photo: Sally F. Nobbs, 2000)

The major characteristic that distinguishes *Scaevola spinescens* from other species of *Scaevola* is their branched spines and this pattern is displayed in *Figure 1.2.1.2.*[12]

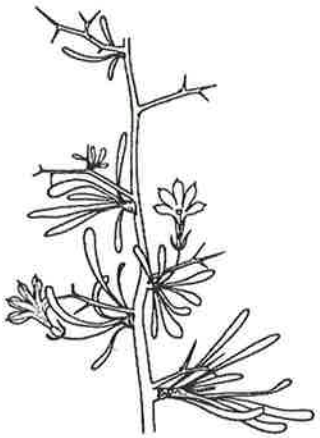


Figure 1.2.1.2: Branchlets of Scaevola spinescens [12]

Scaevola spinescens is found in all states of Australia. It occurs in most soil types and is commonly found on hillsides and stony plains.[12,14] A map illustrating the distribution of *Scaevola spinescens* is given in *Figure 1.2.1.3* .[12]

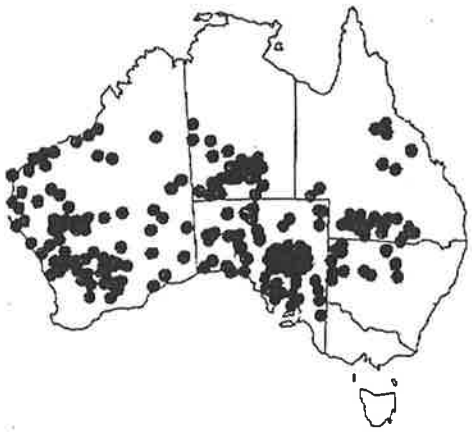


Figure 1.2.1.3: Distribution map for Scaevola spinescens [12]

1.2.2 Use of plants of the *Scaevola* genus as natural medicines

Three species of *Scaevola* have been mentioned as having been used, or are still being used, as natural medicines through their natural range.

1.2.2.1 *Scaevola enantophylla* F. Muell, *Scaevola oppositifolia* sensu F. Mueller

Scaevola enantophylla F.Muell and *Scaevola oppositifolia* sensu F.Mueller are considered to be the same plant and are classified as *Scaevola enantophylla* F.Muell.[12] It is suspected of being poisonous to stock[5,17] and the juice was used as either an arrow poison[5,17] or applied to areas affected by eye and ear inflammation.[18] In Papua New Guinea its leaves were used to treat and cover sore eyes.[19]

1.2.2.2 *Scaevola taccada* (Gaertner) Roxb.

Scaevola taccada (Gaertner) Roxb. is the name given to a species that had previously been classified as *Scaevola sericea* M. Vahl, *Scaevola koenigii* M. Vahl, *Scaevola frutescens* var *koenigii* (M.Vahl) Domin, *Scaevola lobelia* L.ex Vriese, *Scaevola frutescens* K. Krausse, *Lobelia taccada* Gaertner, and *Lobelia sericea* var koenigii.[12]

Jeffrey (1980)[20] established the use of the name *Scaevola sericea* for this plant species, but the nomenclature established by Carolin (1991)[12] for the Australian Biological Resources Study is *Scaevola taccada* and this name will be used in this thesis to refer to all of the above species names.

Scaevola taccada is widely distributed along the coasts of Madagascar (Africa) and other countries of the Indian Ocean, tropical Australia and through the Pacific Ocean from Taiwan through to the Hawaiian Islands.[12]

It has been and is still widely used as a natural medicine throughout its geographical range. It is not unique to Australia and so was not considered for study in this project. It is the most widely documented species of *Scaevola* used as a natural medicine. **Table 1.2.2.1** (on the next page) outlines the places and uses of *Scaevola taccada*.

This information is included because it supports the view that plants from the *Scaevola* genus may possess active compounds of therapeutic value.

Scaevola taccada M. Vahl is one of only two *Scaevola* species that occurs in Southern Africa. It is reported, in Madagascar, to be used as an antidote against the poisonous prick of 'armed' fish.[13, 21] Personal communications[22-25] with four botanists working in the field in Southern Africa indicate no known medical use of *Scaevola taccada* or the other species of *Scaevola* along the coast of South Africa and Mozambique.

1.2.2.3 *Scaevola spinescens* (R.Br.)

Scaevola spinescens has been recorded through most of the arid and semi-arid parts of South Australia (see *Figure 1.2.1.3* - Map of the distribution of *Scaevola spinescens*). It became the plant of interest for this current project because of its widespread use as a medicinal plant by South Australian Aborigines and because of its use as a potential anti cancer treatment in Western Australia.

Scaevola spinescens has been described as:

- an analgesic.
- a treatment for coughs and colds.
- an anti bacterial agent for treatment of wounds, sores and septic ulcers.
- able to be used for skin disorders such as tinea, ringworm, itches and leprosy.
- able to be used for stomach disorders.
- an anti tumour agent by a wide range of independent sources.[8]

My initial interest in the therapeutic value of *Scaevola spinescens* was raised by discussions between Mrs. Pearl McKenzie[26] an elder of the Adnyamathanha people of Hawker, South Australia and Mr John McEntee[16], owner of Erudina station near Lake Frome.

Table 1.2.2.1: Medicinal uses of Scaevola taccada

Place	Use	References
Australia	Crushed fruits were rubbed on the skin to cure tinea	8
	Leaf decoction used by Aborigines externally to treat sores	8, 18, 21
	native cabbage - the young leaves eaten as a vegetable	13, 18
New Guinea	Leaves applied to sores	18, 19
	Leaves used for sore eyes	19
New Ireland	New soft leaves are chewed for colds	27
Malacca	Bitter leaves used to cure indigestion	28
	Leaf poultice applied to head to treat headaches	18, 28
Moluccas	Used to treat diarrhoea	28
Philippines	Fruit juices used to treat cataracts	1
	Roots used to treat syphilis and dysentery	1, 13, 18
	Antidote for fish poison	13, 18
	Root used to treat beri-beri	13, 18, 28
Fiji	Root decoction taken for stomach ache	1, 18
	Leaf softened by beating and scorching, made into a poultice to treat wounds caused by circumcision	13
Samoa	Leaves mixed with leaves of *Tournefortia sp* to treat cold fits (shivering, shaking, chills) and in cases of paralysis and oedematous ulcers	13
Caroline Is	Leaves used as a tonic and an aphrodisiac	18
Hawaii	Deep cuts, cataracts, scaly skin, puncture wounds	29
India	Berry juice used to treat eyes with dim vision	19
Area not indicated	Leaf used as an emollient to promote suppuration of boils and tumours	13
	Decoction of root used to treat beri-beri	13, 28
	Fruit pulp used by native women to induce menstruation	13
	Crushed ripe fruit-pulp is effective for tumours	13
	Medulla of young stems is used to stop diarrhoea, as an oral aphrodisiac and to treat male impotence	13
	Leaf poultice applied to headaches and on swollen legs	28
	Juice used on sore eyes	21, 28
	Antidote for poisonous fish and crabs	21
	Smoked leaf like tobacco	13

Both individuals indicated that *Scaevola spinescens* was a powerful natural medicine still used in certain situations to treat pain and bleeding in the

alimentary canal. Further personal communications by Ms Sue Semple[30] with the Aboriginal people of Oodnadatta and station owners around Lake Eyre supported the view that *Scaevola spinescens* was a powerful natural medicine.

Further research from a variety of sources indicated the following uses of *Scaevola spinescens.*

Colds
The whole plant was burnt and the fumes inhaled for colds.[8, 21]

Treatment of sores
The leaves and branches (both dry and green) were placed in a hole in the ground and burnt. A boy who had been recently circumcised squatted over the hole and urinated into the hot ashes so that steam would rise around the penis.[9] Other sores were treated in the same way, but using water on the ashes instead of urine.[8, 9] A decoction of broken stems was used to cure boils, sores and rashes when drunk for three to four days.[8]

Stomach and urinary pains
The Aborigines of South Australia boiled the root of the plant and drank the liquor for stomach ache and urinary complaints.[1,8,9] Aborigines of Western Australia drank an infusion of roots for pain in the alimentary canal.[6] Aborigines in the Murchison region of Western Australia used the woody stems to treat alimentary ulcers.[7]

Analgesic
An infusion of *Scaevola spinescens* was used to alleviate the pain of cancer.[12,15]

Cancer
An infusion of the leaves and twigs of *Scaevola spinescens,* in conjunction with *Codonocarpus cotinifolius,* was reputed to cure cancer. A later investigation of this mixture was inconclusive.[7,8]

Personal correspondence with Dr E. Ghisalberti[31] of the University of Western Australia indicated that the Western Australian Public Health Department had used a decoction of *Scaevola spinescens* as a rudimentary treatment for cancer from the 1930's to the 1970's, with among five to fifteen people using the decoction. Pharmaceutical companies tested a plant extract with no obvious cytotoxic properties being noted.

An article[15], written in 1966, has only recently come to my notice, outlining the 'history' of the use of *Scaevola spinescens* as an anti-cancer agent in WA. The following is a precis of the article.

> In January 1937 a full-blood Aboriginal received medical treatment at a country hospital in WA.
>
> He possessed an ulcerated tongue, which was diagnosed as a possible infection or even cancer. No biopsy was performed. Months later, he appeared with his tongue healed and no pain. He said that it had been treated by swallowing infusions from a local plant - maroon bush - which is the local Aboriginal name for *Scaevola spinescens*.
>
> The rumour of a cancer cure then circulated around Perth and samples, prepared in accordance with the method used by the Aborigines, were forwarded to Perth.
>
> These samples were then sent to the Royal Cancer Hospital, London, where they were tested on various animal tumours but the samples had no obvious effect. As there were still rumours and interest in the plant, samples were tested at the Public Health Laboratory, Perth. These samples were found to have no toxic effects and a preparation was made available to any patient with inoperable cancer.
>
> Further pharmaceutical tests were conducted in 1959, which indicated that the extract was not active against a range of cancer cell lines. Between 1 June 1957 and 27 June 1967, 66 patients were treated with inoperable cancer. All appeared to have died more or less when expected although at least five patients had relief from symptoms.

At present an extract from *Scaevola spinescens* is supplied by the Chemistry Centre of Western Australia to a limited number of patients who have been diagnosed with various terminal cancers.[31] However there is pressure for this to stop.

When I first started the project in January 1994, I was unable to find any reference to *Scaevola spinescens* in the chemical literature. I chose *Scaevola spinescens* as the plant to be investigated because, firstly, it was only found in Australia (unlike *Scaevola taccada*).

Secondly the literature research and personal and close communication with the Adnyamathanha people indicated it was a very powerful natural medicine, which was still being used.

Thirdly, I was able to collect a large amount of the plant material easily (unlike *Scaevola taccada*, which is a tropical plant from Northern Australia).

Fourthly, when I started the project I believed that no one had worked on *Scaevola spinescens* as there was no published literature on the plant, apart from a paper written in 1971[32], outlining pharmacological testing of the aqueous

extract. I proceeded to collect 12.7 kg of the aerial parts of the plant and to work on the more polar fractions of the plant, as the Aboriginal people prepare the plant by boiling it.

A colleague of mine attended a conference in August 1995 and learnt that a group in Perth, associated with Curtin University had previously worked on *Scaevola spinescens* although they had not published any of their results. I arranged to go to Perth in January 1996 and met Dr E. Ghisalberti who informed me that a PhD student supervised by Dr R. Longmore of Curtin University was currently working on *Scaevola spinescens*.

Discussions with Dr Ghisalberti[31] and Dr Longmore[33] were very fruitful, for it appeared that they were concentrating on the non-polar fractions. Not only that, but it was clear that their anecdotal evidence for the efficacy of *Scaevola spinescens* supported my information from the Adnyamathanha people and that this plant has real potential as a source of therapeutically active compounds. It was also pointed out that there might be variation in the plants collected from sources as widely separated as Western Australia and South Australia.

They were also able to confirm that samples of the plant had been sent to and tested by the US National Cancer Institute, but were not forthcoming with any results of this investigation.

I have proceeded with my investigation of the compounds isolated from *Scaevola spinescens* by investigating the polar extracts of the plant.

1.2.3 Biological testing of *Scaevola* species

There have been some investigations into the pharmacological activity of plants from the *Scaevola* genus.

1.2.3.1 General screening

In 1990 the CSIRO published a book - "Plants for Medicines"[11] - which is a survey of all phytochemical and chemical investigations of plants in the Australian region. Eleven species of *Scaevola* were investigated in an alkaloid and anti tumour screening, with the results outlined in **Appendix B**.

In summary, of the eleven *Scaevola* species tested, three showed the presence of alkaloids (*Scaevola aemula, Scaevola hispida* and *Scaevola densevestita*) while six were tested for anti tumour activity (*Scaevola aemula, Scaevola albida,*

Scaevola angustata, Scaevola hispida, Scaevola taccada and *Scaevola oppositifolia*) but all gave a negative result.[11] *Scaevola spinescens* was not one of the species tested.

1.2.3.2 *Scaevola taccada*

From the data given above (phytochemical screening) it would appear that *Scaevola taccada* does not possess anti tumour activity.

Ishii et al[34] in 1984 tested *Scaevola taccada* as well as over 303 different plant extracts for bio-antimutogens (agents that suppress cellular mutagenesis) using the criteria of suppressing UV induced mutations in *E.coli* WP2 trp and the mutator activity in *B. subtilis* NIG1125 (*his met*). The *Scaevola taccada* extract did not appear to have any effect.

In 1995 extracts of *Scaevola taccada* were investigated for anti viral, anti fungal and anti bacterial activity in vitro by Locher et al.[29] Their research showed that the acetonitrile fraction of *Scaevola taccada* showed selective anti viral activity, while the dichloromethane fraction exhibited anti fungal activity to a lesser extent.

1.2.3.3 *Scaevola spinescens*

The earliest investigation of the effects of aqueous extracts of the wood of *Scaevola spinescens* was published in 1971 by Goss et al[32] where it was reported that this extract contained an antagonist of 5-hydroxy tryptamine (5HT).

Kerr and Longmore[35] in 1996 reported in an abstract to a conference the isolation of a number of known compounds and that:
"Results of screening on the US National Cancer Institute (NCI) tumour panel assay, together with the constituents isolated and/or found to be present in the plant, provide evidence in support of the anecdotal reputation of the Scaevola bush as a medicinally active, potentially important cancer chemotherapeutic agent or source material."
They did not give any details of the results of this screening.

In December of 1996 Kerr, Longmore and Betts[36] published a paper identifying a number of pentacyclic triterpenoid compounds. They used the GC-MS technique with peak matching of libraries of MS data, and hence were able to identify previously isolated compounds. These compounds were isolated from non-polar fractions.

In 1998, Semple[37] reported on her investigation into the anti viral activity of the extracts of forty different Australian plants that the Aboriginal people used in their traditional medicines. She found that the methanol fraction of *Scaevola spinescens* was active against human cytomegalovirus (HCMV) at therapeutic levels.

Kerr, Longmore and Yench[38] (1999) tested extracts of *Scaevola spinescens* for their ability to inhibit tumour formation in the potato Crown Gall Tumour (CGT) assay and for possible anti microbial activity against *Bacillus subtilis*. Their conclusion was that the aqueous and methanolic extracts of *Scaevola spinescens* were significantly active in the CGT assay "*indicating a strong potential for further investigation for anti-cancer activity.*"

1.3 Classes of compounds previously isolated from plants of the *Scaevola* genus.

As has been detailed previously, the CSIRO, through its general chemical and pharmacological screening program of indigenous Australian plants, has indicated the presence of alkaloids in three out of eleven of the *Scaevola* species tested, while six out of the eleven were investigated for anti tumour activity with negative results.[11]

Isolation and structure determination of compounds from species of *Scaevola* have revealed a range of organic compounds, which can be classified into six different classes, iridoids, alkaloids, coumarins, fatty acids and hydrocarbons, flavonoids and terpenoids.

1.3.1 Iridoids

Iridoids are monoterpenoids that contain the cyclopentane-[c]-pyran skeleton and that are natural constituents of a large number of plant families. Iridoids are usually, but not always, found as a glycoside. They have been widely implicated in the biosynthesis of indole and isoquinoline alkaloids.[39]

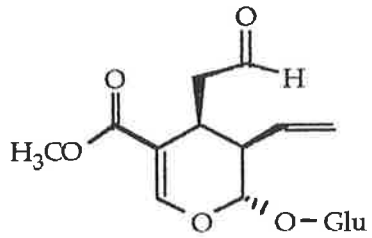
1.3.1.1 Iridoids isolated from *Scaevola* species

The earliest report of a chemical investigation of species of *Scaevola* was by Tammes, who as early as 1909,[40] reported the following:

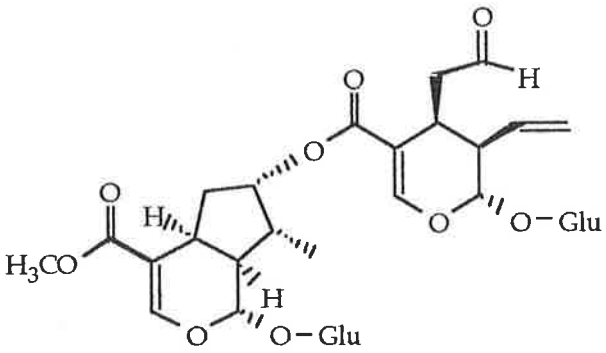
> "Only in the three species of the genus *Scaevola* of the order Goodeniaceae, which were at my disposal, I found after warming parts of the plants in a moist place, that a blue colouring matter occurs which is doubtless dipsacotin".

No structural information for dipsacotin was given, and this reference should be treated carefully.

Jensen[41] (1993), of the Technical University of Denmark, initially identified secologanin (**1**) in *Scaevola suavolens*. When Jensen[41] compared his data with an iridoid previously isolated from *Dipsarcus* sp he identified the compound as cantleyoside (**2**).



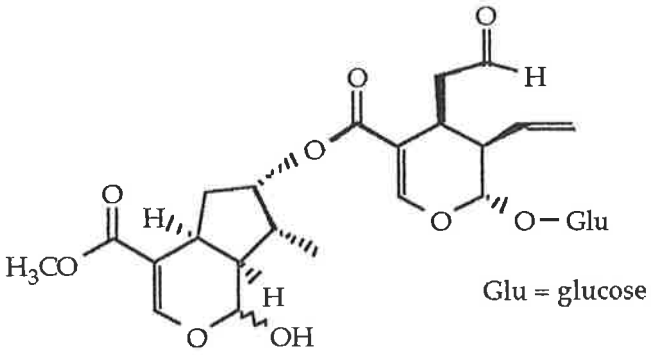
(1)



(2)

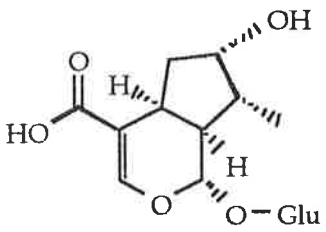
Glu = glucose

Jensen (1993, 1997) also isolated and identified cantleyoside (**2**), sylvestroside III (**3**) and loganic acid (**4**) from *Scaevola taccada*.[41, 42]



Glu = glucose

(3)



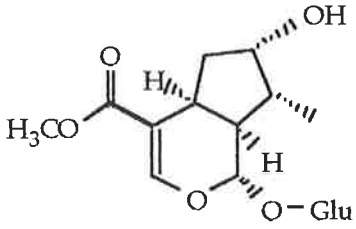
(4)

Jensen (1993)[41] also investigated *Scaevola crassifolia* and *Scaevola ramosissima*, but was unable to isolate iridoids from these two species. None of these investigations have been reported in the literature.

Skaltsounis (1989)[43] isolated five iridoids, including two dimethyl acetal derivatives, from *Scaevola montana* and five iridoids (1989)[44], from *Scaevola racemigera*, while Cambie (1997)[45] isolated a new iridoid from *Scaevola floribunda*.

Table 1.3.1.1 indicates the number and name of the iridoids isolated from five species of *Scaevola*. In general, the iridoids were isolated from the aerial parts of the plant, usually using an extraction with dichloromethane, followed by

methanol. Silica column chromatography of the methanol extract (90% chloroform/10% methanol) was then used to isolate the iridoids.[43, 44, 45]

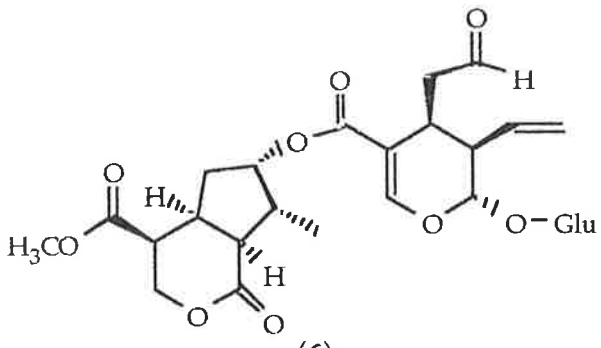


Glu = glucose

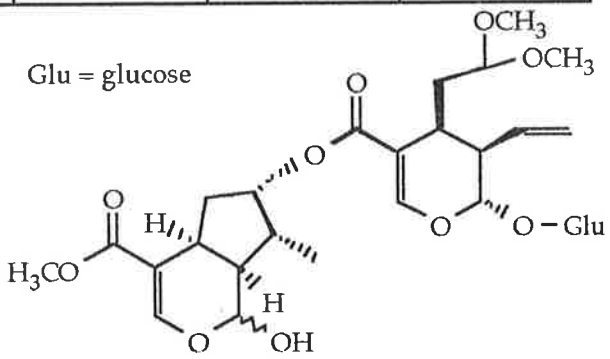
(5)

Table 1.3.1.1: Iridoids from five *Scaevola* species

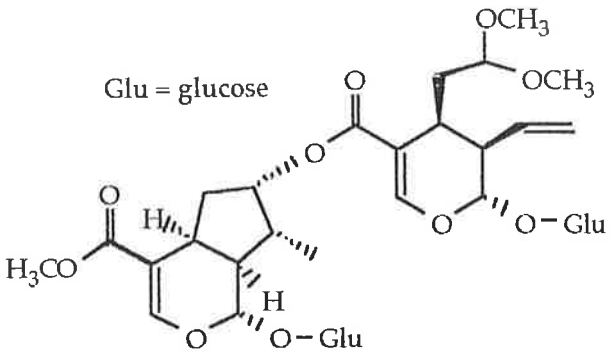
	Scaevola suavolens 41	*Scaevola taccada* 41,42	*Scaevola montana* 43	*Scaevola racemigera* 44	*Scaevola floribunda* 45
Cantleyoside (2)	*	*	*	*	
Sylvestroside III (3)		*	*	*	
Loganic acid (4)		*		*	
Loganin (5)			*	*	
Scaevoloside (6)				*	
Sylvestroside III dimethyl acetal (7)			*		
Cantleyoside dimethyl acetal (8)			*		
Floribundal (9)					*



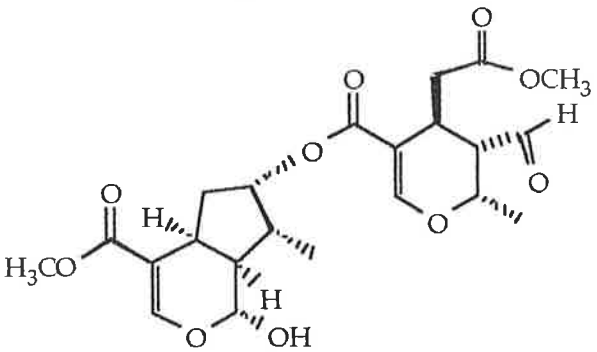
(6)



(7)



(8)



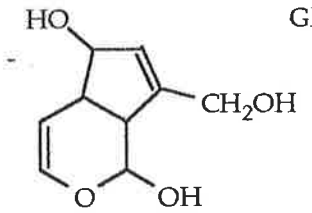
(9)

1.3.1.2 Pharmacological activity of iridoids

The iridoids do not appear to be an especially important class of compounds as physiologically active substances. They have been used for centuries as bitter tonics, sedatives, laxatives, cough medicine, remedy for wounds, against skin diseases and because of their insecticidal or anti-hypotensive effects.[39] Very little investigation of the properties of pure iridoids has been undertaken, and so this summary is a generalised one.

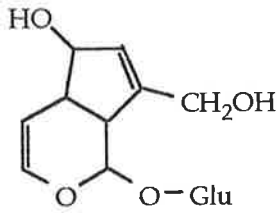
Anti microbial activity

Some iridoids with a very similar structure to those which have been isolated from *Scaevola* genera exhibit remarkable anti microbial activity.[39] Isiguro (1986)[46] indicated that it may be the hemi-acetal structure of the iridoid aglycone, aucubigenin (**10**), that is essential for the manifestation of the anti microbial activities of aucubin (**11**), confirming the previous findings from 1956 - 1966, listed in Sticher.[39]



(**10**)

Glu = glucose



(**11**)

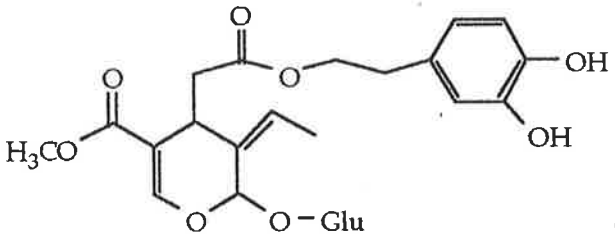
Isiguro (1986)[46] treated aucubin (**11**) with beta-glycosidase and found that the resultant aglycone, aucubigenin (**10**) was very active against *Staphylococcus aureus* while aucubin (**11**) itself was inactive. He was able to confirm the structure of the aglycone from comparisons with previous spectral data.

These results support the pharmacological work of Elich (reported in Sticher[39]) who, in 1962, found that an iridoid aglycone was very effective against *Staphylococcus aureus* . His results showed that 1 mL of a 2% aqueous solution of aucubin (**11**), in the presence of beta-glycosidase, had the same effect as 600 I.U. (international units) of penicillin. However, Elich was unable to isolate the aglycone, as it appeared to be unstable, so that with hydrolysis a poorly soluble dark coloured polymerisation product was formed. It is possible for the aglycone to form a dialdehyde (**12**) structure and that this may be active as an anti bacterial agent.

(12)

Anti hypotensive Effect

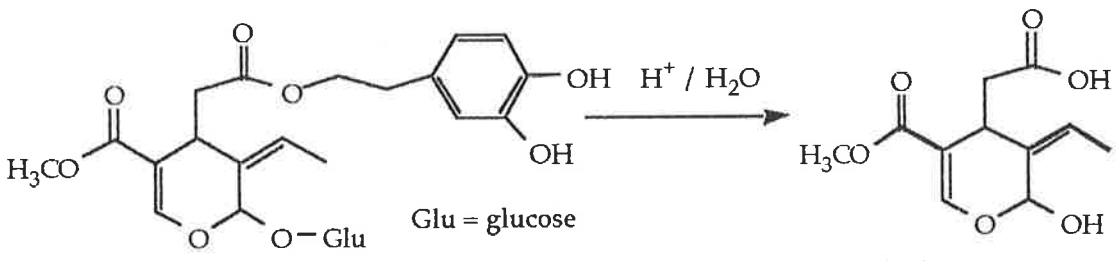
Certain secoiridoids and iridoids exhibit anti hypotensive activity. Oleuropein (13) has been isolated from olive extracts, for which there is both anecdotal evidence and empirical clinical data concerning the healing effect of olive leaves for hypotensive conditions.[39]



(13)

Petkov and Manolov (1975)[47] found that oleuropein (13) is the hypotensive principle of the leaves of the olive tree when they undertook a detailed pharmacological analysis. They found that an extract of the leaves, which contained oleuropein (13), exhibited hypotensive, coronary dilating, anti arrhythmic, anti histamine and anti serotonin activity. When oleuropein (13) is hydrolysed with acid one of the products of the degradation process is elenolic acid (14), which has been shown to possess hypotensive activity. This degradation process is outlined in **Scheme 1.3.2** (Walter, in Sticher[39]).

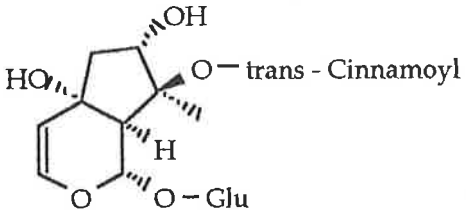
Scheme 1.3.2: Degradation of Oleuropein (13) to form elenolic acid (14)



(13) (14)

Analgesic properties

Harpagocide (**15**) was isolated from the root of *Harpagophytum procumbers*, a South African plant used as a native medicine and its pharmacological activity was investigated. It exhibited some analgesic properties.[39]



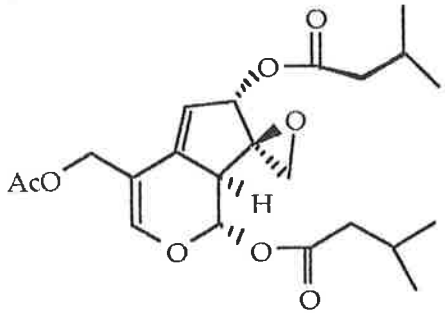
(15)

Bitter tonic

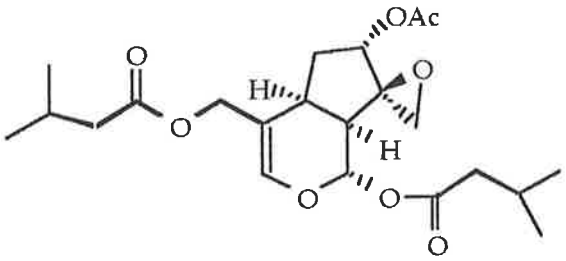
Crude extracts containing Loganin (**5**) are used as a bitter tonic.[39]

Sedative agents

Petkov and Manolov (1975)[47] found a clear CNS depressive action (relaxant, anti convulsive and hypnotic) associated with a crude extract from *Valeriana officinalis* that contained a mixture of "valepotriates", with the main component being valtratum (**16**). This group of non-glucosidic iridoids has been developed by pharmaceutical companies as weak sedatives, known under the collective name "valepotriates" with the general structure of dihydrovaltratum (**17**). They possess a tranquillising effect and an improved co-ordination capacity.[39, 46]



(16)



(17)

Laxative properties

Inouye (1978)[48] investigated the relative purgative activities of thirteen naturally occurring iridoid glycosides, one of which was Loganin (**5**). Loganin has a laxative effect, given as ED_{50} = 0.54 g/kg (50% cathartic dose), with an onset of diarrhoea after four hours.

Anti inflammatory agent

Recio et al (1993)[49] evaluated twelve iridoid glycosides to determine their anti inflammatory activity, using two models. Loganic acid (**4**) was the most active on the carrageenan-induced mouse ear edema test, while loganin (**5**) showed the

highest activity on the TPA induced mouse ear edema test. Recio et al also reported on the hemodynamic (Circosta, 1984), cholenergic (Miyagashi, 1984) and hepatoprotective (Chang, 1983) properties of certain unnamed iridoids.

Their findings suggested only a modest anti inflammatory effect by the iridoids tested. They indicated that certain structural features associated with the iridoid structure determined the anti inflammatory response.

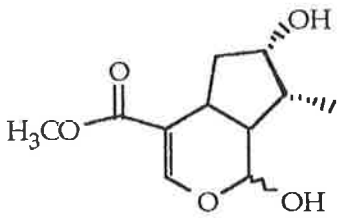
Choleretic action

Takeda (1980)[50] investigated the effect of iridoids on bile secretion and concluded that the hemiacetal moiety of iridoid compounds plays an important role in exerting a strong choleretic activity. All the aglycones tested increased bile secretions after intravenous administration, while the effect of the glycosilated iridoid moiety was not as marked.

Anti tumour activity

Isiguro (1986)[46] investigated the anti tumour activity of iridoid glycosides and their aglycones in mice bearing the experimental tumour Leukaemia P388. They used loganin (**5**) and aucubin (**11**) as well as other glycosides and their aglycones with anti tumour activity evaluated in terms of life prolongation. An *in-vitro* growth inhibition assay was also conducted.

None of the glycosides were active against mice bearing the experimental tumour Leukemia P388, while most of the aglycones were active. The value for loganin aglycone (**18**) was significantly high with a value of 129 % (given as maximum total/control (T/C values) at 100 mg/kg), while aucubinegin (**10**) recorded the highest activity with a value of 162% (given as maximum total/control (T/C values) at 100 mg/kg).



(18)

Their conclusion was that the hemiacetal of the aglycone structure is important for the anti tumour activity of the iridoids tested. They also suggested that it is possible that the role of iridoid compounds as active principles in some medicinal plants may be due to a synergistic activity with other substances.[46]

1.3.2 Alkaloids

Alkaloids have been isolated from *Scaevola racemigera* (Daniker).[51,52] They are members of the monoterpene alkaloid group of plant alkaloids, formed biosynthetically from the iridoids loganin (**5**) and secologanin (**1**) by condensation with ammonia. These alkaloids have been reported previously as genuine plant metabolites.[51,52] It is interesting to note that plants of the *Scaevola* genera possess both iridoids and monoterpene alkaloids.

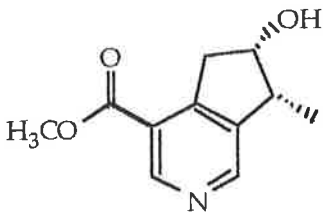
1.3.2.1 Alkaloids from *Scaevola racemigera*

Nine alkaloids (1985)[51] and four dimeric alkaloids (1987)[52] have been isolated from the aerial parts of *Scaevola racemigera* (Daniker), collected from New Caledonia, by analysing the more polar fractions of the extract and using an acid/base extraction.

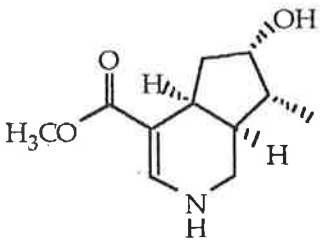
Initially, 4.5 kg of leaves and branches were treated with 10% ammonium hydroxide and cold extracted with dichloromethane, giving a yield of 8 g (0.18% total plant) of alkaloid extract. A second, novel, cold extract of the same material with methanol gave a further 3 g, giving a total of 12 g (0.25% of total plant). Successive silica column chromatography allowed the separation of the nine alkaloids.

However, ammonium hydroxide was used in the initial extraction process so it is not clear whether these alkaloids are plant metabolites or are a consequence of the extraction process.

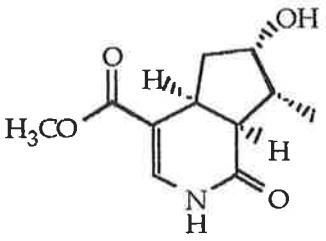
The following alkaloids (**19**) - (**31**) have been isolated from *Scaevola racemigera* (Daniker).[51,52]



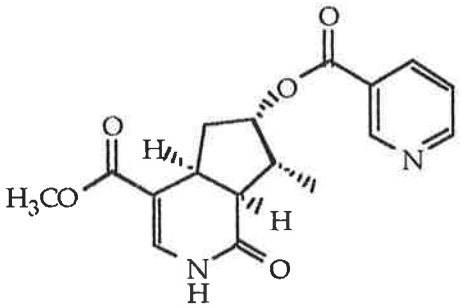
(**19**)
Cantleyine



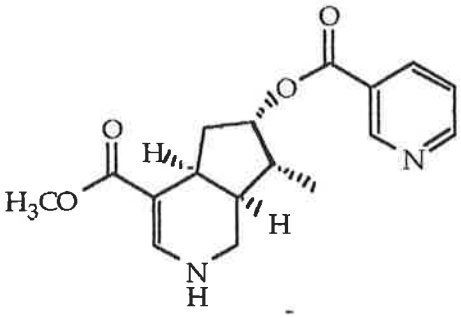
(**20**)
Tetrahydrocantleyine



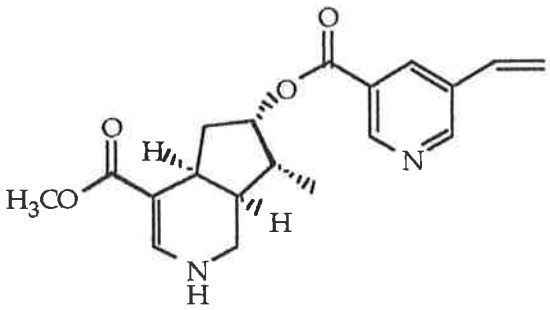
(21)
Strychnovoline



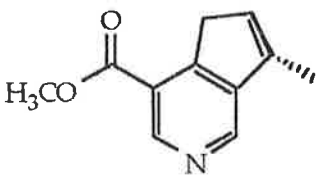
(22)
6-*O*-Nicotinoyl strychnovoline



(23)
6-*O*-Nicotinoyl tetrahydrocantleyine



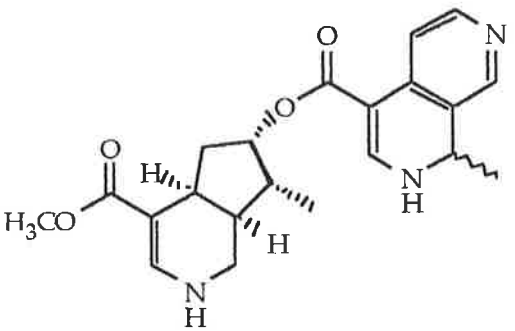
(24)
6-*O*-(5-vinyl nicotinoyl)-6-tetrahydrocantleyine



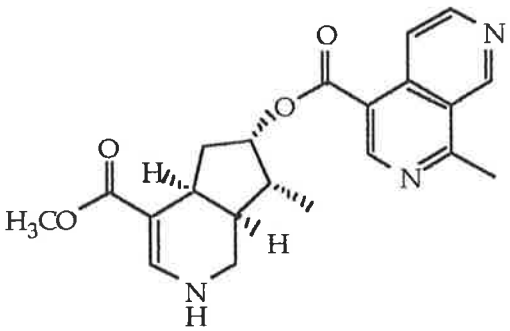
(25)
Racemigerine

(26)
6,7-epoxy racemigerine

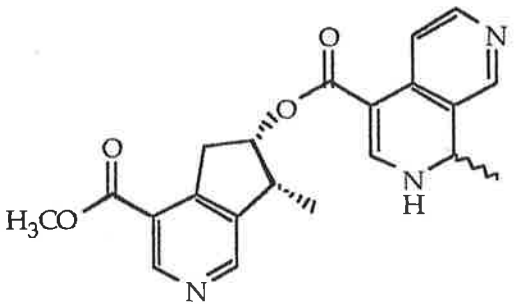
(27)
Scaevoline



(28)
Scaevodimerine A

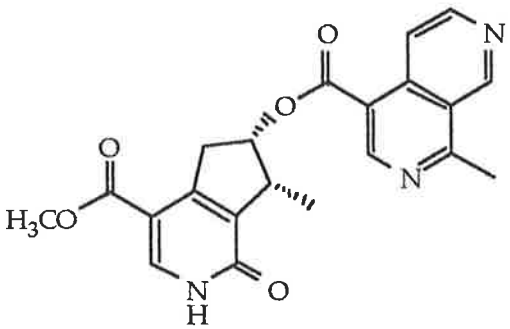


(29)
Scaevodimerine B



(30)

Scaevodimerine C



(31)

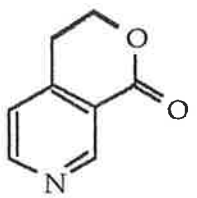
Scaevodimerine D

Skaltsounis[44] reported the synthesis of cantleyine (**19**) and tetrahydrocantleyine (**20**) from loganin (**5**) with the first step being the hydrolysis of the glycoside to form the loganin aglycone (**18**) followed by condensation of the hemiacetal with ammonia.

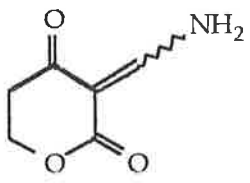
1.3.2.2 Pharmacological activity of alkaloids

There are many instances of alkaloids being biologically active. Of interest are examples of monoterpene alkaloids that possess structural similarities to the alkaloids that have been isolated from *Scaevola* species and which appear to have been formed biosynthetically from iridoids.

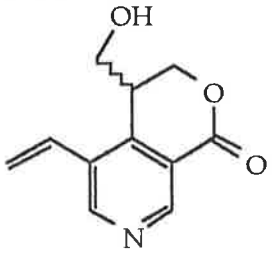
Gentianadine (**32**) is very mildly toxic and has hypotensive, hypothermic, anti inflammatory and muscular relaxant activity, while gentianaine (**33**) and gentianamine (**34**) both possess anti inflammatory activity. Gentianine (**35**) is a CNS stimulator, which at higher doses has a paralysing effect. It also has hypotensive, anti inflammatory and muscular relaxant activity. Tecomaine (**36**) is used to treat diabetes in Mexico as it has hypoglycaemic properties.[53a]



(32)

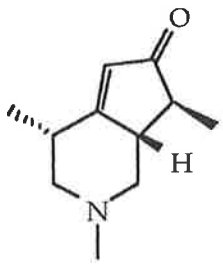


(33)



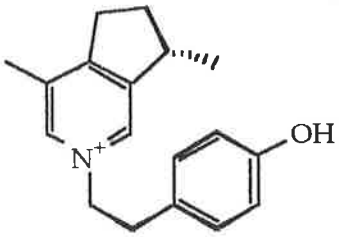
(34)

(35)

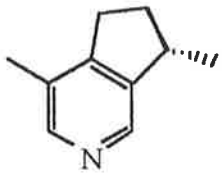


(36)

N-(*p*-Hydroxyphenethyl) actinidine (**37**) is a highly active inhibitor of cholinesterase activity, while actinidine (**38**) itself does not appear to have pharmacological activity. Actinidine does cause excitation in cats and occurs in defensive secretions of certain ants and beetles.[53a]

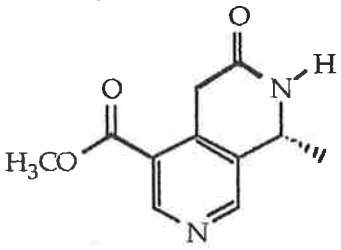


(**37**)

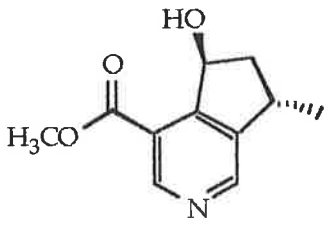


(**38**)

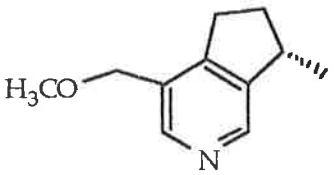
Jasminine (**39**), rhexifoline (**40**) and valerianine (**41**) have very similar structures to the iridoids isolated from *Scaevola* species but do not appear to possess any pharmacological activity.[53a]



(**39**)



(**40**)



(**41**)

1.3.3 Coumarins

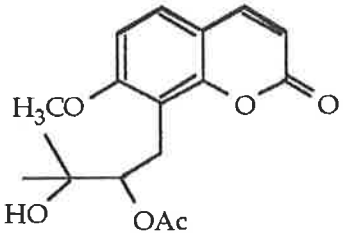
Coumarins are widely distributed within the plant world and are an important class of natural products. Coumarin itself is the sweet-smelling constituent of white clover, and a large number of hydroxyl and methoxy coumarins and their glycosides have been isolated from plant sources.[53b]

1.3.3.1 Coumarins from plants of the *Scaevola* genus

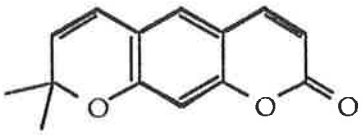
Furanocoumarins are specifically mentioned as the possible biologically active constituents of *Scaevola spinescens*[6,8,13] and were also mentioned by Wohlrabe[54] in a report on *Scaevola taccada* although specific compounds were not reported.

The three classes to which coumarins found in plants of the *Scaevola* species belong are firstly, ones where hydroxyl or methoxy groups are substituted on the ring eg. Unnamed coumarin (**42**). Secondly there are isoprenoid substituents

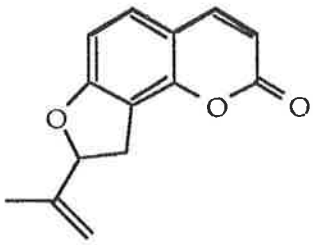
attached to the ring eg. xanthyletin (**43**), while the third class of compound are the furano coumarins that are coumarins with an annulated furan or dihydrofuran ring attached eg. angenomalin (**44**).[53b]



(**42**)



(**43**)



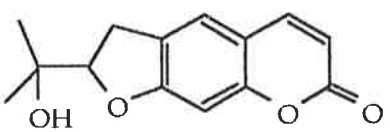
(**44**)

Coumarins are generally extracted with non-polar solvents such as benzene or dichloromethane and then isolated by silica column chromatography using a mixture of non-polar solvents.

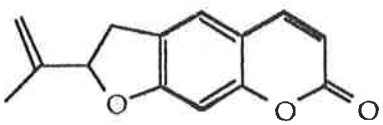
Seven coumarins have been reported as having been isolated from species of the *Scaevola* genera.[35,36, 54, 55, 56] Wohlrabe (1977)[54] and Bohlmann (1975)[55] investigated *Scaevola frutescens* while Kikuchi (1974)[56] investigated *Scaevola lobelia*. (*Scaevola frutescens* and *Scaevola lobelia* are now classified as *Scaevola taccada*). Therefore the coumarins that have been reported from these two sources have all been isolated from the same species, *Scaevola taccada*. This data is displayed in **Table 1.3.3.1**.

Table 1.3.3.1: Coumarins from two species of *Scaevola*

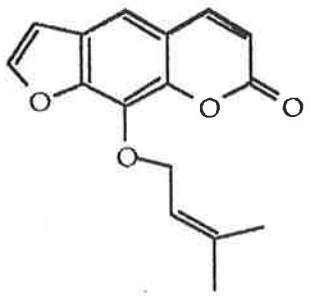
Compound	*Scaevola spinescens* [35, 36]	*Scaevola taccada* [54, 55, 56]
Unnamed coumarin XI (**42**)		* 56
Xanthyletin (**43**)	*35	* 56
Angenomalin (**44**)		* 55
Nodakenetin/marmesin (**45**)	*36	* 54
Isoangenomalin (**46**)		* 55
Imperatorin (**47**)		* 54
Unnamed coumarin IX (**48**)		* 56



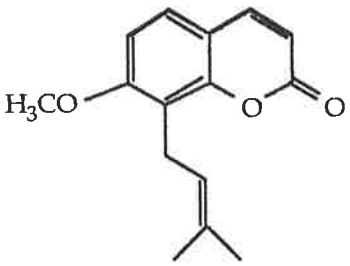
(**45**)



(**46**)



(47)



(48)

1.3.3.2 Pharmacological activity of coumarins from *Scaevola*.

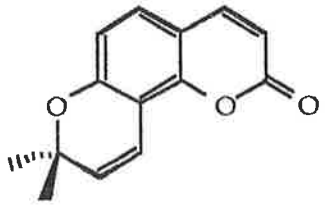
Coumarins are often isolated from higher plants, and many are known to possess pharmacological activity. Of particular interest is the biological activity of coumarins that have previously been isolated from plants of the *Scaevola* genus.

Imperatorin (**47**) is known to have weak activity against Hela-cell proliferation and some anti-mutagenic properties[53b], while Kumar[57] indicated that (**47**) possesses insecticidal activity.

Nodakenetin (**45**) is known to inhibit human platelet aggregation in vitro.[53b] Namba[58] investigated a number of coumarins, including nodakenetin, with respect to their ability to inhibit Ca^{2+} influx by blocking Ca^{2+} channels. Namba found that nodakenetin (**45**) was very successful and had the potential to be developed as a Ca^{2+} channel blocker.

Xanthyletin (**43**)[53b] has been shown to exhibit powerful anti tumour and anti bacterial activity. Gunatilaka[59] tested xanthyletin (**43**) and seselin (**49**) - a structural analogue of xanthyletin - against a mechanism-based bioassay, employing DNA - repair deficient (rad 6 and rad 52Y) and repair proficient (RAD^+) yeast strands The rad 6 represents a yeast mutant deficient in the error-prone repair pathway and the rad 52Y is deficient in a recombinational pathway associated with repair of double-strand breaks and meitotic recombination.

Both (**43**) and (**49**) had detectable activity to rad 52Y strain at a dose of 500 ug/ml. When tested with other yeast strains, (**49**) showed selective activity against the rad 52Y yeast as compared to the RAD^+ strain, *"indicating that it functions as a DNA-damaging agent."*[59]



(49)

Takeuchi's investigations (1991)[60] showed that xanthyletin (**43**) exhibited a dose-related increase in vertebral, carotid and femoral blood flow in guinea pigs, as well as decreasing blood pressure and increasing heart rate. His results indicate that seselin (**49**) is a more effective analogue than xanthyletin (**43**).

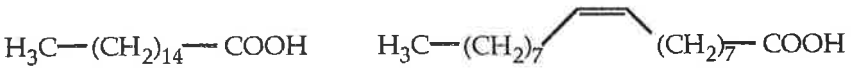
1.3.4 Fatty acids, lipids and hydrocarbons

Fatty acids, lipids and hydrocarbons are generally simple in structure and there are a large number of different compounds found in the plant kingdom. Fatty acids occur as the free acids, as esters with long alkyl chains (wax esters) and as glycerol esters.

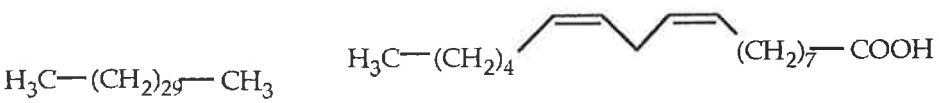
They usually comprise a C_{12} - C_{20} hydrophobic tail with a carboxylic acid functional group as the 'head', which then forms an ester with glycerol. If the three fatty acids that bind to the glycerol are identical, a 'simple' triglyceride is formed. However, if the fatty acids are different the more common 'mixed' triglyceride is formed. There are several hundred fatty acids that have been isolated from plants, of which a few are consistently found.

1.3.4.1 Fatty acids, lipids and hydrocarbons isolated from *Scaevola* species.

Palmitic acid (**50**), oleic acid (**51**) and hentriacontaine (**52**) have been isolated from *Scaevola spinescens* (1996)[36] while linoleic acid (1993)[61] (**53**) has been isolated from *Scaevola taccada*.

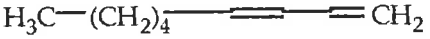


(50) (51)



(52) (53)

Bohlmann (1975)[55] also reported a diene (**54**) from *Scaevola taccada*.



(54)

1.3.4.2 Pharmacological activity of fatty acids and hydrocarbons from plants of the *Scaevola* genus

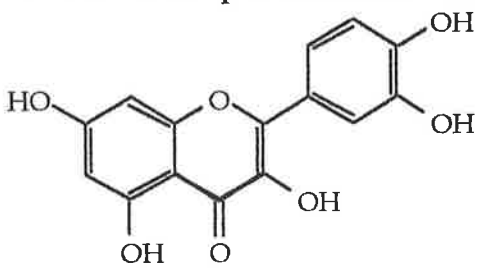
Palmitic acid (**50**) is a major component of most fats and is therefore a constituent of plant lipids. It is a major component, for example, of palm oil. It is used as a lubricant and as an emulsifying agent, but does not appear to possess any pharmacological activity.[53c] Oleic acid (**51**) is also widespread in vegetable fats and oils and is used in assisting the absorption of drugs through the skin.[53c]

Hentriacontaine (**52**) occurs in many plant waxes and is used in lipsticks and other cosmetics, in chewing gum and as a protective coating for certain citrus fruits.[53c] Linoleic acid (**53**) occurs widely in plant lipids.

1.3.5 Flavonoids

There are a range of flavonoids that have been found widely distributed in plants and are found as co-pigments to the anthocyanins in petals (to give the colour) and in the leaves of higher plants.

Both the flavones and flavonols occur as aglycones but they are often glycosylated, with a wide range of glycosides. There are over 135 different glycosylated compounds derived from quercetin (**55**).[53d]



(**55**)

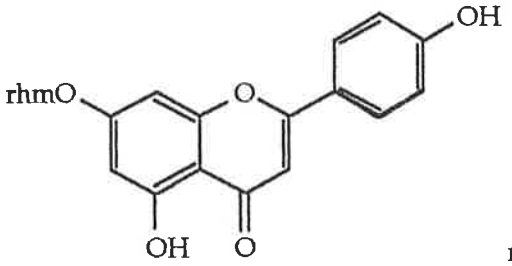
One technique used for the initial identification of flavonoids is that of using their unique UV absorption, chromatographic mobility (TLC) and colour reactions. These techniques are outlined in Harbourne.[62]

1.3.5.1 Flavonoids identified from *Scaevola* genus

Patterson (1984)[63] used the UV absorption and chromatographic characteristics of flavonoids to investigate the adaptive radiation of seven species of *Scaevola* found in the Hawaiian Islands. In most cases, adaptive radiation is investigated using such factors as leaf size, leaf patterns, inflorescence and features of the flower. Patterson, however, used the occurrence of secondary metabolites - in this

case flavonoids - to attempt to outline the adaptive radiation of the seven species of *Scaevola* found on different islands of Hawaii, as well as one hybrid species.

The eight species of *Scaevola* studied are outlined in **Table 1.3.5.1.1** along with the flavonoids identified within each species. Patterson found seven flavonoid compounds readily apparent on chromatograms of the crude extracts of the species.[63] The only flavone isolated was apigenin-7-*O*-rhamnoside (**56**).



rhm = rhamnose

(56)

Table 1.3.5.1.1: Flavonoids isolated from *Scaevola* species[63]

	Species of *Scaevola*							
Flavonoid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Apigenin-7-*O*-rhamnoside	x	x	x	x	x	x	x	x
Quercetin-3-*O*-glucoside	x	x	x	x	x	x	x	x
Quercetin-3-*O*-rhamnoside		x	x	x	x	x	x	x
Quercetin-3-*O*-rutinoside	x	x	x	x	x	x	x	x
Quercetin-3,7-*O*-diglucoside	x	x	x	x	x	x	x	x
Quercetin-3-*O*-glu-7-*O*-rhm	x	x	x		x	x	x	x
Quercetin-3-*O*-gal-7-*O*-rhm		x						

1 = *Scaevola chamissoniana* Gaudich
2 = *S. coriacea* Nutt
3 = *S. gaudichaudiana* Cham
4 = *S. gaudichaudii* Hook & Arn
5 = *S. kilaueau* Degenes
6 = *S. mollis* Hook & Arn
7 = *S. procera* Hillebrand
8 = hybrid - *S. gaudichaudiana* x *mollis*

glu = glucoside
rhm = rhamnoside
gal = galactoside

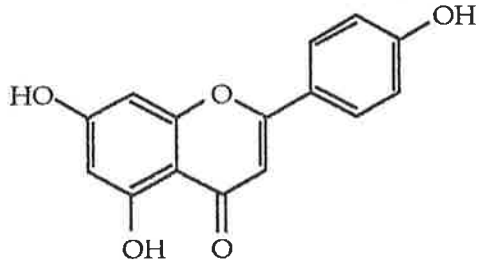
The other six compounds identified were glycosylated quercetin (**55**) derivatives classified as flavonols. These quercetin (**55**) derivatives differed in both the type of sugar attached and the point of attachment. Three of the quercetin (**55**) derivatives were mono-glycosylated with glucose, rhamnose and rutinose at the 3 position, while the other three derivatives were di-glycosylated at the 3 and 7 position with a combination of glucose, rhamnose and galactose.

Patterson's data indicated that the flavonoid features are more or less uniform for the entire group, including the hybrid, even given a wide range of habitat variation and morphological diversity.

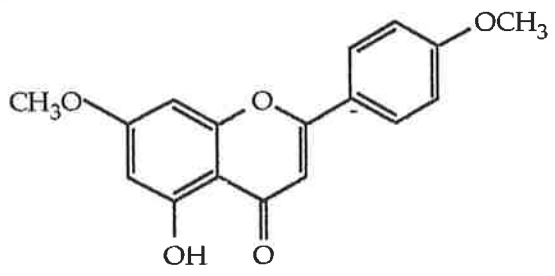
1.3.5.2 Pharmacological activity of flavonoids from plants of the *Scaevola* genus

Many flavones and flavonols are biologically active in a range of ways.

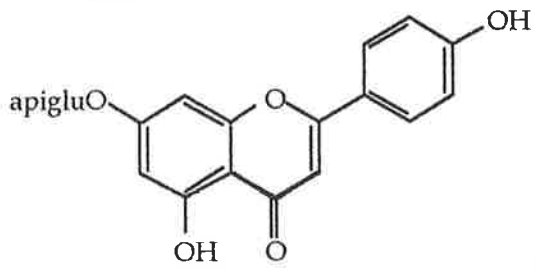
Apigenin (5,7,4′-trihydroxylflavone) (**57**) has been shown to have anti bacterial, anti inflammatory, diuretic and hypotensive activities. It has also been shown to inhibit many enzymes and to promote smooth muscle relaxation. Apigenin-7,4′-dimethyl ether (**58**) has both anti inflammatory and anti nuclear activities, while in terms of glycosylated apigenin-type flavones, apigenin-7-apiosylglucoside (**59**) is an inhibitor of lens aldose reductase.[53d]



(**57**)



(**58**)



apiglu = apiose-glucose

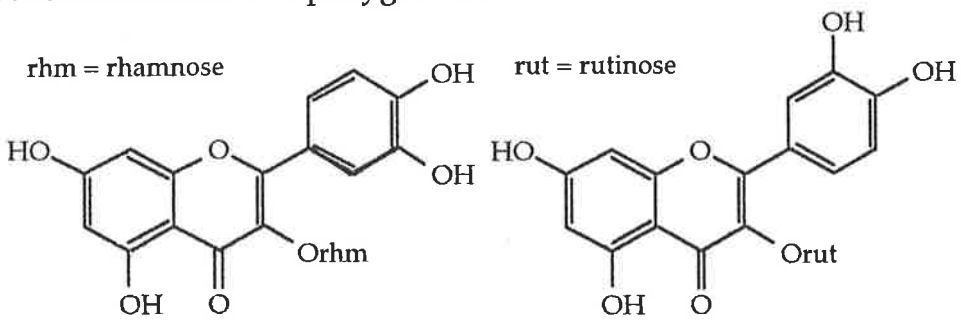
(**59**)

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) (**55**) is considered to be the most common flavonoid to be found in higher plants and has a range of pharmacological properties. It is known to inhibit many enzymes, such as protein kinase C, lipogenases, lens aldose reductase and 3′, 5′-cyclic adenosine monophosphate phosphodiesterases. It is a radical scavenger and is also able to inhibit smooth muscle contraction. It is known to be anti gonadotropic, anti inflammatory, anti bacterial, anti viral and shows some mutagenic activity and allergenic properties.[53d]

The glycosides of quercetin are also biologically active. Quercitrin (**60**) (quercetin-3-*O*-rhamnoside) has anti mutagenic, anti ulcer, anti viral, anti haemorrhage and

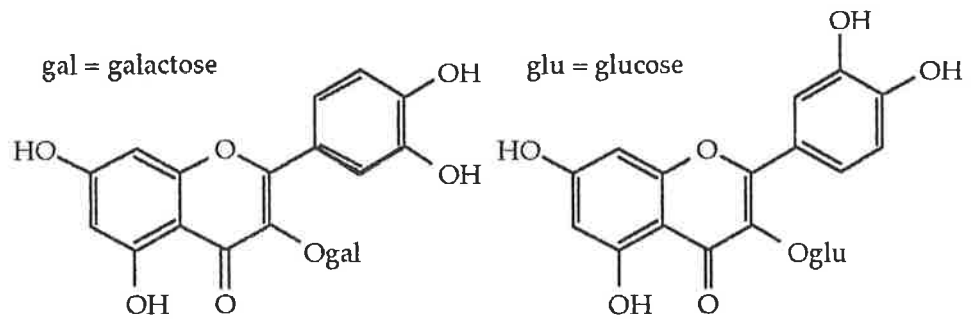
anti hepatotoxic activities, as well as anti bacterial activity against *Pseudomonas maltophilia* and *Enterobacter cloacae*. Quercitrin (**60**) is also known to be a strong inhibitor of lens aldose reductase.[53d]

Rutin is the common name for quercetin-3-*O*-rutinoside (**61**) and it has a wide variety of biological uses. It is used medicinally against capillary fragility and varicosis. Rutin also exhibits anti viral and anti bacterial activities and it inhibits lens aldose reductase and 5-lipoxygenase.[53d]

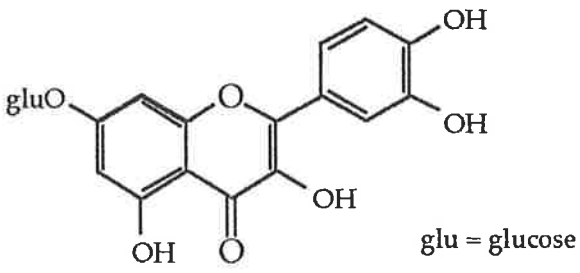


(**60**) (**61**)

Quercetin-3-*O*-galactoside (**62**) is also known as hyperin and has been known to be a potent inhibitor of lens aldose reductase and it has anti bacterial activity against *Pseudomonas maltophilia*.[53d] Isoquercitrin (**63**) (quercetin-3-*O*-glucoside) does not occur in the seven *Scaevola* species tested, but is known to also inhibit lens aldose reductase as well as show anti bacterial activity against *Pseudomonas maltophilia*.[53d] Quercetin-7-*O*-glucoside (**64**) is known as quercimeritrin and appears to have no pharmacological properties (as reported in Harbourne).[53d]



(**62**) (**63**)



(64)

The types of glycosylated flavones and flavonols (in particular) which have been found in the seven species of *Scaevola* from Hawaii indicate a range of biological activities.

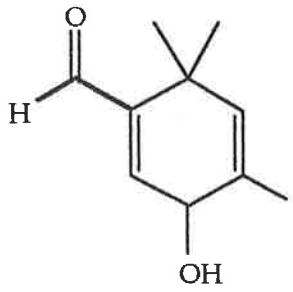
1.3.6 Terpenoids

There are a large number of classes of organic compounds, which are based on the biosynthetic pathway associated with the isoprene-repeating unit. Terpenoid compounds are defined as a 'group of natural products whose structure may be divided into isoprene units ... (the) ... classification of the terpenes depends upon the number of isoprene units'[64] (monoterpenes - C_{10}, sesquiterpenes - C_{15}, diterpenes - C_{20} and triterpenes - C_{30}).

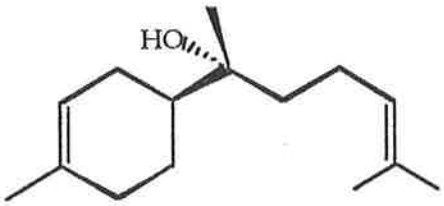
1.3.6.1 Terpenoids isolated from species of *Scaevola*

Bohlmann,[55] in 1975, reported isolating a number of esters of the monoterpene ferulol (**65**). He isolated them from *Scaevola lobelia*, which has since been systematically named *Scaevola taccada*. However, no other information is available about which esters were isolated.

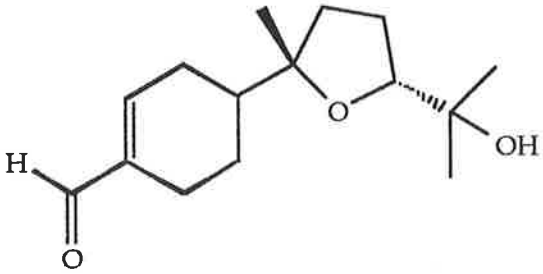
Kerr, Longmore and Yench (1999)[38] report the isolation of two sesquiterpenes, one of which is a novel compound, isolated from the methanol fraction of *Scaevola spinescens*. The two compounds are α-bisabolol (**66**) and scaevolal (**67**) (7,10-epoxy-11-hydrobisabolol-3-en-15-al).



(65)

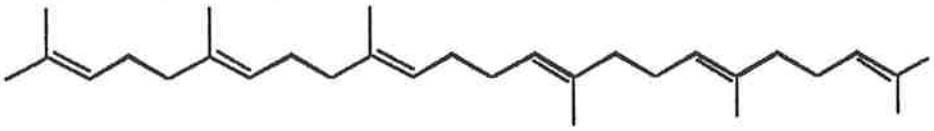


(66)



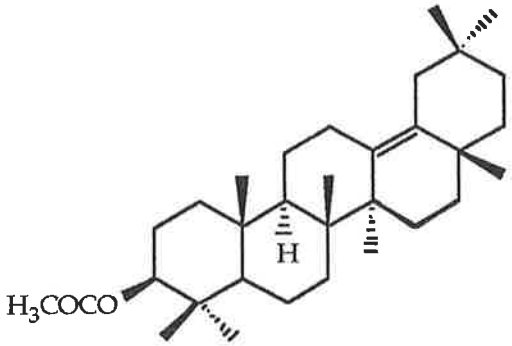
(67)

The triterpenes are based on the cyclization of squalene (**68**) to form either tetracyclic or pentacyclic ring systems.



(68)

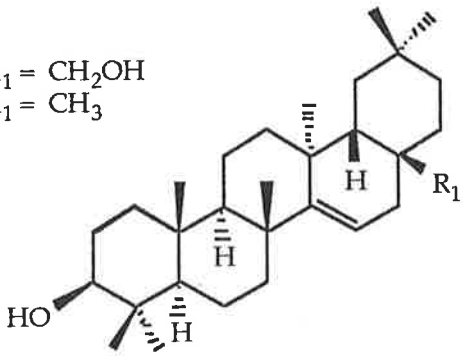
Bohlmann (1975)[55] reported the isolation of 19-H-13-(18)-dehydrogermanicol acetate (**69**) from *Scaevola taccada*.



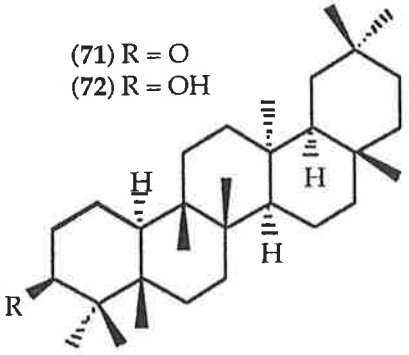
(69)

Kikuchi (1974)[56] reported the isolation of seven triterpenes from *Scaevola frutescens* (now known as *Scaevola taccada*). They were identified as myricadiol (**70**), friedelin (**71**), epifriedelanol (**72**), taraxerol (**73**), α-amyrin (**74**), γ-amyrin acetate (**75**), and γ-amyrone (**76**).

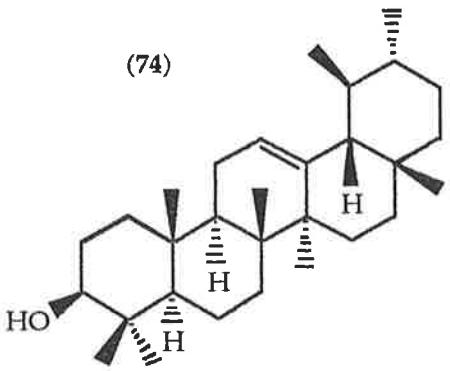
(70) R_1 = CH_2OH
(73) R_1 = CH_3



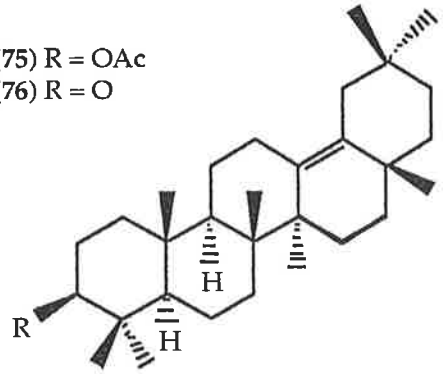
(71) R = O
(72) R = OH



(74)

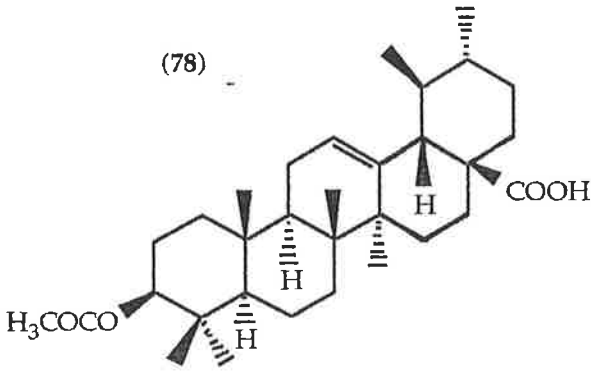


(75) R = OAc
(76) R = O

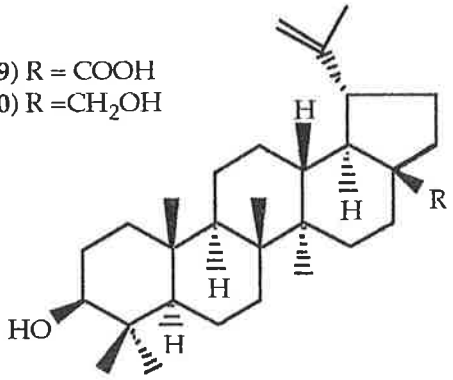


Cambie (1997)[45] reported the isolation of five triterpenes and six novel γ-amyrin fatty esters with C_{20} - C_{30} acid moieties from *Scaevola floribunda*, a Fijian plant. The triterpenes isolated were γ-amyrin acetate (**75**), γ–amyrin (**77**), ursolic acid acetate (**78**), betulinic acid (**79**) and betulin (**80**).

(78)

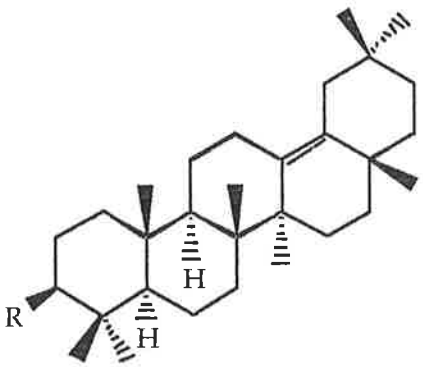


(79) R = COOH
(80) R = CH_2OH



The six novel fatty acids attached to the γ-amyrin (**77**) molecule formed the following amyrinic fatty acids: γ-amyrin eicosanoate (**81**), γ-amyrin docosanoate (**82**), γ-amyrin tetracosonanoate (**83**), γ-amyrin hexacosanoate (**84**), γ-amyrin octacosanoate (**85**) and γ-amyrin triacontanoate (**86**).

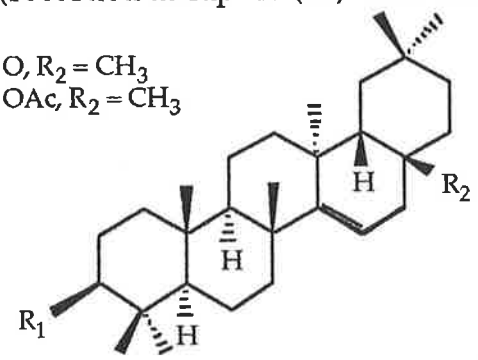
(77) R = OH
(81) R = $OCOC_{19}H_{39}$
(82) R = $OCOC_{21}H_{43}$
(83) R = $OCOC_{23}H_{47}$
(84) R = $OCOC_{25}H_{35}$
(85) R = $OCOC_{27}H_{55}$
(86) R = $OCOC_{29}H_{59}$



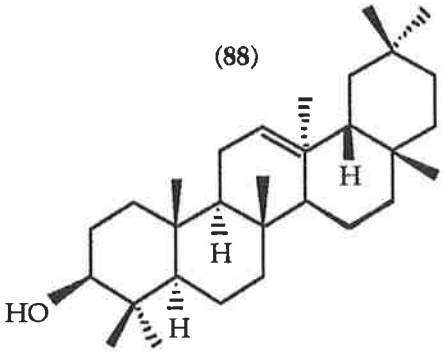
Kerr (1996)[36] and (1997)[35] reported, using MS peak-matching techniques, the identification of a total of nine triterpenes and two phytosterols from *Scaevola spinescens*. The nine triterpenes isolated were squalene (**68**), myricadiol (**70**),

taraxerol (**73**), taraxerone (**87**), β-amyrin (**88**), lupeol (**89**), lupeol acetate (**90**) lupenone (recorded as lup-20-(29)-en-3-one) (**91**) and taraxerol acetate (**92**).

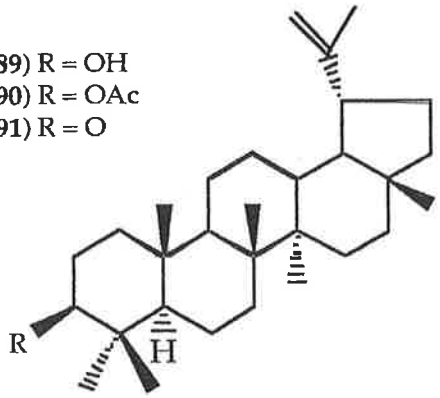
(87) $R_1 = O, R_2 = CH_3$
(92) $R_1 = OAc, R_2 = CH_3$



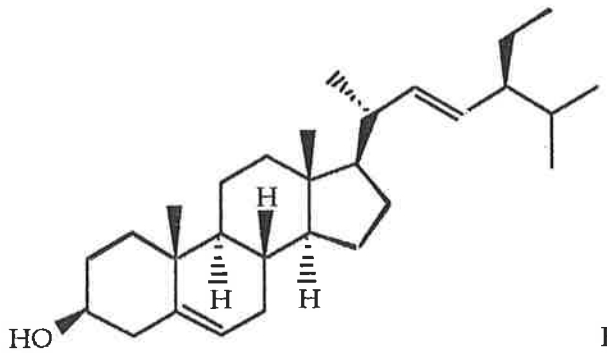
(88)



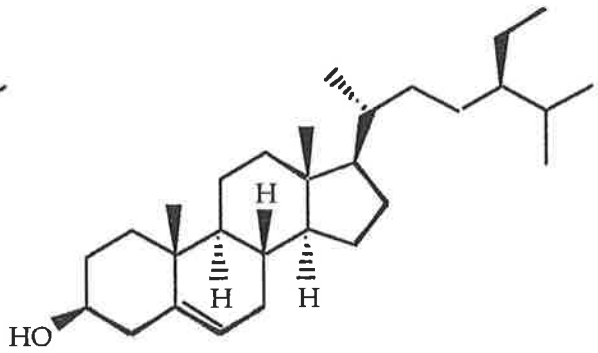
(89) R = OH
(90) R = OAc
(91) R = O



The two phytosterols reported were (3β,22E)-stigmasta-5-22-dien-3-ol (**93**) and (3β,24S)-stigmast-5-en-3-ol (**94**). Kerr also reported isolating β-sitosterol in the same paper but β-sitosterol is the common name for (3β, 24S)-stigmast-5-en-3-ol (**94**) so that these two compounds would appear to be the same.



(**93**)



(**94**)

All the terpenoids identified from three species of *Scaevola* are outlined in **Table 1.3.6.1** with a total of thirty terpenoids being reported.

Table 1.3.6.1: Terpenoids from three species of*Scaevola*

		Scaevola		
		taccada	*spinescens*	*floribunda*
Monoterpenes	Esters of Ferulol (**65**)	*55		
Sesquiterpenes	α-Bisabolol (**66**)		*38	
	Scaevolal (**67**)		*38	
Triterpenes:	Squalene (**68**)		*35 *36	
	19-H,13(18)dihydroxygermanicol acetate (**69**)	*55		
	Myricadiol (**70**)	*56	*35 *36 *38	
	Friedelin (**71**)	*56		
	Epifriedelanol (**72**)	*56		
	Taraxerol (**73**)	*56	*35 *36	
	α-Amyrin (**74**)	*56		
	γ-Amyrin acetate (**75**)	*56		*45
	γ-Amyrone (**76**)	*56		
	γ- Amyrin (**77**)		*36	*45
	Ursolic acid acetate (**78**)			*45
	Betulinic acid (**79**)			*45
	Betulin (**80**)			*45
	γ-Amyrin eicosanoate (**81**)			*45
	γ-Amyrin docosanoate (**82**)			*45
	γ-Amyrin tetracosanoate (**83**)			*45
	γ-Amyrin hexacosanoate (**84**)			*45
	γ-Amyrin octacosanoate (**85**)			*45
	γ-Amyrin triacotanoate (**86**)			*45
	Taraxerone (**87**)		*35 *36	
	β-Amyrin (**88**)		*36	
	Lupeol (**89**)		*35 *38	
	Lupeol acetate (**90**)		*35	
	Lupenone(**91**)		*35	
	Taraxerol acetate (**92**)		*35 *36	
Phytosterols:	Stigmasterol (**93**)		*36	
	β-sitosterol (**94**)		*36	

*35 = reference in the literature

1.3.6.2 Pharmacological activity of terpenoids isolated from species of *Scaevola*

In December of 1996 Kerr, Longmore and Betts[36] identified a number of pentacyclic triterpenoid compounds isolated from *Scaevola spinescens*. They used the GC-MS technique with peak matching of libraries of MS data, and hence were able to identify previously isolated compounds. These compounds were isolated from non-polar fractions.

They identified myricadiol (**70**) as the major component of their fractionation process, yielding 0.56% of the dry weight of the plant. They state that this is considerably higher than the yield of myricadiol (**70**) in other species. Kerr, Longmore and Betts therefore have postulated the use of the compound within the plant as a potential anti cancer drug.[36]

Kerr (1999)[38] reported the testing of extracts from *Scaevola spinescens* for possible anti microbial activity against *Bacillus subtilis* and other representative micro-organisms, as well as investigating the extracts for their ability to inhibit tumour formation in the potato Crown Gall tumour (CGT) assay . This assay indicates anti tumour activity of test compounds by their inhibition of the formation of characteristic crown galls induced in wounded potato tissue by the organism *Agrobacterium tumefaciens*.

The results of the CGT assay indicated that the most active fraction was a freeze-dried aqueous extract of 68.8% (percentage inhibition of tumour formation) while myricadiol (**70**) and scaevolal (**67**) had an inhibition of 53.2% and 50.0% respectively (concentrations of (**67**) and (**70**) are not given).

The results for the anti microbial tests indicated that α-bisabolol (**66**) and scaevolal (**67**) displayed inhibitory activity against *B. subtilis* although the individual results are not recorded as the sample tested was a mixture.

Their overall conclusion was that the most significantly active compounds in the CGT assay included scaevolal (**67**), myricadiol (**70**) and lupeol (**89**). Kerr also reported that these three terpenoids, as well as α-bisabolol (**66**), were also mildly inhibitory against *Bacillus subtilis*, but not in the presence of tetrazolium red (a redox chromogenic reagent used to visualise live bacterial cells and colonies).

Kerr (1996)[35] also reported the isolation of the following compounds from *Scaevola spinescens* - squalene (**68**), myricadiol (**70**), taraxerol (**73**), taraxerone

(87), lupeol (89), lupeol acetate (90), lupenone (91) and taraxerol acetate (92) and that these compounds exhibited the following:

"Bioactivities, where previously reported include the following: mineralocorticoid, bactericidal, immunostimulatory, anti tumour, anti ulcer, gastric anti secretory, and hypotensive activities."

He also suggested that the results of screening on the US National Cancer Institute (NCI) tumour panel assay provide evidence:

"In support of the anecdotal reputation of Scaevola as a medicinally active, potentially important cancer chemotherapeutic agent or source material."

There are many reports on the biological activity of terpenoids that have been isolated from *Scaevola* species. **Table 1.3.6.2** gives a summary of the types of biological activity associated with the terpenoids isolated from *Scaevola* species.[53e]

Table 1.3.6.2: Pharmacological activity of triterpenes from *Scaevola* species

Compound	Activity
Squalene (68)	Bactericidal and anti tumour activities. Immunostimulant.
Friedelin (71)	Diuretic activity.
Taraxerol (73)	Anti ulcer and gastric anti secretory activity.
Betulinic acid (79)	Anti tumour activity: active against the Walker carcinoma 256 tumour system.
Betulin (80)	Anti tumour activity: active against the Walker carcinoma 256 tumour system.
Lupeol (89)	Anti tumour activity: active against the Walker carcinoma 256 tumour system. Anti hyperglycaemic and hypotensive activity.
Lupeol acetate (90)	Anti hyperglycaemic and anti ulcer activity.
β-Sitosterol (94)	Anti hyperlipoproteinaemic activity.

CHAPTER 2 GENERAL PROCEDURES

2.1 Collection

Scaevola spinescens is very widespread throughout semi-arid and arid South Australia. After initial consultation with the South Australian State Herbarium, a small sample was collected from a site 10 km west of Morgan, on the Morgan-Eudunda Road. A picture of the collection site is given in *Figure 2.1*.

Figure 2.1: Picture of collection site (Photo: Sally Nobbs, 2000)

The sample was sent to the State Herbarium, where it was verified as being *Scaevola spinescens* – Voucher Number AD 99702040.

12.7 kg of the aerial parts of the plant (leaves and branches) was then collected from this site.

2.2 General treatment of collected samples

The sample (12.7 kg) of *Scaevola spinescens* was ground to a powder using a ball mill. The powder was then sequentially soaked in four different solvents for two

weeks each. Initially it was soaked in hexane, then ethyl acetate, then methanol and finally water. The solvents were removed using a cyclone evaporator.

The methanol fraction was then subjected to separation by flash chromatography, which is outlined in Chapter 3 in more detail.

Each fraction initially isolated was analysed to determine its purity by TLC - either under UV light or by using specific reagents. A series of TLC plates were used to follow the elution of compounds from the flash columns (using silica) as in normal procedure.

Flash chromatography was conducted on the crude fractions, with continual fractionation until such time as the samples appeared pure (1 dot on TLC under at least two different solvent conditions).

It was often necessary, initially, to use large scale flash chromatography (up to 150 g) and a useful reference for large-scale chromatographic techniques is to be found in Claeson[65] that deals with samples up to 200 g. Still[66] was also informative in determining optimum conditions for large scale flash chromatography.

Preparative TLC plates were also used when the compounds to be separated:
(a) were stable
(b) could be removed off the silica
(c) were UV active
(d) were separated by 1 cm on the plate and
(e) if there was between 5 - 10 mg of sample to be separated.

If there was sufficient sample (>50 mg) recrystallization techniques were used to purify the sample. The melting point (MP) of the crystals was then measured and purity assigned if the MP range was very small. However, if there was less than 50 mg of sample, this step was not attempted until all spectroscopic experiments had been completed and a final structure had been deduced.

If there was less than 50 mg of sample, or if the sample would not crystallize, analytical HPLC - either normal or reverse phase, depending on the compound's polarity - was used to analyse the purity of the sample. UV detection was by fixed wavelength. The sample was deemed to be pure if it was one peak under at least two different solvent conditions - not only different solvents, but also using gradient conditions.

Analytical and reverse phase HPLC was also used to purify mixtures, with the aim being the development of a particular solvent protocol so that the compounds to be separated would elute with at least a one minute gap between each peak.

Semenova[67] was very useful in determining optimum HPLC separation conditions (reverse phase HPLC, using methanol/water gradient systems) for a number of separations.

A chloroform/methanol/water extraction was used on a number of the more polar extracts, as a means of removing non-polar compounds with the resultant water extract subjected to reverse phase HPLC.

All solvents were distilled before use with drying and purification of solvents and reagents performed using standard laboratory procedures.[68,69]

2.3 Structure determination

Once a compound was isolated its purity was assessed by consideration of the following criteria:
(a) the compound having a narrow melting point range
(b) the compound being one peak on analytical HPLC (under at least two different solvent conditions) or
(c) the compound being one spot on TLC (under at least two different solvent conditions).

A range of spectroscopic experiments was then conducted, in a reasonably set order. A major factor to consider was the amount of sample purified, as this would determine the order in which certain functional group tests were conducted. If over 50 mg of sample was isolated and purified, then the order of spectroscopic analysis was not important, as the fear of loss of compound was not as evident. However, many of the samples that were separated were less than 10 mg and the following order of testing was adopted:
1. UV absorption
2. All NMR experiments that were necessary
3. MS analysis
4. Optical Rotation
5. Infra Red
6. Melting point (if possible)

2.3.1 UV and visible spectrum

Some classes of compounds, such as the flavonoids, can be identified using only their UV spectrum, although this usually applies to known compounds. This is the technique that was used by Patterson[63] to identify the flavones and flavonols that were present in eight species of *Scaevola* found on the Hawaiian Islands, and is based on the known chromophore for a particular compound (see Chapter 1.3.5.1). Harbourne[62] is used as the reference for this type of identification. The chromophore for flavones, flavonols[62] and coumarins[70], for example, is quite diagnostic for the general structure.

UV is a simple technique that does not use much sample (1 mg in 100 mL) and the sample is recoverable. It relies upon the excitation and relaxation of electrons, generally between a bonding or lone-pair orbital and an unfilled non-bonding or anti-bonding orbital. The wavelength of absorption will be a measure of the energy level separation of the orbitals concerned and will be unique for different natural products. The resulting chromophore can be analysed to determine the types of functional groups present.

The λ_{max} (the wavelength of ultra violet light that gave the maximum absorbence) of a mixture and of a pure sample was routinely recorded to aid in setting the detector wavelength when doing HPLC.

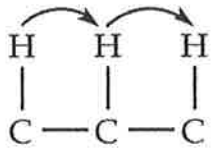
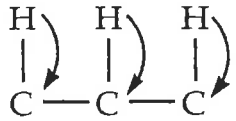
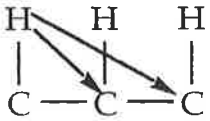
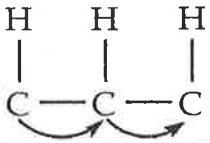
UV was also used when analysing TLC plates (preparative or analytical) to follow the position of a sample if it was UV active.

2.3.2 Nuclear Magnetic Resonance

The University of Adelaide has available Nuclear Magnetic Resonance Instruments operating at 200 MHz, 300 MHz and, most importantly for this field of study, at 600 MHz. This has made the analysis of very small quantities of material possible.

There are many different experiments that can be used. What is germane to this thesis is the order and the use of different pulse sequences to the analysis and structure determination of natural products. There was a set order in both acquiring NMR data and in using the raw data to identify compounds and this is outlined in **Table 2.3.2.1** (on the next page).

Table 2.3.2.1: NMR techniques for structure determination

1	^{1}H SPECTRUM	
2	^{13}C SPECTRUM	
3	$^{n}J_{HH}$ CORRELATIONS 	COSY TOCSY
4	$^{1}J_{CH}$ CORRELATIONS 	APT / DEPT HETCOR HMQC
5	$^{2/3}J_{CH}$ CORRELATIONS 	COLOC HMBC
6	C - C CORRELATIONS 	INADEQUATE
7	STEREOCHEMISTRY	COUPLING CONSTANTS nOe (1D or 2D) ROESY

Initially a standard ^{1}H NMR experiment was run, and the purity of the sample assessed in terms of the integration of the proton spectrum. This experiment gave information on ^{1}H chemical shift values (hence the types of protons present) on spin-spin coupling and on integration.

The second routine experiment to be run was a standard ^{13}C NMR experiment to record a ^{13}C NMR spectrum with proton broad-band decoupling, so as to get ^{13}C chemical shift values for the ^{13}C nuclei. Very rarely was a ^{13}C experiment run that was not decoupled, as there were better experiments that could be run which gave the same information.

The next step was to establish the $^{n}J_{HH}$ correlations between the protons present in the standard ^{1}H spectrum. Although coupling constants and line widths gave information concerning adjacent protons, in many cases the spin-spin coupling information was obscured by overlapping multiplets, and a COSY experiment was usually much more informative.

The COSY (COrrelation SpectroscopY) pulse sequence generated a 2D NMR spectrum, so that ^{1}H NMR signals were correlated against each other and cross peaks were formed if spin - spin coupling was present. It was also possible to determine long range COSY correlations by changing the normal pulse sequence of the COSY experiment by inserting an additional fixed delay before and after the second radio frequency pulse.

gCOSY experiments using pulse field gradients were an advantage because they collected data in a much shorter period of time than routine COSY experiments. Even more advanced and effective were DQF-COSY pulse sequences [Double Quantum Filtered] which had the added advantage of giving good solvent suppression. The TOCSY in principle gave a total correlation of all protons of a chain with each other.

Once ^{1}H - ^{1}H correlations had been established the next step was to determine $^{1}J_{CH}$ correlations. If sufficient material was available then an APT or DEPT experiment could be run to determine the multiplicity of the ^{13}C spectrum. However, this is time consuming, considering the inherent insensitivity of ^{13}C experiments (due to the low natural abundance of ^{13}C) and other experiments gave the same information in a quicker time.

The experiments of choice were the HMQC and gHMQC. These two experiments correlated a selected carbon with its attached proton by one bond C, H coupling. As these experiments were inverse detected techniques (that is, it is the ^{1}H nucleus that is observed rather than the ^{13}C nucleus), they were considerably more sensitive than directly detected experiments such as HETCOR. This allowed for each protonated carbon to be assigned and therefore it was also possible, assuming a ^{13}C spectrum had been run, to determine quaternary carbons as well.

HMQC stands for Heteronuclear Multiple Quantum Coherence and is based on inverse detection of the carbon by proton observation. The normal HMQC experiment was able to suppress undesired signals from ^{12}C, and by use of pulsed field gradients it was able to give artefact free C,H correlation spectrum in a very

small amount of time. It was also possible to use an HSQC experiment when the ^{13}C NMR spectrum was particularly crowded.

Once the $^{1}J_{CH}$ correlations had been established, then it was possible to observe $^{2}J_{CH}$ and $^{3}J_{CH}$ correlations by the use of COLOC, HMBC and gHMBC pulse sequences. To observe $^{2}J_{CH}$ or $^{3}J_{CH}$ correlations it was necessary to suppress any $^{1}J_{CH}$ correlations. It was possible to achieve this aim by changing the time delays of the pulse sequence. The original experiments of this type were dependent on ^{13}C sensitivity and so needed a large amount of sample or a large amount of time. COLOC (COrrelation spectroscopy via LOng range Coupling) incorporated the ^{1}H chemical shift information into the earlier signals, but still relied upon the observed nuclide being ^{13}C and so was not very sensitive.

The HMBC (Heteronuclear Multiple Bond Correlation) was a pulse sequence that was able to suppress $^{1}J_{CH}$ correlations, while being an inverse H,X correlation experiment in which the proton signal was used to determine the ^{13}C data, thus being much more sensitive and therefore less time consuming. The gHMBC experiment also gave $^{2}J_{CH}$ and $^{3}J_{CH}$ correlations, usually with better results in a fraction of the time taken for the HMBC.

Although there are NMR experiments that give $^{1}J_{CC}$ correlations (INADEQUATE), the inherent insensitivity of ^{13}C spectroscopy meant that it was not possible to use these experiments on samples with a small amount of mass.

However, by establishing $^{n}J_{HH}$, $^{1}J_{CH}$, $^{2}J_{CH}$ and $^{3}J_{CH}$ correlations it was possible to determine the structure of the natural product, with the next step being the establishment of the stereochemistry of the molecule.

Coupling constants within the spin-spin relationship in the ^{1}H NMR spectrum were useful, not only for establishing $^{n}J_{HH}$ relationships but also in establishing stereochemistry and conformation if the coupling constant value could be determined. The Karplus equation was then used as a guide to determine the stereo relationships between adjacent protons. Not all coupling constant values were recorded as some overlapping multiplets made it difficult to determine the actual coupling constant value.

nOe (nuclear Overhauser effect) spectroscopy was used to determine stereo chemical problems that arose if there were no suitable coupling constants

observed. nOe correlations indicate protons that are close in space but not necessarily adjacent to or even close to each other in terms of bonding.

The NOESY is a 2 dimensional version of the nOe experiment and gave correlation signals that were due to nuclei in a close spatial relationship, ie. they were correlations through space, not via bonds. The ROESY experiment gave similar information. When considering the data of a ROESY experiment it was important to realise that the ROESY may also show ^{1}H - ^{1}H correlations that are visible in the COSY experiment, and that these correlations may be through bonds, between protons that were adjacent to each other and so should be ignored in terms of other spatial correlations.

The last NMR experiment that could be run routinely was a D_2O exchange experiment, which removed any exchangeable signals from the ^{1}H spectrum. This often made the ^{1}H spectrum easier to analyse, but may have had the resultant disadvantage of a large water peak appearing in the spectrum.

All these techniques and the theory of NMR spectroscopy have been covered in many publications in greater depth. Of particular use (assuming the NMR instrument has the capacity to run advanced pulse sequences) were Braun, Kalinowski, and Berger - "100 and more Basic NMR Experiments"[71], Derome's "Modern NMR techniques for chemistry research"[72] and Sanders and Hunter's "Modern NMR spectroscopy".[73]

"Carbon-13 NMR Spectroscopy"[74] by Breitmaier and Voelter was a very good summary of ^{13}C spectrum, with analysis of the ^{13}C spectrum of different classes of natural products, including coumarins, flavonoids, triterpenes and sugars and was an excellent reference.

2.3.3 Mass spectrometry

Once all NMR spectral data had been collected and a tentative structural assignment had been achieved, a small amount of the sample was sent to the Central Science Laboratory at the University of Tasmania for an accurate mass (HRMS) measurement so that the molecular formula could be determined. The molecular formula was then used, in conjunction with the ^{1}H and ^{13}C NMR spectral data, to determine the double bond equivalents (DBE) and therefore whether double bonds and/or ring systems were present in the molecule.

Other information could be determined from the mass spectrum obtained for each sample. The mass spectrum could be compared to those of known compounds, as there are a number of MS "libraries" and peak matching techniques allow for the assignment and structure determination of known compounds.

Fragmentation patterns and metastable ion formation could also be analysed, but by far the most important information to be gained from MS analysis was the M^+ and hence the molecular formula.

Usually, the acquisition of the M^+ was only obtained when all NMR spectroscopy had been completed and was essentially only used to establish the molecular formula. The NMR data and MS data were used to determine the structure of the natural product, with the UV spectrum being recorded mainly as a tool to help in purification.

2.3.4 IR, optical rotation and MP

The IR, optical rotation and MP were then recorded where possible. IR was used to confirm the presence of functional groups.

The UV, IR, $[\alpha]_D$, MS and MP values were very important when a compound that was isolated appeared to be a previously recorded natural product. These values were compared to the recorded literature values.

CHAPTER 3 CRUDE EXTRACT

3.1 Extraction, separation and purification of compounds from 12.7 kg of *Scaevola spinescens*.

12.7 kg of *Scaevola spinescens* was cut up and masticated to a powder, using a grinder and a ball mill. The sample was then placed into four large containers and a total of 80 L of solvent, which had been distilled, was added to the four containers (20 L each container) and left to steep for two weeks. The solvents that were added to the container were, sequentially, hexane, ethyl acetate, methanol and water.

For each solvent the resultant solution was drained from the four containers and the solvent removed using a cyclone evaporator, leaving a residue. The hexane, ethyl acetate and water residues were stored under nitrogen. The methanol extract was then subjected to a series of steps to isolate organic compounds.

A total of 828 g of the methanol extract was then subjected to flash chromatography in seven separate columns. Each of the seven flash chromatography columns used the same sequential solvent system, starting from 90% hexane/10% ethyl acetate through to 50% ethyl acetate/50% methanol. The flash column used was 14 cm in diameter; 200 g of silica was used in each of the columns. The extract was pre-loaded onto silica and 2 L of each solvent was used for each elution (4 column volumes). **Table 3.1.1** indicates the amount of the methanol extract loaded onto each of the seven separate columns.

Table 3.1.1: Mass of methanol extract per flash column

Flash column	Mass of methanol extract (g)
1	50
2	130
3	120
4	120
5	144
6	122
7	124
TOTAL	828

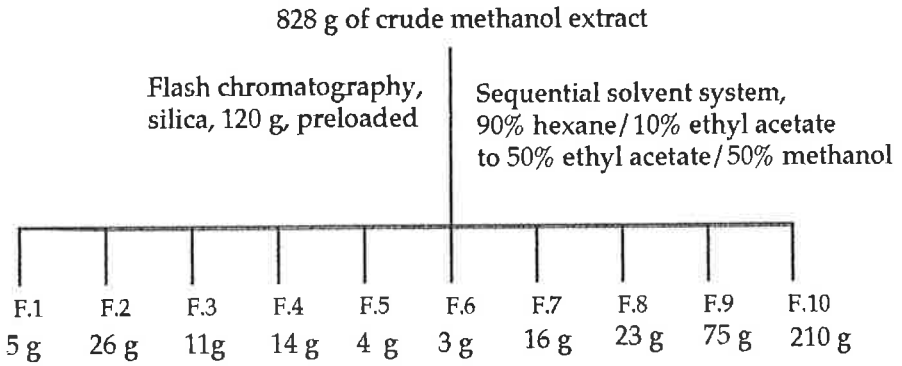
Each of the seven columns underwent the same solvent elution sequence and the equivalent fractions from each column were then combined. Initially, 2 L of 90% hexane/10% ethyl acetate was run through the column and a total of 5 g was collected (after the solvent was removed). This crude fraction was labelled Fraction 1. The next nine fractions, the eluting solvents and the total mass of each fraction (when all seven columns were combined) are outlined in **Table 3.1.2.**

Table 3.1.2: Solvent system for extraction of 828 g of the methanol fraction

Fraction	Eluting solvent	Mass combined (g)
1	90% hexane/10% ethyl acetate	5
2	75% hexane/25% ethyl acetate	26
3	50% hexane/50% ethyl acetate	11
4	100% ethyl acetate	14
5	99% ethyl acetate/1% methanol	4
6	98% ethyl acetate/2% methanol	3
7	95% ethyl acetate/5% methanol	16
8	90% ethyl acetate/10% methanol	23
9	75% ethyl acetate/25% methanol	75
10	50% ethyl acetate/50% methanol	210

This extraction process is also displayed in the following flow chart (**Diagram 3.1.1**).

Diagram 3.1.1: The separation process of 828 g of the methanol extract.



All crude fractions were tested with Meyers reagent[69] to determine whether there were any alkaloids present. All tests were negative.

Fractions 1, 3-9 were then subjected to a range of separation techniques, including prep TLC, flash chromatography, HPLC and recrystalisation, leading to the isolation of eighteen pure compounds.

- Fraction 1 yielded four pure compounds. They were labelled Compound A, Compound B, Compound C and Compound D. The discussion of their structure determinations are outlined in Chapter 4.
- Fraction 2 has not been analysed to date.
- Fraction 3 yielded one pure compound, Compound E, and this compound is discussed in Chapter 5.
- Fraction 4 contained two purified compounds (Compounds F and G) and these compounds are discussed in Chapter 6.
- A white crystalline solid was isolated from Fraction 5 and labelled Compound H. It is analysed in Chapter 7.
- Fractions 6 and 7 were combined as they had very similar TLC profiles. This fraction was relabelled Fraction 7. Four pure compounds (Compounds I to L) were isolated and these are outlined in Chapter 8.
- Fraction 8 yielded three pure compounds - Compounds M to O (Chapter 9)
- Fraction 9 yielded Compounds P and Q (Chapter 10).
- Fraction 10 has not been analysed to this date.

Each of the compounds purified was written up in a consistent manner as outlined in the following section, Chapter 3.2.

3.2 Analysis and write up of purified compounds

Initially, an explanation of the initial treatment, which resulted in the separation and purification, will be given, along with a flow chart to explain each step. Then the derivation of each of the structures of the purified compounds will be outlined.

The mass spectral data in the form of the M^+ value - experimental (calculated value, +/- difference) will be given, along with the molecular formula. The molecular formula will then be analysed for double bond equivalents (DBE). Any useful fragmentation patterns from the MS will be analysed and a mass spectral peak matching process will be routinely conducted. Any IR, UV or melting point data will be given.

The proposed structure will be given, with carbons labelled according to the standard numbering system for the particular type of compound.

If the structure is a known compound then a brief description of the spectral data will be given. With a novel compound or where stereochemical or conformational analysis is required a more detailed analysis of the structure determination will be presented. A summary of the occurrence of the compound in *Scaevola spinescens* and in the *Scaevola* genera will be given.

A table outlining all data - ^{13}C chemical shift values, HMQC correlations to respective protons, the ^{1}H chemical shift values, COSY correlations nOe correlations and HMBC correlations - will be displayed. This method is outlined in **Table 3.2.1.**

Table 3.2.1: Example of Table of NMR data#

C	δC	H	δ_H	I	Mult.	J values	HMBC	nOe
C_1	125.2	H_1	7.685	1	dd	2.4, 8.0	C_2, C_3	H_2
C_2	114.5	H_2	6.962	1	d	8.0	C_1, C_3	H_1
C_3	112.4	H_3	7.244	1	d	2.4	C_1, C_2	

600 MHz (^{1}H), 150 MHz (^{13}C) : δ (ppm) from internal TMS in $CDCl_3$

I = Integration Mult. = Multiplicity

Any non-protonated carbons will not show up in an HMQC spectrum, but they can be deduced by comparison with the routine ^{13}C NMR experiment, where they do appear. Protons, which are not directly bonded to a carbon, will also be determined by comparison of the routine ^{1}H NMR data with the HMQC data.

Each individual COSY correlation ($^{n}J_{HH}$) spin system will be outlined, where necessary, and this will be illustrated using the following type of spin map showing all COSY correlations (see *Figure 3.2.1).*

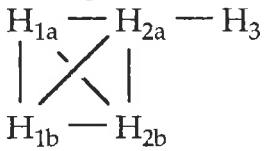


Figure 3.2.1: Diagram illustrating COSY spin systems

The $^{1}J_{CH}$ correlations established by the HMQC experiments will be added to the COSY spin system information to form a part structure (see *Figure* 3.2.2).

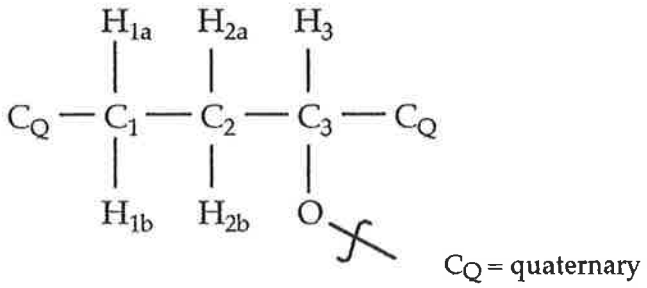


Figure 3.2.2: Diagram of $^{1}J_{CH}$ correlations of COSY spin systems to give a part structure

The part structures and any non-protonated carbons, or any protons not bonded to a carbon, will be connected via $^{2/3}J_{CH}$ correlations established using either an HMBC or gHMBC experiments.

This bonding will be illustrated (where necessary) by using bold lines to indicate the bond(s) connecting the proton under consideration to the carbon to which it has a $^{2/3}J_{CH}$ correlation. The proton and carbon will also be in bold. The HMBC correlations will be outlined as shown below. In this example, H_{1a} has an HMBC correlation to C_3 (see *Figure 3.2.3*).

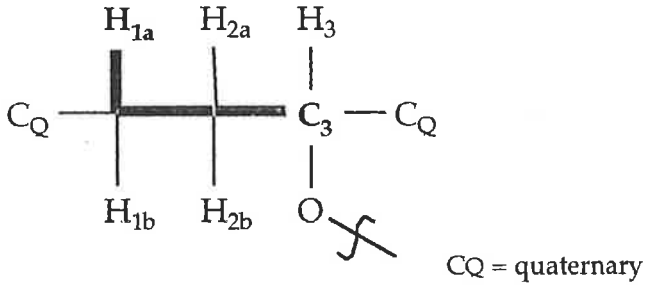


Figure 3.2.3: HMBC correlations in bold.

Stereochemistry and conformation will be determined by use of coupling constants, initially, and by using nOe data established by conducting NOESY and ROESY experiments, where required. The stereochemistry will be illustrated using connecting lines. This is illustrated in *Figure 3.2.4*. In this compound, most methyl protons can be assigned as on the same side of the ring of a pentacyclic compound due to the extensive nOe correlations that were observed.

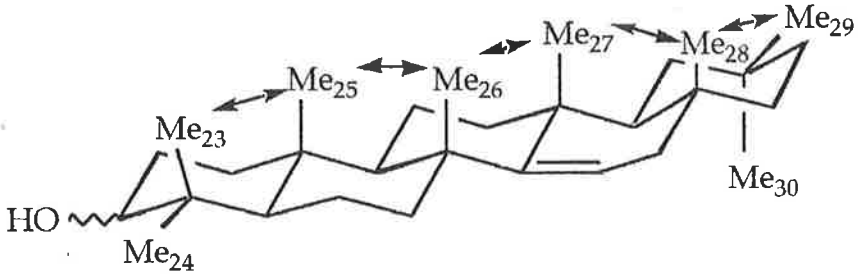


Figure 3.2.4: Illustration of stereochemistry

The final structure of the compound will then be illustrated and a comparison with previously recorded compounds made (where possible).

CHAPTER 4 FRACTION 1

4.1 Initial treatment

Treatment of Fraction 1 yielded four compounds, as outlined in **Diagram 4.1,** pg 53.

5 g of Fraction 1 was separated from the original methanol extract (828 g) using 90% hexane/10% ethyl acetate as the eluting solvent.

Fraction 1 (5 g) was then subjected to flash chromatography (silica, sequential, 100% hexane - 50% hexane/50% ethyl acetate) to yield ten fractions.

Fraction 1.2 (5 mg) was subjected to a flash column (silica, isocratic, 100% hexane) and yielded three fractions. Fraction 1.2.3 was found to be pure when applied to GC/MS and was labelled Compound A (less than one mg). The structure determination of Compound A is given in Chapter 4.2, pg 54.

Fraction 1.4 (150 mg) was subjected to flash chromatography (silica, isocratic, 98% hexane/2% ethyl acetate) to yield seven fractions. Fraction 1.4.1 (50 mg) was subjected to flash chromatography (silica, isocratic, 98% hexane/2% ethyl acetate) to yield Fraction 1.4.1.4 (22 mg) which was found to be a mixture with two major peaks when analysed using GC/MS.

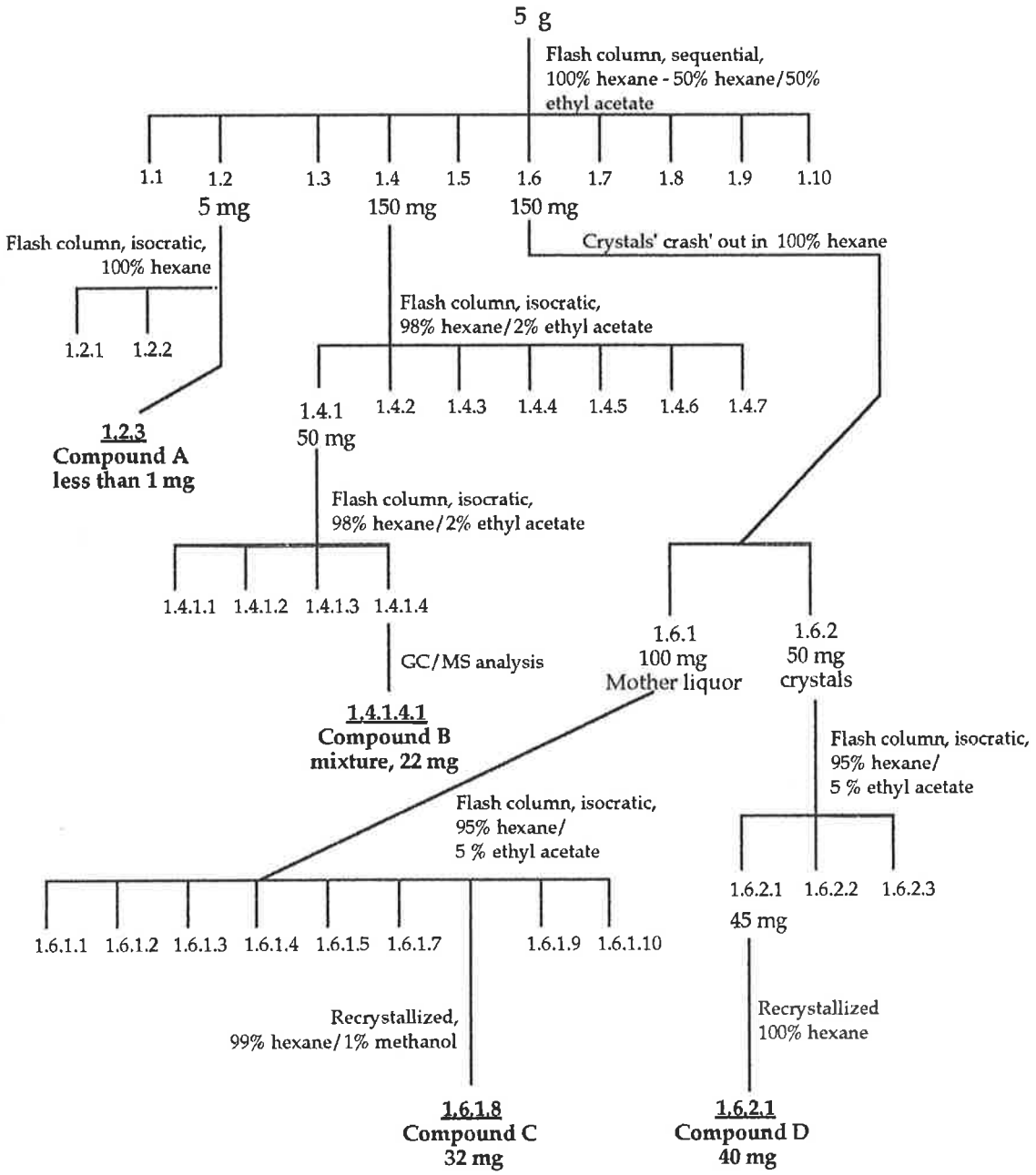
Fraction 1.4.1.4.1 - the first major peak by GC/MS- was analysed and was labelled Compound B - see Chapter 4.3, pg 56 for its structure determination. The identity of the second major peak could not be established

Fraction 1.6 (150 mg) formed crystals in 100% hexane that were labelled 1.6.2 (50 mg).

The mother liquor (100 mg) was labelled 1.6.1. It was subjected to flash chromatography (silica, isocratic, 95% hexane/5% ethyl acetate) and was separated into ten fractions. When Fraction 1.6.1.8 was left standing, a white solid crystallized out of solution (100% hexane). This solid (42 mg) was then recrystallized in 99% hexane/1% methanol to give fine white crystals. It was labelled Compound C (32 mg) (see Chapter 4.4, pg 59).

Fraction 1.6.2 (50 mg) was subjected to flash chromatography (silica, isocratic, 95% hexane/5% ethyl acetate) and separated into three fractions. When fraction 1.6.2.1 was left standing, a white solid crystallized out of solution (100% hexane). This solid (45 mg) was then recrystallized in 99% hexane/1% methanol to give fine crystals. This pure sample was labelled Compound D (40 mg) (see Chapter 4.5, pg 61).

Diagram 4.1: Isolation of Compounds A to D



4.2 Compound A

Compound A (less than one mg) was isolated as a white amorphous solid and was found to be pure when applied to GC/MS (see Chapter 4.1, **Diagram 4.1**, pg 53). It was not possible to undertake NMR experiments on the sample.

When injected into the GC-MS system, it was one peak and it was possible to do a library match with a known compound, with a 99% correlation. The compound was found to have the molecular formula of $C_{17}H_{34}O_2$, giving it a molecular mass of 270 and the structure of hexadecanoic acid, methyl ester (**95**) (see *Figure 4.2.1*).

This compound has not been reported from *Scaevola spinescens* or any other plant from any species of the *Scaevola* genera. However (**95**) is the methyl ester of palmitic acid (**50**), reported previously from *Scaevola spinescens*[36] (see Chapter 1.3.4.1, pg 26).

Figure 4.2.1: Hexadecanoic acid, methyl ester (95)

The overall fragmentation pattern observed in the MS is summarised in *Figure 4.2.2* where the major peak observed was at 74 amu followed by a peak at 227 amu.

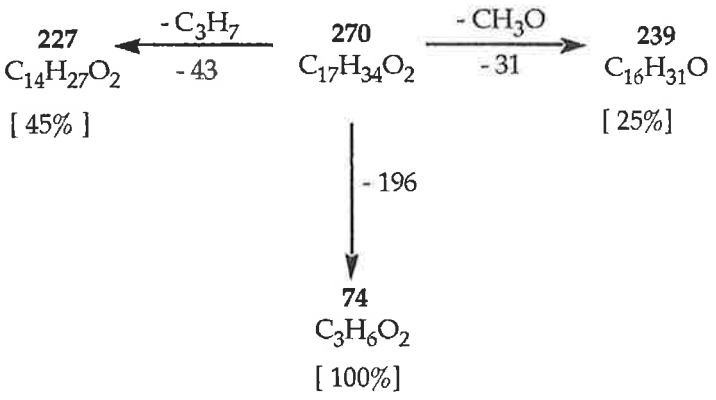


Figure 4.2.2: Major fragmentation patterns observed for hexadecanoic acid, methyl ester (95)

The rest of the fragmentation pattern was indicative of a saturated long chain fatty acid ester. The first fragmentation from the parent ion was the loss of 31

amu (*Figure 4.2.3*), which corresponds to the loss of the methoxy functional group.

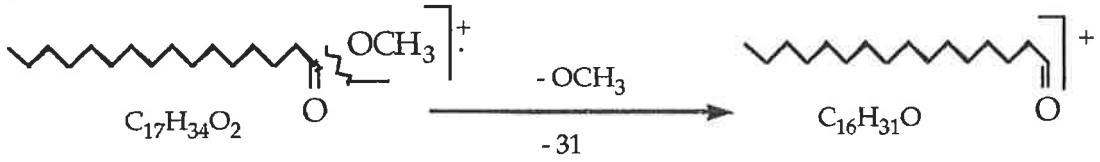


Figure 4.2.3: Initial fragmentation, with the loss of a methoxy functional group

The rest of the fragmentation exhibited a consistent loss of 14 amu (see *Figure 4.2.4*), which corresponds to the loss of successive methylene groups. This is very characteristic of long chained fatty acids and it occurs with gradually decreasing intensity.[75a]

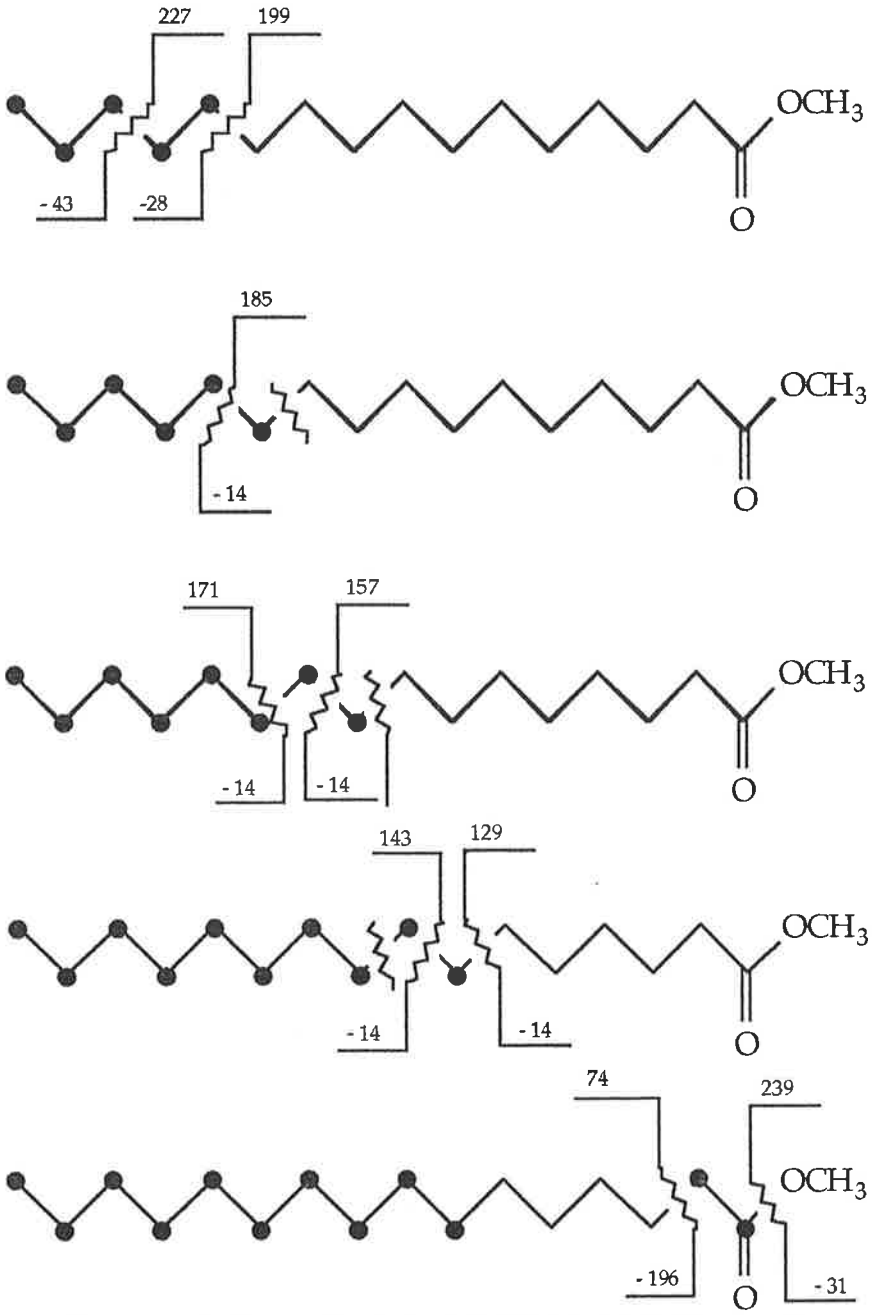


Figure 4.2.4: Progressive loss of methylene and ethylene units

4.3 Compound B

Fraction 1.4.1.4 (22 mg) was a white crystalline solid, with a broad melting point range (see Chapter 4.1, **Diagram 4.1**, pg 53).

It had 60 ^{13}C carbon signals and a very complex ^{1}H spectrum that was difficult to interpret. The sample was sent for MS, with a GC-MS indicating a mixture with two major peaks and four very small peaks. One of the major peaks was labelled Compound B. The accurate mass for Compound B was 468.3964 and therefore the molecular formula for Compound B was $C_{32}H_{52}O_2$ (calculated = 468.3957, +/- 0.0007). This indicated seven DBE.

In the ^{13}C NMR spectrum there was one carbonyl and two sp^2 hybridised carbons, accounting for two DBE, with the other DBE being associated with ring structures. Thus Compound B was a pentacyclic triterpene with a carbonyl and a double bond functional group.

The MS fragmentation of Compound B was diagnostic of taraxerol acetate (**92**) (comparison of mass spectral data, in the Eight Peak Index of Mass Spectra[76], to within 99% analysis of the MS and by comparison with spectrum from an authentic sample). The structure of taraxerol acetate (**92**) is given in *Figure 4.3.1* (the numbering system is that commonly used in the literature for triterpenes).

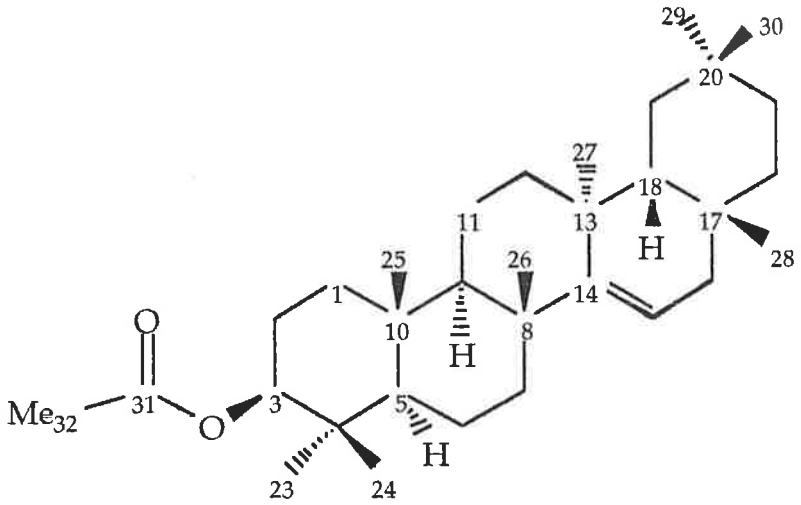


Figure 4.3.1: Structure of taraxerol acetate (92)

The fragmentation pattern for taraxerol acetate (**92**) was 468 (M^+ 20%), 204 (100%), 269 (61%), 189 (49%), 344 (44%), 329 (42%) and 135 (38%). An analysis of this MS data indicates the following:

- There is no loss of water (17 amu), indicating that there was no hydroxyl present in the molecule.
- The fragmentation loss of –15 can be accounted for by the loss of a methyl.
- The major fragmentation patterns of triterpenes are associated with the presence and position of the double bond, as the molecular ion will undergo the equivalent of a retro Diels-Alder fragmentation to give very characteristic peaks. With taraxerol acetate (**92**), the double bond is positioned at Δ^{14} and the retro Diels-Alder fragmentation gives a very characteristic and diagnostic peak at 344 (40%)[75b] (*Figure 4.3.2*).

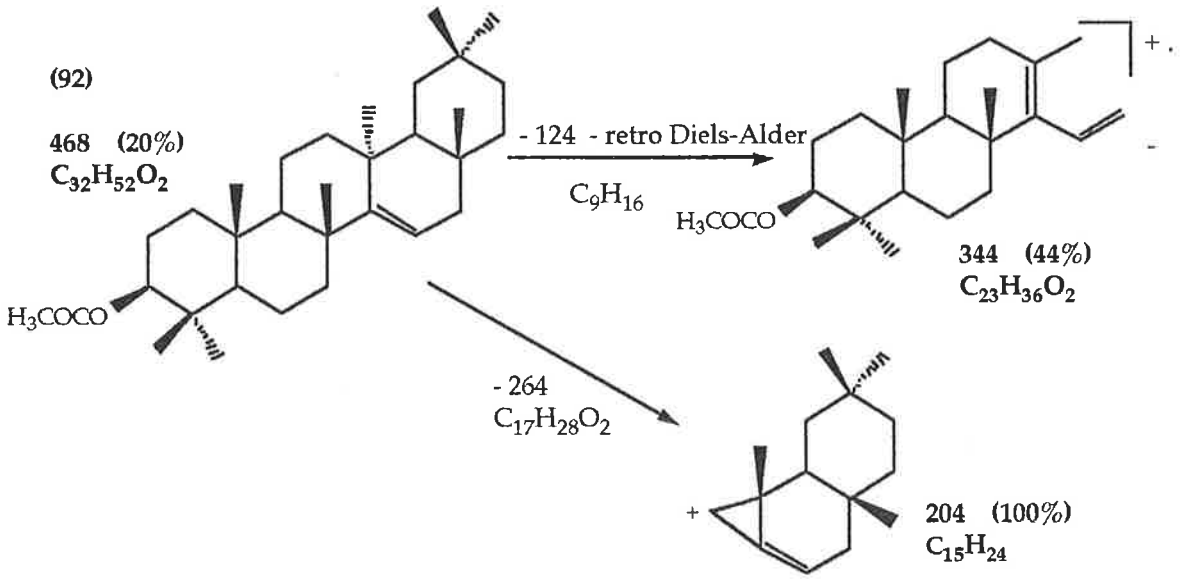


Figure 4.3.2: Fragmentation of (92).[75b, 77]

^1H, ^{13}C and DEPT NMR experiments were undertaken on (**92**) and this NMR data was compared to the recorded values for (**73**).[77] The ^{13}C NMR data is outlined in **Table 4.3.1**. The ^{13}C chemical shift values of δ 158 – 117 are diagnostic for a $\Delta^{14\text{-}15}$ double bond pattern, and there was a very close correlation between the ^{13}C NMR values of (**92**) in comparison with previously published data.[77]

The second major peak (1.4.1.4.6) in the GC/MS gave a fragmentation pattern of 468 (10%), 229 (100%), 289 (94%), 241 (31%), 393 (31%), 230 (29%) and 290 (28%). The only compound in the Eight Peak Index of Mass Spectra[76] that has the major fragment at 229 (100%) is lupeol acetate (**90**) that is listed as 468 (M+), 229 (100%), 297 (55%), 365 (35%), 203 (30%), 218 (20%) and 408 (10%). This is not a good

comparison. As well as this, the ^{13}C NMR data for 1.4.1.4.6 indicated a carbonyl at δ 171.6 and two sp^2 hybridised carbons at δ 146.0 and δ 116.7. The ^{13}C chemical shift values reported in the literature for the equivalent carbon signals in **(90)** are δ 176.2, δ 150.3 and δ 110. Therefore it was not possible to positively identify 1.4.1.4.6.

Table 4.3.1: ^{13}C chemical shift values for (92)# and (73)[77]

C	δC (92)	δC (73)[77]
C_1	37.4	38.1
C_2	26.9	27.3
C_3	79.1	79.2
C_4	38.7	39.1
C_5	56.0	55.7
C_6	19.5	19.0
C_7	35.6	35.3
C_8	39.5	38.9
C_9	49.5	48.9
C_{10}	38.5	37.9

C	δC (92)	δC (73)[77]
C_{11}	18.3	17.7
C_{12}	34.9	35.9
C_{13}	38.2	37.9
C_{14}	158.3	158.1
C_{15}	117.4	117.0
C_{16}	38.3	36.9
C_{17}	38.5	38.1
C_{18}	49.0	49.4
C_{19}	41.3	41.4
C_{20}	28.8	29.0

C	δC (92)	δC (73)[77]
C_{21}	33.9	33.9
C_{22}	32.8	33.2
C_{23}	29.1	28.1
C_{24}	16.4	15.6
C_{25}	16.3	15.6
C_{26}	30.6	30.1
C_{27}	25.2	26.0
C_{28}	30.1	30.1
C_{29}	34.1	33.5
C_{30}	22.1	21.5
C_{31}	166.7	
C_{32}	23.6	

#150 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$

Comparison of ^{13}C values between **(92)** and recorded values of **(73)**[77] is within 1.0 ppm

(73)[77] - 25 MHz (^{13}C): δ (ppm) from TMS in $CDCl_3$

4.4 Compound C

Compound C (32 mg) was isolated as white crystals (see Chapter 4.1, **Diagram 4.1,** pg 53) with a MP of 280 - 284°C and an $[\alpha]_D$ of + 0.69° (c 0.1, $CHCl_3$).

Mass Spectrometry (EI) was conducted on the sample and a library search of the spectrum gave a good match for taraxerol (**73**). A comparison of the fragmentation pattern in the Eight Peak Index of Mass Spectra[76] as well as previously published data[36] gave a very close correlation.

The comparison of the fragmentation pattern of the MS as well as the MP and $[\alpha]_D$ values[78] indicated that Compound C was taraxerol (**73**).

The high resolution MS (HRMS) of Compound C gave a molecular weight of 426.3859 and hence a molecular formula of $C_{30}H_{50}O$ (calculated = 426.3864, +/- 0.0005). This molecular formula gave six DBE. In the initial ^{13}C spectrum, a carbon-carbon double bond was indicated by two ^{13}C chemical shift values, one at δ 159, the other at δ 117, which accounts for one DBE. There were no other sp^2 hybridised carbons present. This suggests that there are five DBE associated with ring systems and that Compound C is a pentacyclic triterpene.

A comparison of the ^{13}C chemical shift values with the recorded values[77] for taraxerol (**73**) also indicated that Compound C is taraxerol (**73**). The ^{13}C chemical shift data of Compound C and taraxerol (**73**)[77] are compared in **Table 4.4.1,** pg 60. Comprehensive analysis of the COSY, HMQC and the HMBC data revealed that Compound C has the basic structure of taraxerol (**73**). An analysis of the ROE data confirmed that Compound C has the stereochemistry of taraxerol (**73**) at all ring junctions.

Therefore Compound C is taraxerol (**73**) and its structure, with stereochemistry, is as shown in *Figure 4.4.1.* (The numbering system is that commonly used in the literature for triterpenes). Taraxerol (**73**) has been reported previously from *Scaevola spinescens.* [35,36,56]

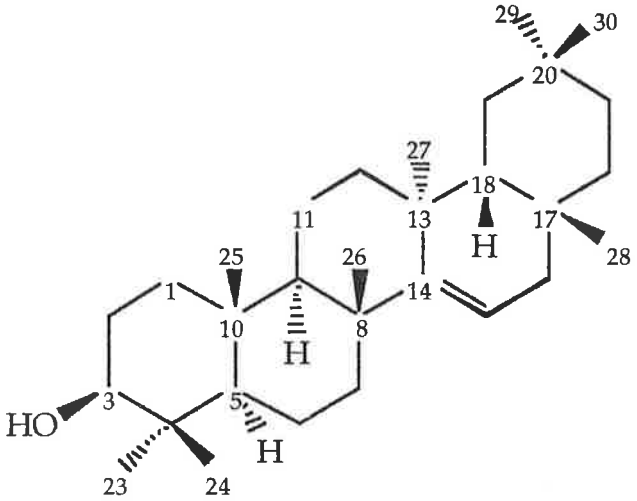


Figure 4.4.1: Taraxerol (73)

Table 4.4.1: ^{13}C chemical shift values for taraxerol (73)

C	δC (73)#	δC (73)77	C	δC (73)#	δC (73)77	C	δC (73)#	δC (73)77
C_1	38.2	38.1	C_{11}	17.8	17.7	C_{21}	33.9	33.9
C_2	27.3	27.3	C_{12}	35.1	35.9	C_{22}	32.8	33.2
C_3	78.9	79.2	C_{13}	37.0	37.9	C_{23}	27.2	28.1
C_4	39.8	39.1	C_{14}	159.1	158.1	C_{24}	15.2	15.6
C_5	55.2	55.7	C_{15}	117.3	117.0	C_{25}	15.2	15.6
C_6	19.1	19.0	C_{16}	36.4	36.9	C_{26}	29.5	30.1
C_7	35.3	35.3	C_{17}	38.6	38.1	C_{27}	25.0	26.0
C_8	38.7	38.9	C_{18}	50.3	49.4	C_{28}	29.7	30.1
C_9	48.2	48.9	C_{19}	41.7	41.4	C_{29}	33.4	33.5
C_{10}	37.9	37.9	C_{20}	29.5	29.0	C_{30}	21.7	21.5

= ^{13}C chemical shift values recorded for Compound C (73)

*150 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$ solution.

Comparison of ^{13}C values between Compound C and recorded values of $(73)^{77}$ is within 1.0 ppm.

$(73)^{77}$ - 25 MHz (^{13}C): δ (ppm) from TMS in $CDCl_3$.

4.5 Compound D

Compound D (40 mg) was isolated as white crystals (see Chapter 4.1, **Diagram 4.1**, pg 53) with a MP of 191 - 193°C and an $[\alpha]_D$ of + 58.6° (c 0.1, $CHCl_3$).

The high resolution MS (HRMS) of Compound D gave a molecular weight of 426.3876 and hence a molecular formula of $C_{30}H_{50}O$ (calculated = 426.3874, +/- 0.0002). This molecular formula gave six DBE. The IR spectrum revealed hydroxyl (3440 cm^{-1}) and carbon-carbon double bond signals (1660 cm^{-1}). Mass spectrometry (EI) was conducted on the sample and a library search of the spectrum gave a good match for taraxerol (**73**) - see Chapter 4.4, pg 59 - with some differences. When taraxerol (**73**) and Compound D were co- spotted on TLC they moved to significantly different positions on the TLC plate. The literature value for the melting point of taraxerol (**73**) is 282 - 283° C[78] while the measured melting point of Compound D was 191 - 193°C.

The main MS fragmentation peaks observed for Compound D and taraxerol (**73**)[35,76] are displayed in **Table 4.5.1**, pg 61, listed in order of the percentage values of each fragmentation in decreasing order. There is a close correlation between the two mass spectra. However, Compound D has fragmentations at 133, 135 and 189 amu that are not present in taraxerol (**73**).

Table 4.5.1 Mass spectral data for Compound D and taraxerol (73)[35,76]

Compound D	Taraxerol (73)[35, 76]
204 (100 %)	204 (100 %)
133 (88 %)	
189 (78 %)	
302 (40 %)	302 (33 %)
287 (38 %)	287 (25 %)
135 (39 %)	

Given the difference in melting point, the difference in their movement on silica and the differences in the fragmentation ions at 133 and 189 it would appear that Compound D is not taraxerol (**73**) but is an isomer of taraxerol (**73**).

The ^{13}C, ^{1}H, HMQC, COSY and HMBC spectral data for Compound D and the ^{13}C literature values for (**73**)[77] are given in **Table 4.5.2**, pg 62.

Table 5.4.2: Spectral data for Compound D# - an isomer of taraxerol (73).[77]

C	δ C	H	δ H	I	M	J (Hz)	COSY	HMBC	(73)
C_1	37.7	$H_{1\alpha}$ $H_{1\beta}$	0.890 1.531	1 1	m m		$H_{1\beta}$, $H_{2\alpha}$, $H_{2\beta}$ $H_{1\alpha}$, $H_{2\alpha}$, $H_{2\beta}$	C_{10} C_{10}	38.1
C_2	27.1	$H_{2\alpha}$ $H_{2\beta}$	1.522 1.486	1 1	m m		$H_{1\alpha}$, $H_{1\beta}$, $H_{2\beta}$, $H_{3\alpha}$ $H_{1\alpha}$, $H_{1\beta}$, $H_{2\alpha}$, $H_{3\alpha}$	C_1, C_3 C_1, C_3	27.3
C_3	79.0	$H_{3\alpha}$	3.125	1	dd	4.8 11.4	$H_{2\alpha}$, $H_{2\beta}$	C_2, C_{23}, C_{24}	79.2
C_4	38.7								39.1
C_5	55.5	$H_{5\alpha}$	0.715	1	dd	2.4 12.0	$H_{6\alpha}$, $H_{6\beta}$	C_{10}, C_{23}, C_{24}, C_{25}	55.7
C_6	18.8	$H_{6\alpha}$ $H_{6\beta}$	1.547 1.412	1 1	m m		$H_{5\alpha}$, $H_{6\beta}$, $H_{7\alpha}$ $H_{6\alpha}$	C_7 C_7	19.0
C_7	41.3	$H_{7\alpha}$ $H_{7\beta}$	1.960 1.271	1 1	dd m	3.0, 12.6	$H_{6\beta}$, $H_{7\beta}$ $H_{7\alpha}$	C_6, C_8, C_{26} C_5, C_6	35.3
C_8	38.9								38.9
C_9	49.2	$H_{9\alpha}$	1.349	1	m		$H_{11\alpha}$	$C_1C_8C_{10}C_{11}C_{25}C_{26}$	48.9
C_{10}	38.0								37.9
C_{11}	17.5	$H_{11\alpha}$ $H_{11\beta}$	1.563 1.412	1 1	m m		$H_{9\alpha}$, $H_{11\beta}$, $H_{12\alpha}$ $H_{11\alpha}$	C_9, C_{12} C_9, C_{12}, C_{13}	17.7
C_{12}	33.7	$H_{12\alpha}$ $H_{12\beta}$	1.560 1.475	1 1	m m		$H_{11\alpha}$, $H_{12\beta}$ $H_{12\alpha}$	C_9, C_{11}, C_{18}, C_{27} C_{11}, C_{13}, C_{27}	35.9
C_{13}	37.5								37.9
C_{14}	158.0								158.1
C_{15}	116.8	H_{15}	5.464	1	dd	3.0 7.4	$H_{16\alpha}$, $H_{16\beta}$	C_8, C_{13}, C_{16}	117.0
C_{16}	37.7	$H_{16\alpha}$ $H_{16\beta}$	1.575 1.841	1 1	dd m	8.4 15.0 3.0 15.0	H_{15}, $H_{16\beta}$ H_{15}, $H_{16\alpha}$	$C_{14}C_{15}C_{17}C_{18}C_{28}$ C_{14}, C_{15}, C_{17}, C_{28}	36.9
C_{17}	35.7								38.1
C_{18}	48.7	$H_{18\alpha}$	0.902	1	m		$H_{19\alpha}$	$C_{12}C_{13}C_{16}C_{19}C_{20}C_{28}$	49.4
C_{19}	36.6	$H_{19\alpha}$ $H_{19\beta}$	0.920 1.224	1 1	m m		$H_{18\alpha}$, $H_{19\beta}$ $H_{19\alpha}$	C_{18}, C_{21} C_{20}	41.4
C_{20}	28.8								29.0
C_{21}	33.3	$H_{21\alpha}$ $H_{21\beta}$	1.172 1.271	1 1	m m		$H_{21\beta}$, $H_{22\alpha}$ $H_{21\alpha}$	C_{17}, C_{20}, C_{29}, C_{30} C_{17}, C_{20}	33.9
C_{22}	35.1	$H_{22\alpha}$ $H_{22\beta}$	0.952 1.305	1 1	m m		$H_{21\alpha}$, $H_{22\beta}$ $H_{22\alpha}$	 C_{20}, C_{28}	33.2
C_{23}	28.0	$Me_{23\alpha}$	0.910	3	s			C_3, C_4, C_5, C_{24}	28.1
C_{24}	15.4	$Me_{24\beta}$	0.734	3	s			C_3, C_4, C_5, C_{23}	15.6
C_{25}	15.4	$Me_{25\beta}$	0.860	3	s			C_1, C_5, C_9, C_{10}	15.6
C_{26}	25.9	$Me_{26\beta}$	1.022	3	s			C_7, C_8, C_9, C_{14}	30.1
C_{27}	29.9	$Me_{27\beta}$	0.841	3	s			C_{12}, C_{13}, C_{14}, C_{18}	26.0
C_{28}	29.7	$Me_{28\beta}$	0.754	3	s			C_{16}, C_{17}, C_{18}, C_{22}	30.1
C_{29}	33.1	$Me_{29\alpha}$	0.881	3	s			C_{19}, C_{20}, C_{21}, C_{30}	33.5
C_{30}	21.3	$Me_{30\beta}$	0.841	3	s			C_{19}, C_{20}, C_{21}, C_{29}	21.5

600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$

(73)[77] - 25 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$

I = Integration M = Multiplicity

The numbering system used is the one commonly found in the literature for triterpenes, with the structure given that of taraxerol (73) without the stereochemistry (see *Figure 4.5.1*).

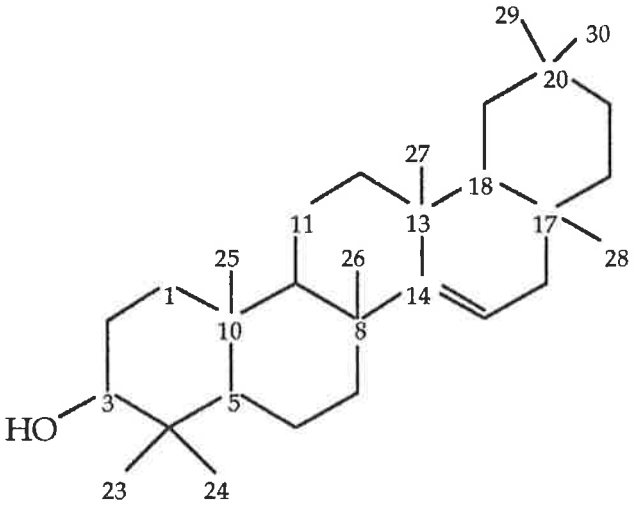


Figure 4.5.1: Compound D - isomer of taraxerol (73)

Compound D is a stereoisomer of taraxerol (73). It is a pentacyclic structure with eight methyl signals, five methine protons and ten methylene pairs that can be assigned as either α or β to the ring structure. The structure has eight chiral carbons. The methylene pairs of hydrogens are diastereotopic and therefore they have different ^{1}H chemical shift values.

The five rings are labelled from A to E and the chiral centres shown in *Figure 4.5.2*.

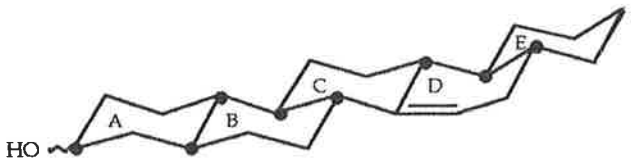


Figure 4.5.2: Labelled rings and chiral centres for the structure Compound D

The stereochemistry and conformation were investigated using both the coupling constant data (where available) and ROE relationships determined by a ROESY experiment. These correlations are displayed in **Table 4.5.3**, pg 64.

The stereochemistry of the methyl protons was apparent in the ROESY experiment, where methyl signals on the same side of the molecule strongly interact. Ring junctions in this rigid system are either trans or cis ring junctions and this relationship can be established according to ROE interactions between the methine protons and the methyl protons.

Table 4.5.3: ROESY correlations#

Pro.	ROESY	Pro.	ROESY	Pro.	ROESY
$H_{1\beta}$		$H_{11\beta}$	$H_{12\beta}Me_{25\beta}Me_{26\beta}$	$H_{21\alpha}$	
$H_{1\alpha}$	$H_{3\alpha}$, $H_{5\alpha}$	$H_{11\alpha}$		$H_{22\beta}$	$H_{16\beta}$
$H_{2\beta}$		$H_{12\beta}$	$H_{11\beta}$	$H_{22\alpha}$	
$H_{2\alpha}$	$H_{3\alpha}$	$H_{12\alpha}$	$H_{9\alpha}$	$Me_{23\alpha}$	$H5_{\alpha}$, $H_{24\beta}$
$H_{3\alpha}$	$H_{1\alpha}H_{2\alpha}H_{5\alpha}Me_{23\alpha}$	H_{15}	$H_{7\beta}$	$Me_{24\beta}$	$H_{23\alpha}$, $H_{25\beta}$
$H_{5\alpha}$	$H_{1\alpha}H_{3\alpha}H_{7\alpha}H_{9\alpha}Me_{23\alpha}$	$H_{16\beta}$	$H_{22\beta}$, $H_{28\beta}$	$Me_{25\beta}$	$H_{6\beta}$ $H_{11\beta}$ $Me_{24\beta}$ $Me_{26\beta}$
$H_{6\beta}$	$Me_{25\beta}$, $Me_{26\beta}$	$H_{16\alpha}$		$Me_{26\beta}$	$H_{6\beta}H_{7\beta}H_{11\beta}Me_{25\beta}Me_{27\beta}$
$H_{6\alpha}$		$H_{18\alpha}$	$H_{9\alpha}$	$Me_{27\beta}$	$Me_{26\beta}$, $Me_{28\beta}$
$H_{7\beta}$	H_{15}, $Me_{26\beta}$	$H_{19\beta}$	$H_{30\beta}$	$Me_{28\beta}$	$H_{16\beta}H_{21\beta}Me_{27\beta}Me_{30\beta}$
$H_{7\alpha}$	$H_{5\alpha}$, $H_{9\alpha}$	$H_{19\alpha}$		$Me_{29\alpha}$	$Me_{30\beta}$
$H_{9\alpha}$	H_5, $H_{7\alpha}$, $H_{12\alpha}$, $H_{18\alpha}$	$H_{21\beta}$	$H_{28\beta}$, $H_{30\beta}$	$Me_{30\beta}$	$H_{19\beta}H_{21\alpha}H_{21\beta}Me_{28\beta}Me_{29\beta}$

600 MHz (^{1}H): δ (ppm) from internal TMS in $CDCl_3$

The ROESY experiment unambiguously assigned the methyl protons $Me_{24\beta}$, $Me_{25\beta}$, $Me_{26\beta}$, $Me_{27\beta}$and $Me_{28\beta}$ to be on the same side of the molecule as there were strong ROE correlations between these methyl groups. There was also a correlation between $Me_{28\beta}$ and $Me_{30\beta}$, which was not as strong as the other ROE correlations, while there was no correlation between $Me_{28\beta}$ and $Me_{29\alpha}$. This relationship is shown in *Figure 4.5.3*.

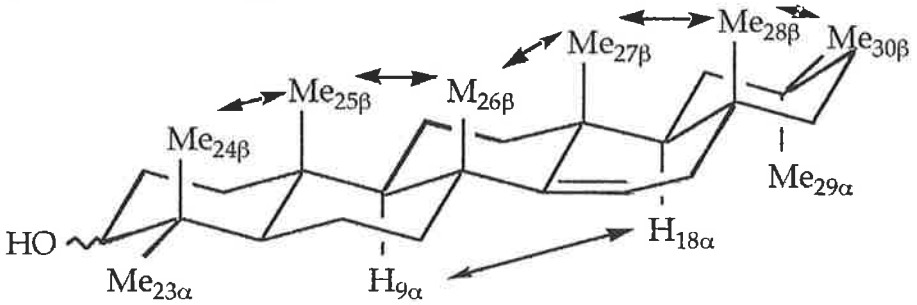


Figure 4.5.3: ROE correlations of the methyl substituents and ring junction protons of Compound D

This arrangement is different to that of taraxerol (**73**) where $Me_{27\beta}$ is below the ring. However, Compound D shows a clear and unambiguous ROE correlation between $Me_{26\beta}$ and $Me_{27\beta}$ that can only occur if they are on the same side of the ring. Also of interest is the fact that there are no ROE correlations between the axial methyl's and $H_{18\alpha}$ that supports the assignment of $H_{18\alpha}$ as being below the ring. This is also different to taraxerol (**73**), as the equivalent proton is above the ring.

There is a weak ROE correlation between $H_{9\alpha}$ (the methine proton at the ring junction of B/C) and $H_{18\alpha}$, which indicates that $H_{9\alpha}$ is also below the ring. This stereorelationship is supported by the lack of ROE correlations between $H_{9\alpha}$ and any of the axial methyl groups, which would be a significant correlation. This stereochemistry is displayed in *Figure 4.5.3*.

The conformation of ring D could be determined using the available ROE data as well as the coupling constant values as the proton signals for both $H_{16\beta}$ (J = 8.4, 15.0 Hz) and $H_{16\alpha}$ (J = 3.0, 15.0 Hz) were unambiguous doublet of doublets. The coupling constants suggest that $H_{16\beta}$ is trans to H_{15} as it has the larger coupling constant (J = 8.4 Hz), however this is not conclusive. This relationship is supported by the ROE correlation from $H_{16\beta}$ to $Me_{28\beta}$, while there is no ROE correlation between $Me_{28\beta}$ and $H_{16\alpha}$, showing that $H_{16\beta}$ is above the ring and $H_{16\alpha}$ is below. This is illustrated in *Figure 4.5.4*.

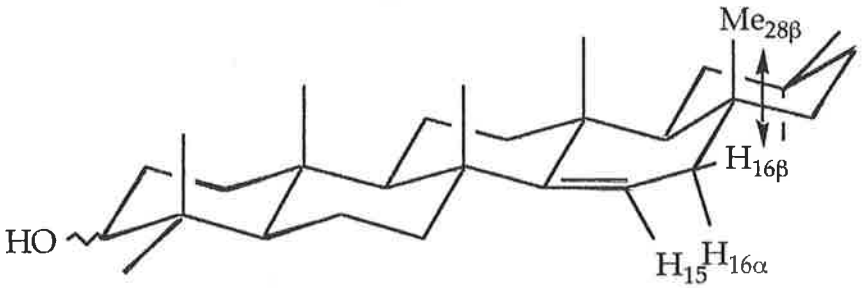


Figure 4.5.4: Stereochemistry of H_{15}, $H_{16\alpha}$ and $H_{16\beta}$.

There is a ROE correlation between H_{15} and $H_{7\beta}$ but not between H_{15} and $H_{7\alpha}$ indicating that H_{15} and $H_{7\beta}$ are close to each other in space. One important correlation is between $Me_{26\beta}$ and $H_{7\beta}$ that places $H_{7\beta}$ on the same face of the molecule as $Me_{26\beta}$. There is no ROE relationship between $Me_{26\beta}$ and $H_{7\alpha}$. As well as this, there is no ROE correlation between H_9 and $Me_{26\beta}$, indicating they are on opposite sides of the ring. $H_{7\alpha}$ has a ROE correlation to $H_{9\alpha}$. These relationships are illustrated in *Figure 4.5.5*.

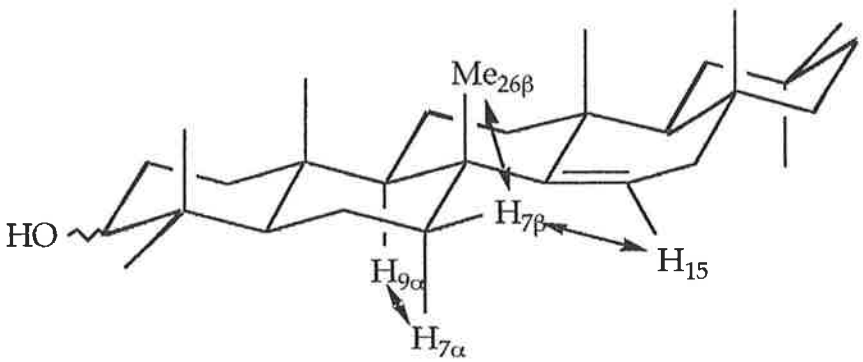


Figure 4.5.5: Stereochemistry of $H_{7\alpha}$, $H_{7\beta}$ and $H_{9\alpha}$

The stereochemistry of $H_{6\beta}$ and $H_{6\alpha}$ can be determined using the ROE correlations between $Me_{26\beta}$ and $H_{6\beta}$ (not $H_{6\alpha}$) and between $Me_{25\beta}$ and $H_{6\beta}$, indicating that $H_{6\beta}$ is on the same side as $Me_{25\beta}$ and $Me_{26\beta}$, while $H_{6\alpha}$ is on the opposite side (see *Figure 4.5.6*).

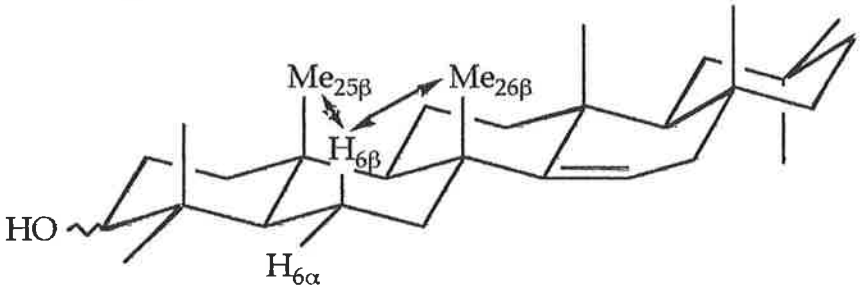


Figure 4.5.6: Stereochemistry of $H_{6\alpha}$ and $H_{6\beta}$

There is a strong ROE correlation between the methine protons at the ring junctions between ring A and ring B - $H_{5\alpha}$ - and between ring B and ring C - $H_{9\alpha}$ (it has been established that $H_{9\alpha}$ is below the ring). Neither of these two protons have any correlations to the methyl protons above the ring. This indicates that $H_{5\alpha}$ and $H_{9\alpha}$ are both below the ring. $H_{5\alpha}$ also has a ROE correlation to $H_{7\alpha}$ that has been established to be below the ring. There is a ROE correlation between $H_{5\alpha}$ and $Me_{23\alpha}$ but not between $H_{5\alpha}$ and $Me_{24\beta}$. These relationships are illustrated in *Figure 4.5.7*.

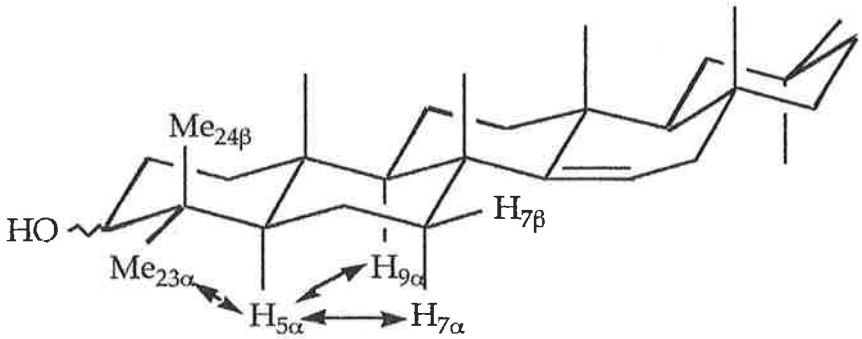


Figure 4.5.7: Stereochemistry of $H_{5\alpha}$ and $H_{9\alpha}$

The stereochemistry of $H_{3\alpha}$ can be established, using the ROE relationship between $H_{3\alpha}$ and $H_{5\alpha}$, while $H_{3\alpha}$ only correlates to $Me_{23\alpha}$, not $Me_{24\beta}$, as illustrated in *Figure 4.5.8*.

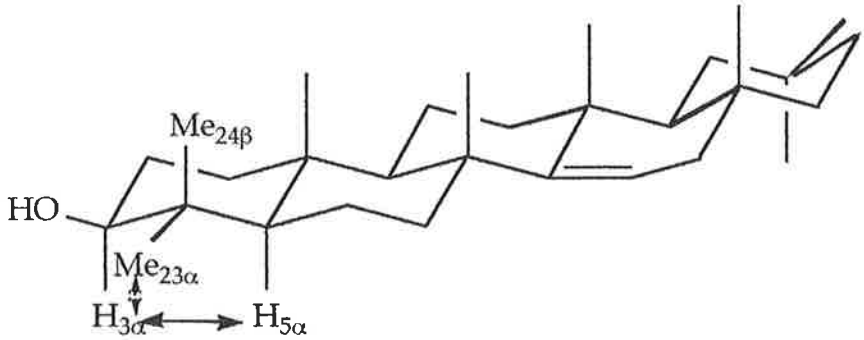


Figure 4.5.8: Stereochemistry of $H_{3\alpha}$

This relationship for $H_{3\alpha}$ (dd, J = 4.8, 11.4 Hz) is also supported by the coupling constants that indicate that $H_{3\alpha}$ must be axial (11.4 Hz indicates an axial/axial relationship with $H_{2\beta}$).

$H_{5\alpha}$ also has a ROE correlation to $H_{1\alpha}$, but not $H_{1\beta}$. $H_{3\alpha}$ has a ROE correlation to $H_{2\alpha}$, but no correlation to $H_{2\beta}$ (see *Figure 4.5.9*).

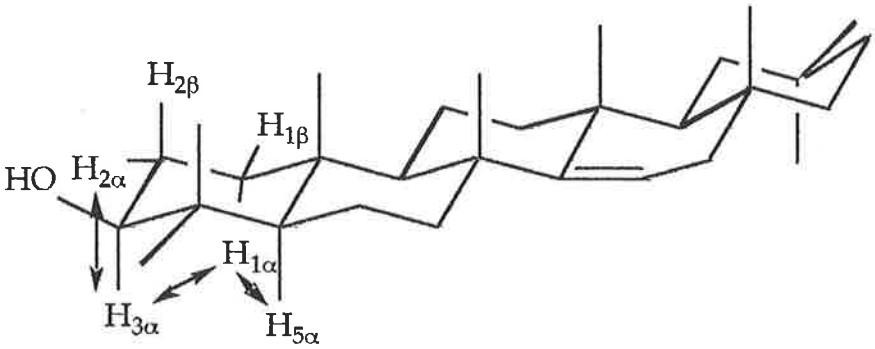


Figure 4.5.9: Stereochemistry of $H_{1\alpha}$ and $H_{2\alpha}$

The full assignment (including stereochemistry) of ring A and ring B has therefore been established.

Ring C has $H_{9\alpha}$ below the ring (at the ring junction between ring B and ring C), while the stereochemistry of the methylene pairs $H_{11\alpha}$ /$H_{11\beta}$ and $H_{12\alpha}$ /$H_{12\beta}$ needs to be established. $H_{11\beta}$ has a strong ROE correlation with both $Me_{25\beta}$ and $Me_{26\beta}$, indicating that $H_{11\beta}$ is above the ring, while there are no ROE correlations between $H_{11\alpha}$ and $Me_{25\beta}$. $H_{12\beta}$ has a ROE correlation to $Me_{26\beta}$ indicating it is above the ring and $H_{12\alpha}$ correlates to $H_{9\alpha}$ (See *Figure 4.5.10*). These assignments confirm the stereochemistry of ring C.

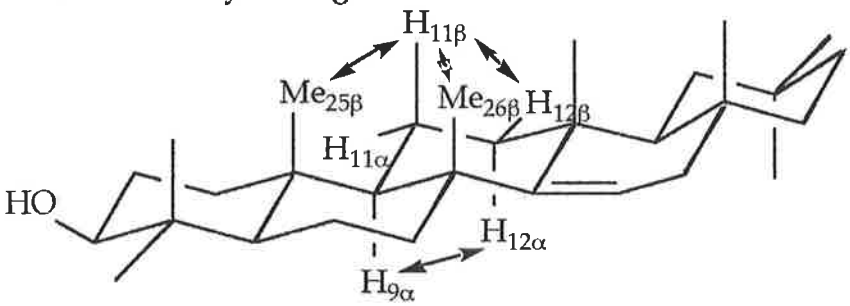


Figure 4.5.10: Assignment of $H_{11\alpha}$, $H_{11\beta}$, $H_{12\alpha}$ and $H_{12\beta}$

The correlations of $H_{18\alpha}$ are important for establishing the stereochemistry of ring E, remembering that $H_{18\alpha}$ has already been established as being below the ring and that the stereochemistry of H_{15}, $H_{16\alpha}$ and $H_{16\beta}$ have also been established so that the stereochemistry of ring D has been completed. Ring E contains three geminal proton pairs $H_{19\alpha}$/$H_{19\beta}$, $H_{21\alpha}$/$H_{21\beta}$ and $H_{22\alpha}$/$H_{22\beta}$ as well as the methyl protons $Me_{28\beta}$, $Me_{29\beta}$ and $Me_{30\beta}$. This arrangement is shown in *Figure 4.5.11*.

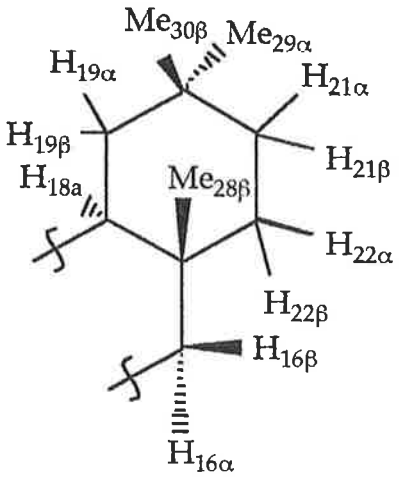


Figure 4.5.11: Protons on ring E

$H_{16\beta}$, a proton on ring D, has a weak ROE correlation to $H_{22\beta}$ (none to $H_{22\alpha}$), indicating they are on the same side of the ring. $H_{16\beta}$ has no ROE correlations to either $H_{21\beta}$ or $H_{21\alpha}$, confirming the positioning of these two methylene carbons (see *Figure 4.5.12*).

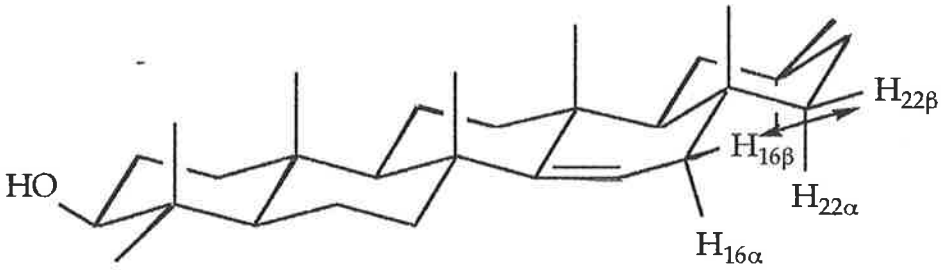


Figure 4.5.12: Conformation of $H_{22\alpha}$ and $H_{22\beta}$

$Me_{28\beta}$ has a strong ROE correlation to $H_{21\beta}$, indicating $H_{21\beta}$ is above the ring. There is no ROE correlation from $H_{22\alpha}$ to $Me_{28\beta}$ (see *Figure 4.5.13*).

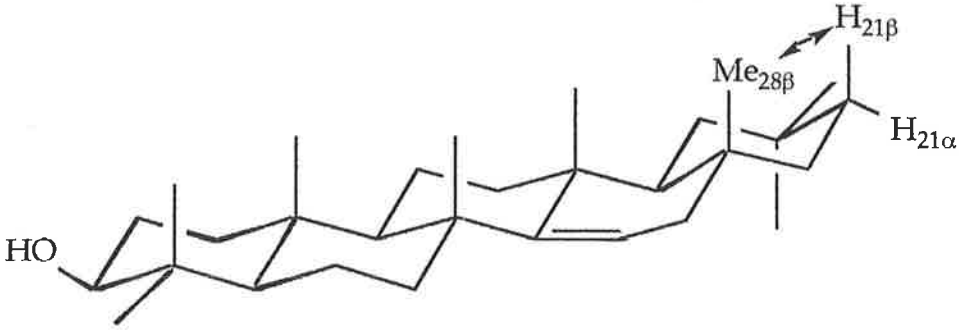


Figure 4.5.13: Conformation of $H_{21\alpha}$ and $H_{21\beta}$

The final assignment is that concerning $H_{19\alpha}$ and $H_{19\beta}$. $Me_{30\beta}$ has a ROE correlation to $H_{19\beta}$, but not to $H_{19\alpha}$, while $Me_{30\beta}$ also has a ROE correlation to $H_{21\beta}$.This suggests that $H_{19\beta}$ is above the ring, while $H_{19\alpha}$ is below the ring (see *Figure 4.5.14*).

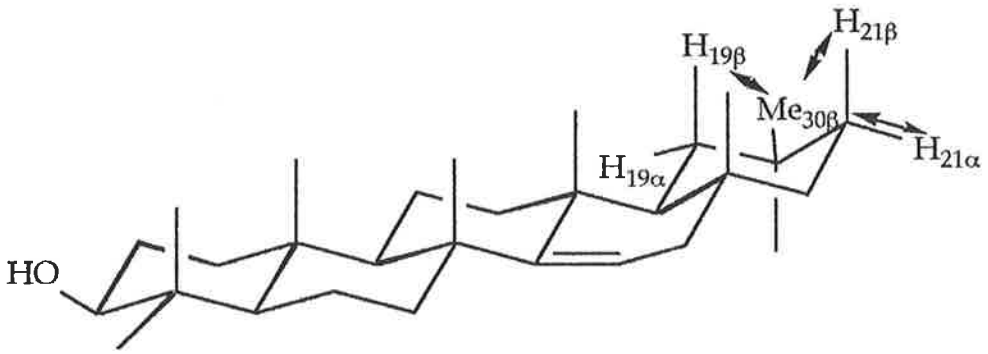


Figure 4.5.14: Conformation of $H_{19\alpha}$ and $H_{19\beta}$

The ROE correlations therefore have allowed for the assignment of all methylene protons and this confirms the proposed stereochemistry.

This structure is an isomer of taraxerol (**73**). The methyl group, $Me_{27\beta}$, is clearly above the ring and $H_{18\alpha}$ is below the ring in Compound D. In comparison, the stereochemistry of the equivalent methyl and proton in taraxerol (**73**) is reversed. The two compounds have differences in their MS fragmentation pattern, they have significantly different melting points and they run to different positions on TLC when added together. Extensive literature research indicates that Compound D had not previously been reported.

It is therefore a new compound that has been named judarrylol (96).

Its structure is illustrated in *Figure 4.5.15*.

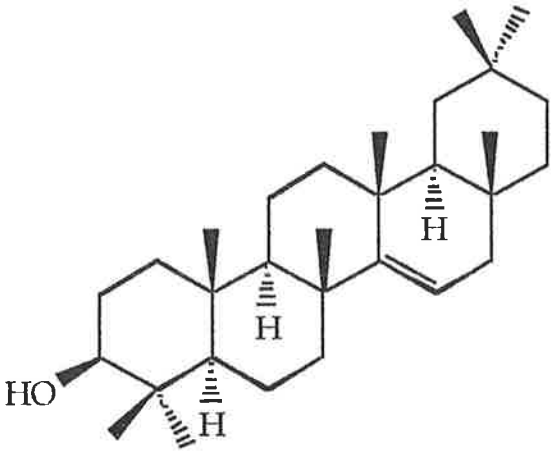


Figure 4.5.15: Judarrylol (96)

CHAPTER 5 FRACTION 3

5.1 Initial treatment

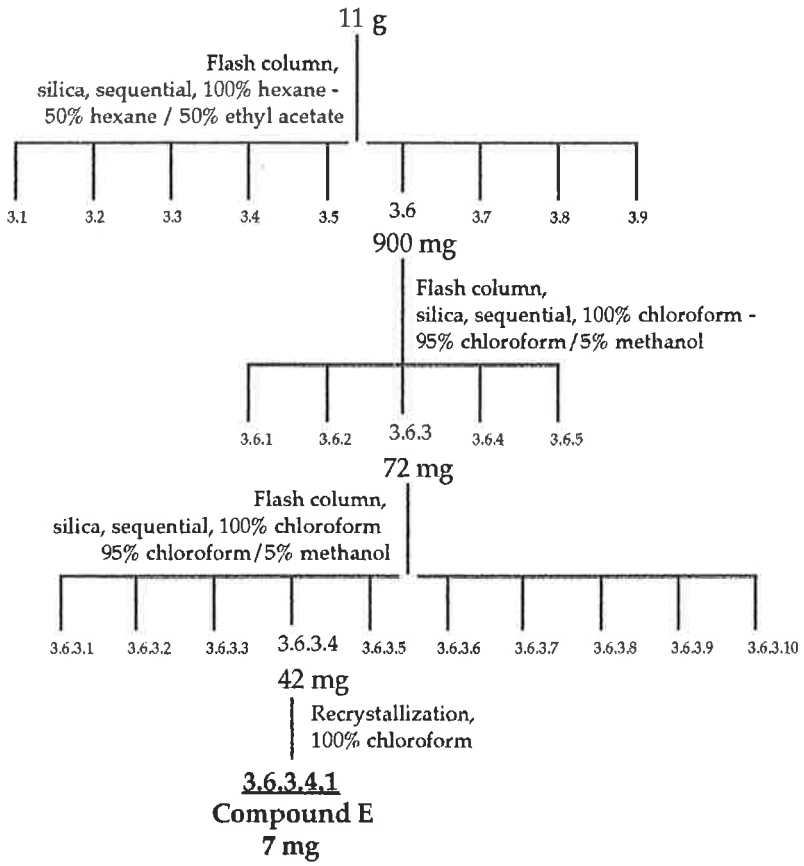
Treatment of Fraction 3 yielded one compound, as outlined in **Diagram 5.1.**

Fraction 3 (11 g) was separated from the original methanol extract (828 g) using 50% hexane/50% ethyl acetate as the eluting solvent.

Fraction 3 was initially subjected to flash chromatography (silica, sequential, 100% hexane - 50% hexane/50% ethyl acetate) to give nine fractions. Fraction 3.6 (900 mg) was then flash chromatographed (silica, sequential, 100% chloroform - 95% chloroform/5% methanol) to yield five fractions.

Fraction 3.6.3 (72 mg) was then separated using flash chromatography (silica, sequential, 100% chloroform - 95% chloroform/5 % methanol) to give ten fractions. Fraction 3.6.3.4 (42 mg) underwent a further purification step (recrystallization, 100% chloroform) to give Fraction 3.6.4.3.1 that was labelled Compound E (7 mg). Its structure determination is discussed in Chapter 5.2, pg 71.

Diagram 5.1: Isolation of Compound E



5.2 Compound E

Compound E (7 mg) was isolated as white crystals (see Chapter 5.1, **Diagram 5.1,** pg 70) with a MP of 282 - 285°C and an $[\alpha]_D$ of + 69° (c 0.1, $CHCl_3$).

The high resolution mass spectrum (HRMS) gave an M^+ of 456.3599 that gave a molecular formula of $C_{30}H_{48}O_3$ (calculated = 456.3605, +/– 0.0006). The molecular formula revealed the presence of seven DBE. The IR indicated the presence of a carbonyl (1720 cm^{-1}), and a carbon-carbon double bond (1660 cm^{-1}) which accounts for two DBE, leaving five DBE to be associated with rings. This indicated that Compound E was a pentacyclic compound. There was also a broad peak at 3450 cm^{-1} that indicated the presence of hydroxyl groups.

The presence and position of a double bond in a triterpene are very diagnostic, as the molecular ion formed undergoes a retro Diels-Alder fragmentation to give a very characteristic peak in the mass spectrum.[75b] The MS of Compound E indicated the very characteristic fragmentation pattern associated with an Δ^{12} ene position. The fragmentations observed in the mass spectrum were 248 (100%), 410 (60%), 203 (60%), 438 (38%) with an M^+ of 456. These are identical to those observed for ursolic acid (**97**)[76] and are outlined in *Figure 5.2.1.*

A series of NMR experiments - ^1H, COSY, ROESY, ^{13}C, HMQC and HMBC - were used to confirm that Compound E was ursolic acid (**97**). This data is given in **Table 5.2.1,** pg 73. The numbering system is that commonly used in the literature for triterpenes and is illustrated in *Figure 5.2.2.*

A ROESY experiment was used to establish the stereochemistry of the ring junctions to be that of ursolic acid (**97**), with $Me_{24\beta}$, $Me_{25\beta}$, $Me_{26\beta}$ and $Me_{29\beta}$ being above the ring. $Me_{23\alpha}$ and $Me_{30\alpha}$ were below the ring. The ring protons $H_{5\alpha}$ and $H_{9\alpha}$ were below the ring, while $H_{18\beta}$ was above the ring. The conformation of the methylene protons could not be determined.

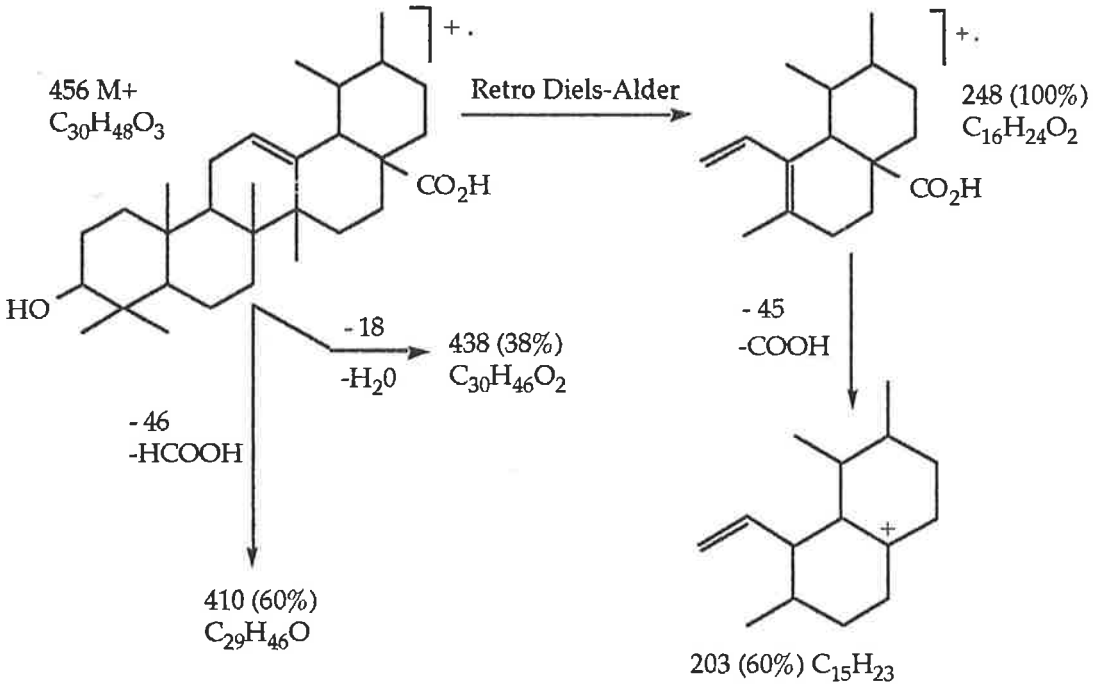


Figure 5.2.1 Fragmentation of Compound E

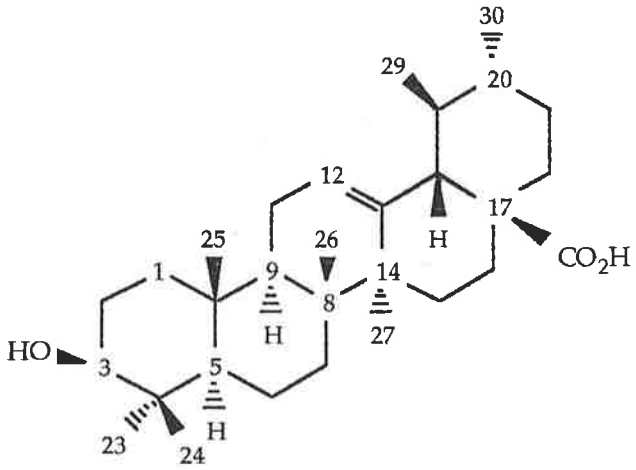


Figure 5.2.2: Structure of (97)

The MS fragmentation, ^{1}H and ^{13}C chemical shift values, MP and $[\alpha]_D$ values for Compound E match the recorded values for ursolic acid (**97**).[76, 78, 79, 80] The systematic name for this compound is 3-β-hydroxy-urs-12-en-28-oic acid. This has not been previously reported from *Scaevola spinescens* or any other plant of the *Scaevola* genera.

However, α-amyrin (**74**), α-amyrinic acid (**75**), and α-amyrone (**76**) have been recorded from *Scaevola taccada*.[56] α-Amyrin is identical to ursolic acid (**97**), apart from the carboxyl functional group that is replaced with a methyl group that is above the ring.

Table 5.2.1: NMR data for Compound E - (97)#

C	δ C	H	δ H	I	Mult.	COSY	ROESY*	HMBC
C_1	38.0	H_{1a}	1.588	1	m			C_2, C_{10}
		H_{1b}	1.466	1	m			C_2, C_{10}
C_2	27.5	H_{2a}	1.461	1	m	H_{2b}, H_3		C_1
		H_{2b}	1.546	1	m	H_{2a}, H_3		C_1
C_3	77.5	$H_{3\alpha}$	2.980	1	br t	H_{2a}, H_{2b}	$Me_{23\alpha}$, $H_{5\alpha}$	C_2, C_{23}, C_{24}
C_4	38.0							
C_5	54.5	$H_{5\alpha}$	0.713	1	m		$H_{3\alpha}H_{9\alpha}Me_{23\alpha}$	$C_6C_7C_{23}C_{24}C_{25}$
C_6	17.6	H_{6a}	1.544	1	m			C_5, C_7
		H_{6b}	1.392	1	m			C_5, C_7
C_7	38.8	H_{7a}	1.435	1	m			C_6
		H_{7b}	0.924	1	m			C_6, C_8, C_{26}
C_8	39.8							
C_9	46.8	$H_{9\alpha}$	1.432	1	m	H_{11a}	$H_{5\alpha}$	C_8, C_{25}, C_{26}
C_{10}	36.2							
C_{11}	22.8	H_{11a}	1.815	1	m	$H_{9\alpha}$, H_{11b}, H_{12}		C_9, C_{12}, C_{13}
		H_{11b}	0.976	1	m	H_{11a}		C_{12}, C_{13}
C_{12}	124.4	H_{12}	5.118	1	br d	H_{11a}		C_{11}, C_{13}
C_{13}	137.6							
C_{14}	41.3							
C_{15}	26.5*	H_{15a}	1.463	1	m			
		H_{15b}	1.081	1	m			
C_{16}	27.5*	H_{16a}	1.613	1	m			
		H_{16b}	1.546	1	m			
C_{17}	32.3							
C_{18}	52.0	$H_{18\beta}$	2.092	1	d,11.4	$H_{19\alpha}$	$H_{26\beta}H_{29\beta}$	$C_{12}C_{13}C_{14}C_{17}C_{18}$
C_{19}	39.0	$H_{19\alpha}$	1.272	1	m	$H_{18\beta}H_{20\beta}Me_{29\beta}$		C_{18}, C_{20}, C_{29}
C_{20}	38.3	$H_{20\beta}$	1.507	1	m	$H_{19\alpha}$, $Me_{30\alpha}$		C_{19}, C_{21}, C_{30}
C_{21}	30.0	H_{21a}	1.503	1	m			C_{20}, C_{22}
		H_{21b}	1.467	1	m			C_{20}, C_{22}
C_{22}	36.0	H_{22a}	1.408	1	m			C_{17}, C_{20}, C_{21}
		H_{22b}	1.267	1	m			C_{20}, C_{21}
C_{23}	28.8	$Me_{23\alpha}$	0.667	3	s		$H_{5\alpha}$	C_3, C_4, C_5, C_{24}
C_{24}	15.1	$Me_{24\beta}$	0.886	3	s		$Me_{25\beta}$	C_3, C_4, C_5, C_{23}
C_{25}	14.7	$Me_{25\beta}$	0.857	3	s		$Me_{24\beta}$, $Me_{26\beta}$	C_1, C_5, C_9, C_{10}
C_{26}	16.4	$Me_{26\beta}$	0.741	3	s		$Me_{25\beta}$	C_8, C_9, C_{14}
C_{27}	23.5	$Me_{27\alpha}$	1.030	3	s			C_8, C_{14}, C_{15}
C_{28}	179.1							
C_{29}	17.6	$Me_{29\beta}$	0.802	3	d,6.6	$H_{19\alpha}$	$H_{18\beta}$	C_{18}, C_{19}, C_{20}
C_{30}	20.5	$Me_{30\alpha}$	0.904	3	d,6.0	$H_{20\beta}$		C_{20}, C_{21}

* = assignments may be reversed

600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$

I = Integration Mult. = Multiplicity

ROESY* = Only those signals that were used to assign stereochemistry of the ring junctions are recorded.

CHAPTER 6 FRACTION 4

6.1 Initial treatment

Treatment of Fraction 4 yielded three compounds, as outlined in **Diagram 6.1.1** and **Diagram 6.1.2.**

Fraction 4 (14 g) was separated from the original methanol extract (828 g) using 100% ethyl acetate as the eluting solvent.

Initially 1 g of Fraction 4 was partitioned between hexane (100 ml) and a methanol (90 ml)/water (10 ml) mixture The methanol/water fraction was then freeze-dried and redissolved in methanol. This was then partitioned with chloroform (100 mL), methanol (90 mL) and water (10 mL) and the chloroform extract labelled 4.E (45 mg).

A TLC of Fraction 4.E (45 mg) revealed two blue spots under UV light. It was subjected to flash chromatography (silica, isocratic, 99% chloroform/1% methanol) to give nine fractions.

Fraction 4.E.6 (22 mg) gave two clear peaks under normal phase analytical HPLC (isocratic, 50% hexane/ 50% ethyl acetate, 330 nm, 1 mL/min., 30 min.). These peaks were then collected using normal phase analytical HPLC under the same conditions and labelled 4.E.6.1 (1 mg) and 4.E.6.5 (2 mg). When both these fractions were reinjected under the same HPLC conditions as previously, they appeared as single peaks at the expected retention time.

It was decided that the rest of Fraction 4 (13 g) would be subjected to an identical partition process to isolate more of these compounds. The chloroform extract of this second partition process was labelled 4.C (200 mg).

4.C was then subjected to flash chromatography (silica, isocratic, 99% chloroform /1% methanol) to give nine fractions.

Fraction 4.C.3 (100 mg) contained a blue spot at the same R_f as 4.E.6.5 and was then flash chromatographed (silica, isocratic, 99% chloroform/ 1% methanol) a second time to give nine fractions.

Fraction 4.C.3.3 (63 mg) was then subjected to normal phase preparative HPLC (isocratic, 50% hexane/50% ethyl acetate, 330 nm, 5 mL/min, 30 min.) to give three fractions.

Fraction 4.C.3.3.2 had the same retention time as 4.E.6.5. Fraction 4.C.3.3.2 was labelled Compound F (25 mg). The structure determination of Compound F is discussed in Chapter 6.2, pg 77.

Fraction 4.C.6 (27 mg) was also subjected to normal phase analytical HPLC (isocratic, 50 % hexane/50 % ethyl acetate, 256 nm, 1 mL/min, 30 min.) where 4 mg of 4.C.6.1 was isolated. This fraction was labelled Compound G (4 mg) (see Chapter 6.3, pg 88).

There was insufficient material to make a definitive determination of the structure of Fraction 4.E.6.1 (1 mg). This is still being pursued.

Diagram 6.1.1: Separation of Compound F

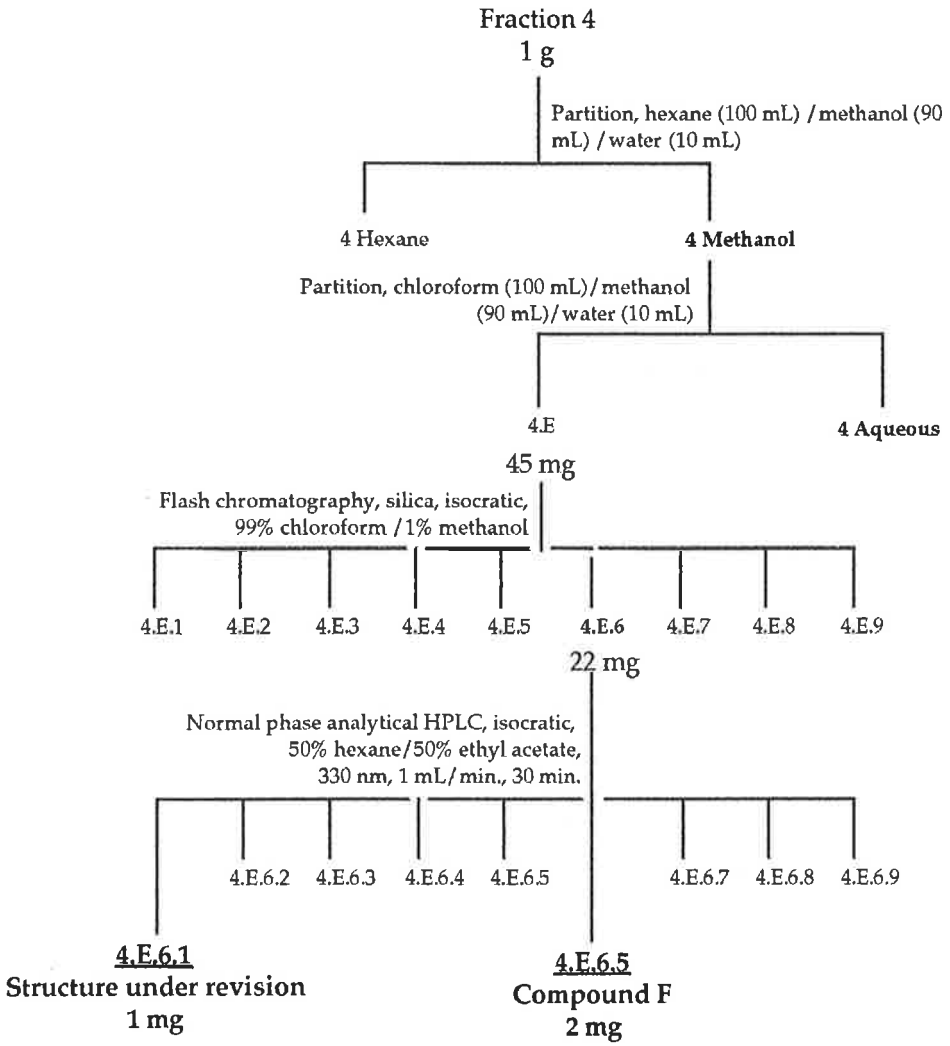
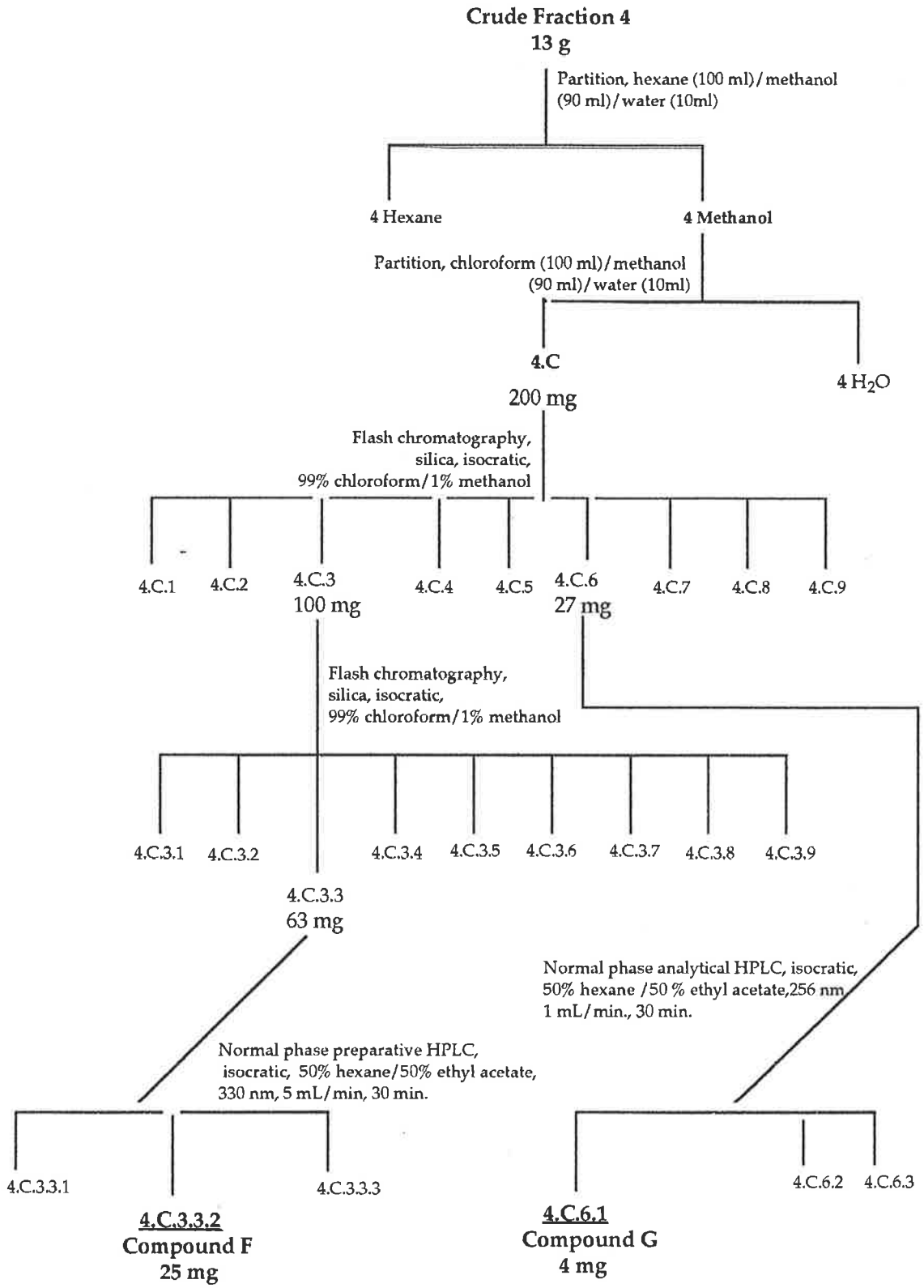


Diagram 6.1.2: Separation of Compounds F and G

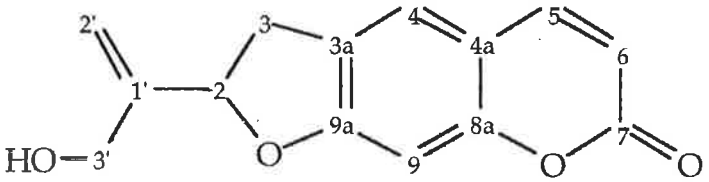


6.2 Compound F

Compound F (25 mg) was isolated as white crystals (see Chapter 6.1, **Diagrams 6.1.1** and **6.1.2**, pgs 75, 76) with a MP of 123 - 125°C and an $[\alpha]_D$ of - 10° (c 0.1, $CHCl_3$).

Compound F was found by HRMS to have an M^+ (EI) of 244.0733 that gave it a molecular formula of $C_{14}H_{12}O_4$ (calculated = 244.0736, +/- 0.0003). There was a total of six DBE to be accounted for. The IR spectrum revealed a carbonyl carbon (1720 cm^{-1}) as well as peaks in the aromatic fingerprint region, and carbons in the alkene region (1660 cm^{-1}). There was also an hydroxyl functional group present (3360 cm^{-1}).

Routine ^1H and ^{13}C NMR experiments were run as well as COSY, HSQC, HSQC-TOCSY, HMBC and ROESY experiments. This led to the elucidation of Compound F as a novel dihydrofurano coumarin, named emmarin (**98**). The numbering system is that commonly used in the literature for dihydrofurano coumarins.



(98)

The NMR data for (**98**) is given in **Table 6.2.1** pg 78. There were eleven ^1H signals recorded and fourteen ^{13}C carbon signals. There were therefore four oxygen atoms and one proton to be accounted for, as required by HRMS.

There was a clear COSY correlation between H_5 and H_6 (9.6 Hz), which, when combined with the HMQC data, were the protons of an α, β unsaturated system. H_5 (δ 7.527) is directly bonded to C_5 (δ 143.9) while H_6 (δ 6.151) is bonded to C_6 (δ 112.6). As H_5 and H_6 are both doublets, C_5 and C_6 must be bonded to a quaternary carbon. This part structure is shown in *Figure 6.2.1*.

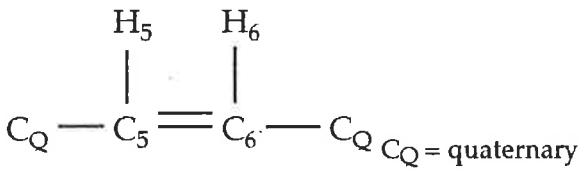


Figure 6.2.1: Part structure A

Table 6.2.1: NMR data for (98)#

C	δ C	H	δ H	Int	M.	J (Hz)	COSY	HMBC
C_2	85.4	H_2	5.394	1	dd	7.2, 9.6	H_{3a},H_{3b},$H_{2'a}$,$H_{2'b}$	$C_{1'}$, $C_{2'}$, $C_{3'}$
C_3	34.1	H_{3a}	3.392	1	dd	9.6, 15.6	H_2, H_{3b}, H_4	C_2, C_{3a}, C_{9a}, $C_{1'}$
		H_{3b}	3.137	1	dd	7.2, 15.6	H_2, H_{3a}, H_4	C_2, C_{3a}, C_{9a}, $C_{1'}$
C_{3a}	124.6							
C_4	123.7	H_4	7.162	1	br s		H_{3a}, H_{3b}	C_3, C_5, C_{8a}, C_{9a}
C_{4a}	116.1							
C_5	143.9	H_5	7.527	1	d	9.6	H_6	C_4, C_6, C_7, C_{8a}
C_6	112.6	H_6	6.151	1	d	9.6	H_5	C_5, C_7
C_7	161.5							
C_{8a}	155.1							
C_9	98.4	H_9	6.703	1	s			C_{3a}, C_{8a}, C_{9a}
C_{9a}	163.0							
$C_{1'}$	146.6							
$C_{2'}$	113.3	$H_{2'a}$	5.321	1	br s		H_2,$H_{2'b}$,$H_{3'}$	C_2, $C_{1'}$, $C_{3'}$
		$H_{2'b}$	5.219	1	br s		H_2,$H_{2'a}$,$H_{3'}$	C_2, $C_{1'}$, $C_{3'}$
$C_{3'}$	63.1	$H_{3'}$	4.209	2	m		$H_{2'a}$, $H_{2'b}$	C_2, $C_{1'}$, $C_{2'}$
		$OH_{3'}$	4.915	1	br s			

600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in d_6-DMSO

Int = Integration M. = Multiplicity

COSY correlations were observed for all but one of the remaining protons within the molecule. This spin system is displayed in *Figure* 6.2.2.

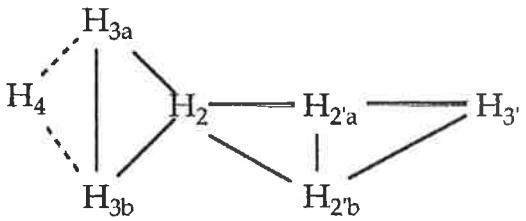


Figure 6.2.2: COSY correlations for spin system B

H_4 was a broadened singlet in the high-resolution ^{1}H spectrum, and had a COSY correlation to H_{3a} and H_{3b} that must be a long-range coupling.

Spin system B (see *Figure* 6.2.2) can be extended by analysing the $^1J_{CH}$ correlations.

H_{3a} and H_{3b} (δ 3.392 and δ 3.137) are methylene protons, both attached to C_3

(δ 34.1). These ^{1}H and ^{13}C chemical shifts are in the alkane range, but with electronegative constituents nearby. H_{3a} and H_{3b} COSY correlate to $H_{2,,}$ which is bonded to C_2. The ^{1}H chemical shift of H_2 is δ 5.394 and this, as well as the ^{13}C chemical shift of C_2 (δ 85.4) indicates that C_2 is directly bonded to oxygen.

The valency of C_3 must be completed by being bonded to a quaternary carbon, as H_{3a} and H_{3b} are both unambiguous doublet of doublets, although they do have a long range COSY correlation to H_4. There are long-range $^{n}J_{HH}$ correlations from H_2 to $H_{2'a}$ (δ 5.321) and $H_{2'b}$ (δ 5.219). However, $H_{2'a}$ /$H_{2'b}$ are a pair of methylene protons. They are directly attached to $C_{2'}$ (δ 113.3 - an sp^2 hybridised carbon) and appear as broadened singlets in the ^{1}H spectrum. The valency of $C_{2'}$ needs to be completed and given the ^{1}H and ^{13}C chemical shift values of $H_{2'a}$, $H_{2'b}$ and $C_{2'}$, it must be attached to another sp^2 hybridised carbon, which is a quaternary carbon.

The valency of C_2 is satisfied with one more bond, so it follows that the COSY correlation between H_2 and $H_{2'a}$/$H_{2'b}$ is long-range, rather than the protons being adjacent. This is consistent with the observation made earlier (ie. there is a weak COSY correlation).

H_2 is also COSY correlated to $H_{3'}$ (two protons). $H_{3'}$ is a methylene pair of protons, with chemical shifts of δ 4.209 and which are bonded directly to $C_{3'}$ (δ 63.1). These ^{1}H and ^{13}C chemical shift values are consistent with $C_{3'}$ also being directly attached to oxygen. The valency of $C_{3'}$ can therefore be satisfied by one further bond to a carbon atom as it is clearly not an acetal carbon.

There are therefore three part structures that are adjacent to each other (see *Figure 6.2.3*).

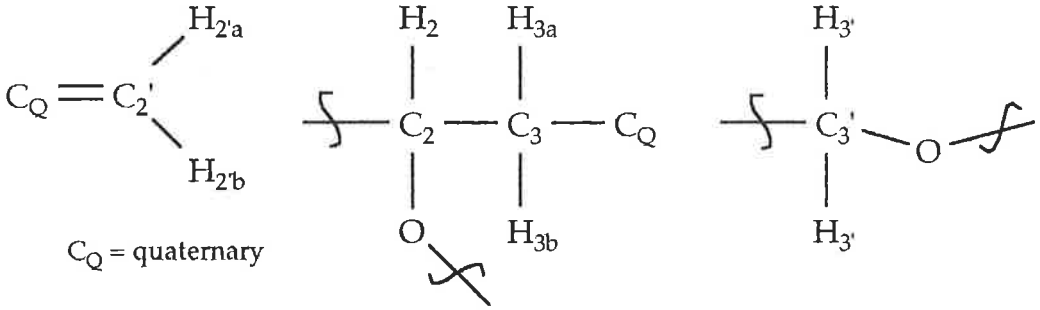


Figure 6.2.3: Part structures B

There are two proton signals in the ^{1}H spectrum that have yet to be considered.

H_4 – a proton directly attached to C_4 - has an ^1H chemical shift of δ 7.162 and is a broadened singlet. There is a very weak COSY correlation between H_4 to both H_{3a} and H_{3b}, which must be due to long-range coupling.

The only other proton not mentioned so far is H_9, a singlet with a chemical shift value of δ 6.703, attached to C_9 (δ 98.4). These ^1H and ^{13}C chemical shift values are consistent with an aromatic proton attached to a carbon adjacent to two carbon atoms, each with an oxygen attached.

C_{3a}, C_{4a}, C_7, C_{8a}, C_{9a} and $C_{1'}$ are all quaternary carbons that are not involved in any of the part structures established so far. The HMBC correlations listed in **Table 6.2.1**, pg 78 were then used to establish connectivity.

Part Structure A

The pair of doublets, H_5 and H_6, have a $^{2/3}J_{CH}$ correlation into C_7 (δ 161.5). C_7 is a quaternary carbon within the range of the carbonyl carbon of an ester functional group. The ^{13}C chemical shift value for C_6 - δ 112.6 - indicates that it is adjacent to a carbon that is directly attached to oxygen while the valency of C_5 is completed by being attached to a quaternary sp^2 hybridised carbon (see *Figure 6.2.4*).

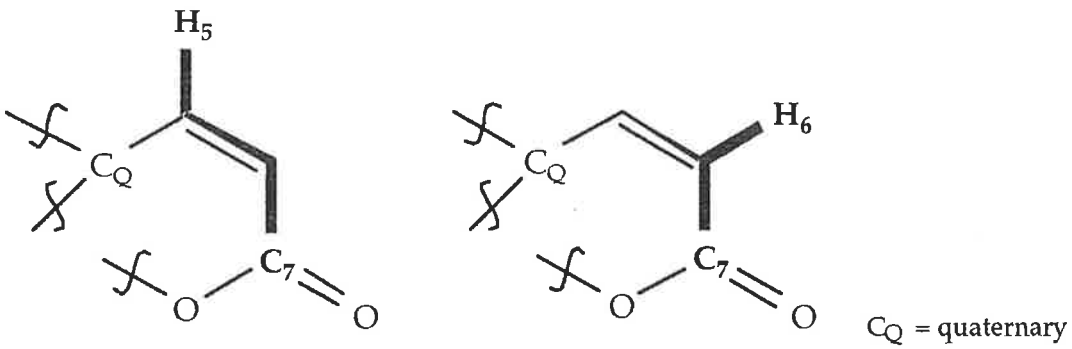


Figure 6.2.4: HMBC correlations for H_5 and H_6

C_{8a} is a quaternary carbon with a ^{13}C chemical shift value within the range of a phenolic carbon (δ 155.1). H_5 has an HMBC correlation into C_{8a}. This indicates that C_{8a} is bonded to the oxygen of the ester, in a position where H_5 can have a $^3J_{CH}$ correlation to C_{8a} (*Figure 6.2.5*)

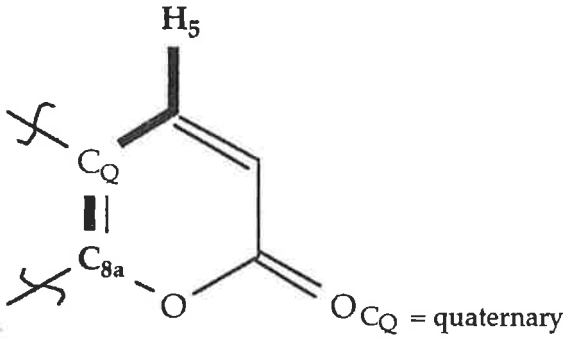


Figure 6.2.5: HMBC correlations from H_5 to C_{8a}

Although there are no HMBC correlations to the other quaternary carbon in the position as shown in the diagram above, this arrangement accounts for one more DBE as it results in the formation of a ring.

H_5 also has an HMBC correlation into C_4 (δ 123.7) - an sp^2 hybridised carbon - which is directly attached to H_4. H_4 (δ 7.162) is a singlet and so C_4 is also bonded to two quaternary carbons. H_4 has a $^{2/3}J_{CH}$ correlation into C_5 and C_{8a}. This confirms the positioning of H_4 adjacent to H_5. The other carbon bonded to C_4 is not only quaternary, but also must be sp^2 hybridised. Also, given the ^{13}C chemical shift value of δ 123.7, C_4 is adjacent to carbons that are not directly bonded to oxygen. The positioning of C_4/H_4 is shown in *Figure 6.2.6*.

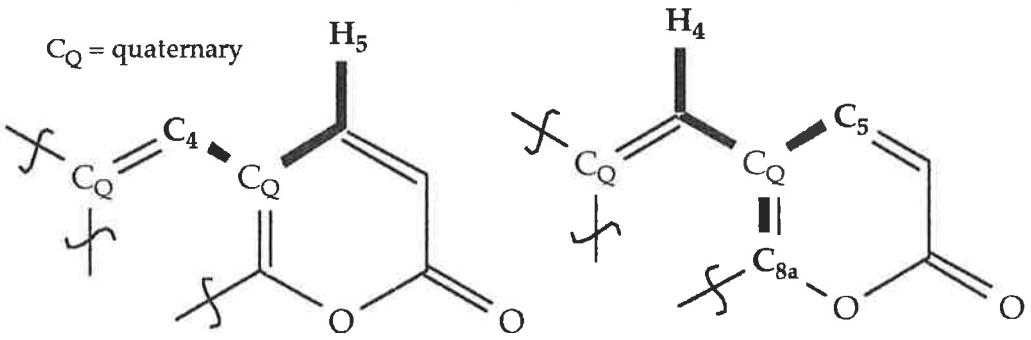


Figure 6.2.6: HMBC correlations from H_5 to C_4; H_4 to C_5 and C_{8a}

Part structure B

There are three part structures that have to be adjacent for their allylic coupling to be observed.

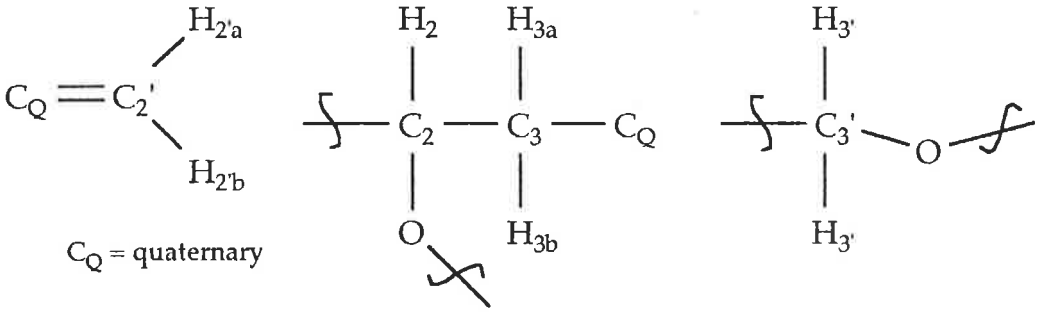


Figure 6.2.3: Part structures B

There are three quaternary carbons that have $^{2/3}J_{CH}$ correlations that can be used to connect the three part structures (see *Figure 6.2.3*).

$H_{2'a}$ and $H_{2'b}$ are the alkene protons that are bonded to $C_{2'}$, which must be bonded to a quaternary sp^2 hybridised carbon. They have a $^{2/3}J_{CH}$ correlation into $C_{1'}$ (a quaternary carbon with a ^{13}C chemical shift value of 146.6 ppm). This suggests that $C_{1'}$ is the other carbon of the double bond (see *Figure 6.2.7*).

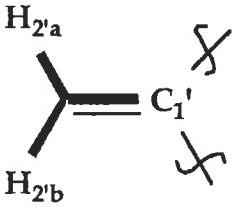


Figure 6.2.7: Correlations of $H_{2'a}$ and $H_{2'b}$ to $C_{1'}$

$H_{2'a}$ and $H_{2'b}$ also $^{2/3}J_{CH}$ correlate to C_2 and $C_{3'}$. The valency of $C_{1'}$ will therefore be completed by being bonded to C_2 and $C_{3'}$ (see *Figure 6.2.8*)

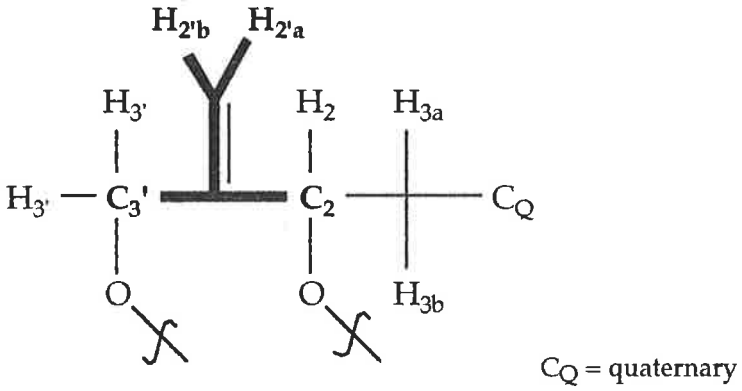


Figure 6.2.8: HMBC correlations for $H_{2'a}$ and $H_{2'b}$

There are other $^{2/3}J_{CH}$ correlations for protons H_2, H_{3a}, H_{3b} and $H_{3'}$ that support this arrangement. H_2 correlates into $C_{1'}$, $C_{2'}$ and $C_{3'}$ while $H_{3'}$ correlates into $C_{1'}$, $C_{2'}$ and C_2. H_{3a} and H_{3b} are geminal protons that have $^{2/3}J_{CH}$ correlations to $C_{1'}$ and C_2 (see *Figure 6.2.9*).

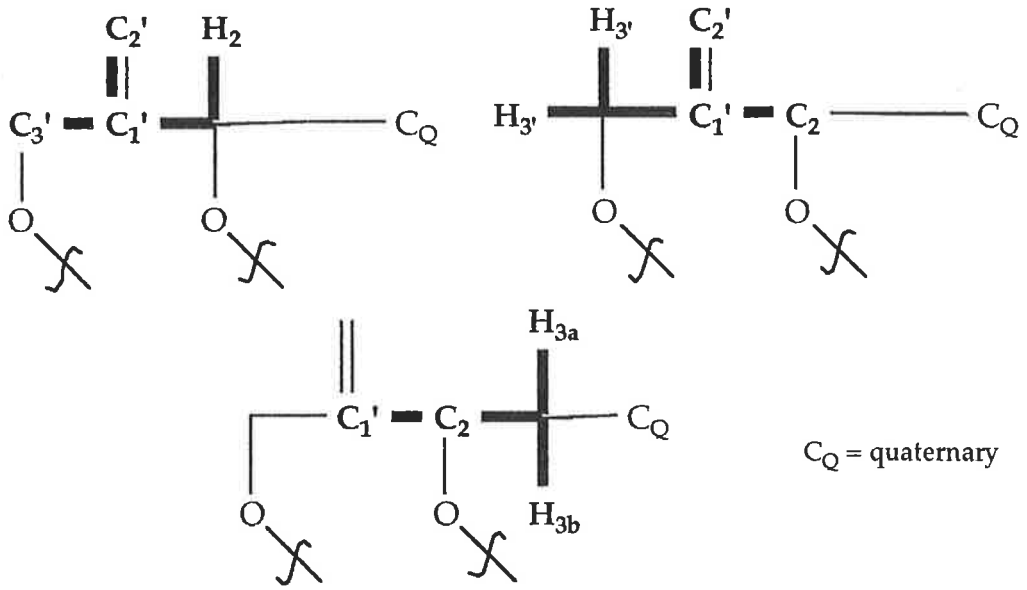


Figure 6.2.9: HMBC correlations for H_2, H_{3a}, H_{3b} and $H_{3'}$

This gives rise to part structure B, illustrated in *Figure 6.2.10*. There are three positions where the valency of atoms has not been completed in this part structure. There are two oxygen atoms that need to be bonded to another atom and one quaternary carbon that must be assigned.

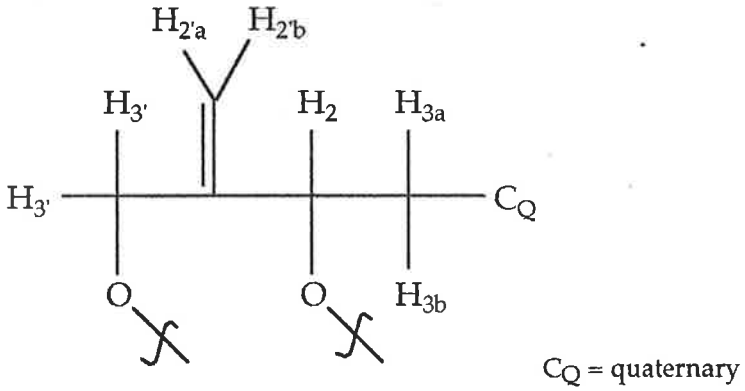


Figure 6.2.10: Part structure B

Connecting part structures A and B

Part structures A and B are illustrated in *Figure 6.2.11*.

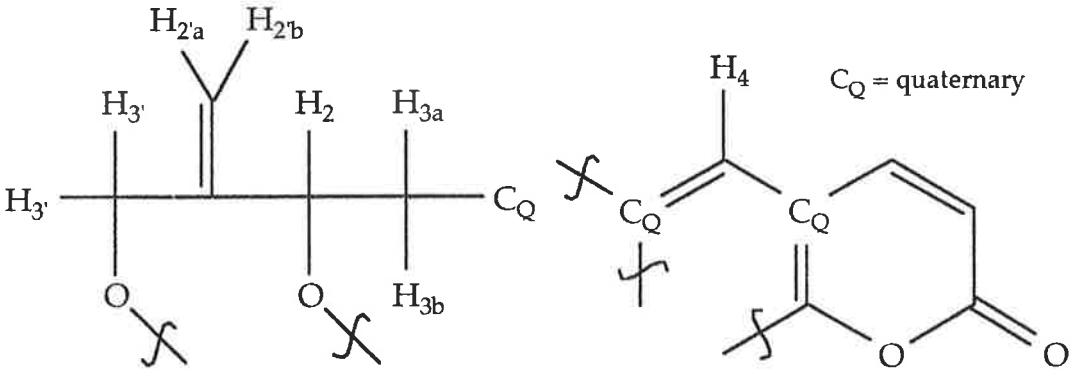


Figure 6.2.11: Part structures A and B

There are four carbon atoms that have not been assigned. C_{3a}, C_{4a} and C_{9a} are all quaternary, sp^2-hybridised carbon atoms while C_9 is bonded to H_9 (δ 6.703) which is a singlet. This indicates that C_9 must be bonded to two quaternary carbons to have its valency requirements met.

H_{3a} and H_{3b} have a weak COSY correlation to H_4 that is part of part structure A and so the quaternary carbon adjacent to both H_4 and the geminal pair H_{3a} and H_{3b} is the same carbon. There are three quaternary carbons to be assigned (see *Figure 6.2.12*).

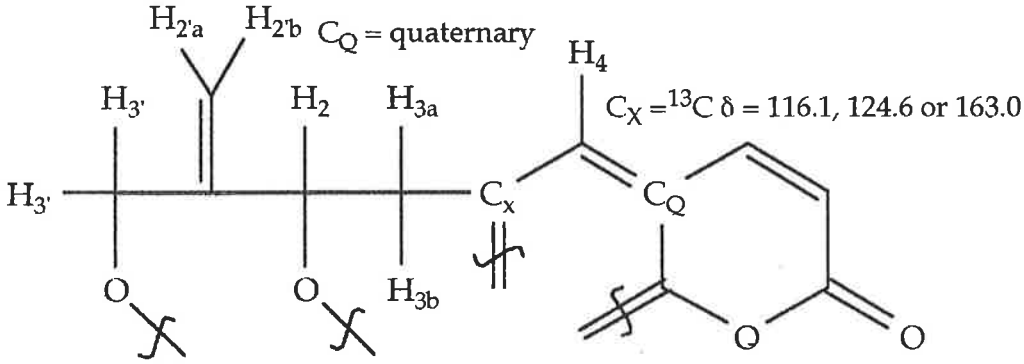


Figure 6.2.12: Possible bonding to C_{3a}

H_{3a} and H_{3b} have $^{2/3}J_{CH}$ correlations to the quaternary carbons C_{9a} (δ 163.0) and C_{3a} (δ 124.6), but not to C_{4a} (δ 116.1), so $C_X = C_{4a}$ can be discarded.

The ^{13}C chemical shift of C_{9a} indicates that it is sp^2 hybridised and bonded to oxygen. It is not consistent with being bonded to C_3 and C_4. Therefore $C_X = C_{9a}$ can also be discarded.

The quarternary carbon adjacent to H_{3a}/H_{3b} and H_4 is therefore C_{3a} (see *Figure 6.2.13*).

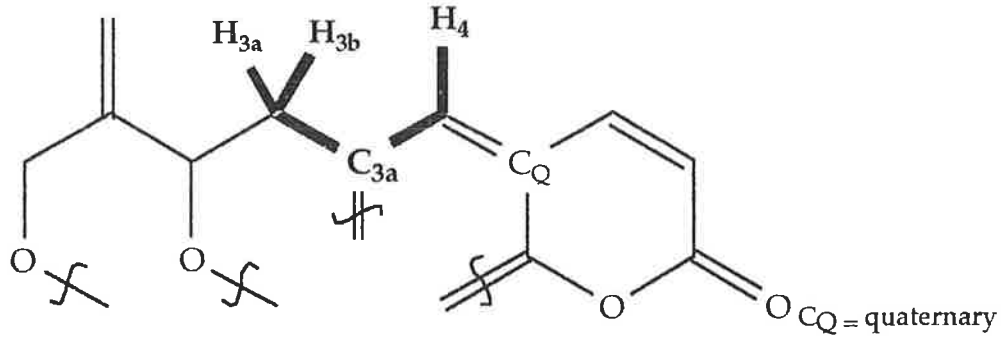


Figure 6.2.13: HMBC correlations to C_{3a}

It also follows that the valency of C_{3a} is met by being bonded to C_{9a} to account for the HMBC correlations between H_4, H_{3a} and H_{3b} and C_{9a} (*Figure 6.2.14*).

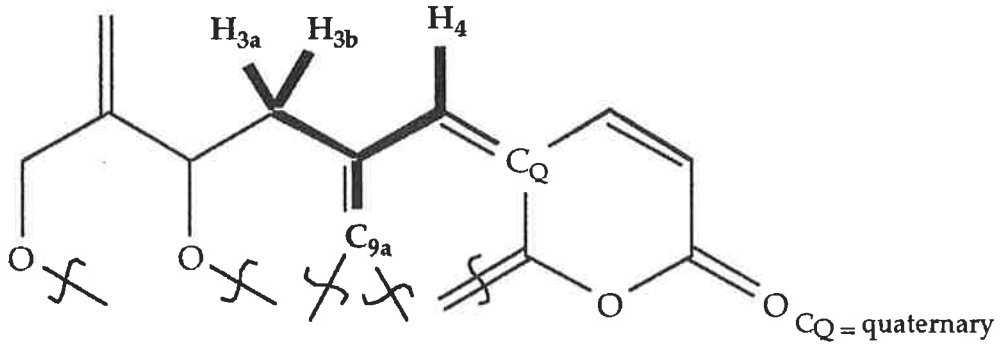


Figure 6.2.14: HMBC correlations to C_{9a}

There is only one more quaternary carbon, C_{4a} (δ 116.1) which does not have any HMBC correlations. Therefore C_{4a} is at the ring junction (see *Figure 6.2.15*).

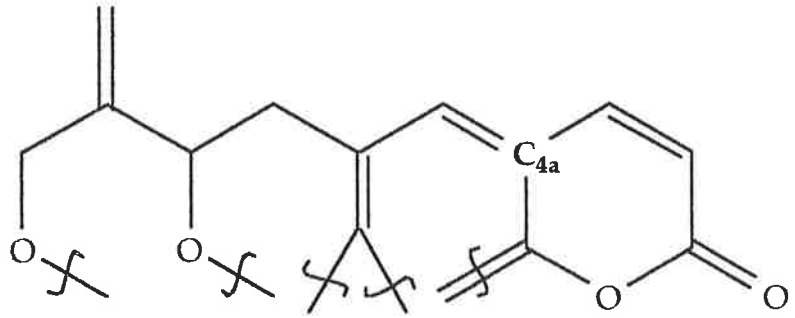


Figure 6.2.15: Position of C_{4a}

H_9 is a singlet (δ 6.703), bonded to C_9 (δ 98.4), indicating that C_9 is bonded to two quaternary carbons to complete its valency. H_9 has HMBC correlations to C_{3a}, C_{8a} and C_{9a}. It follows that C_9 is bonded to C_{8a} and C_{9a}. This will complete a second ring and accounts for another DBE (see *Figure 6.2.16*).

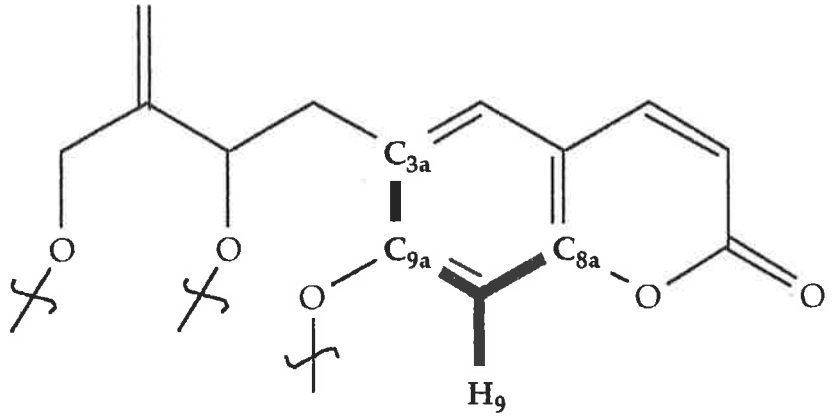


Figure 6.2.16: HMBC correlations for H_9

Given the ^{13}C chemical shift value of C_{9a} (δ 163), it has to have its valency requirements met by being bonded to an oxygen atom.

This structure accounts for all the carbon signals. However it does not account for one hydroxyl proton and, as drawn, it contains one too many oxygen atoms. Therefore, one of the oxygen atoms must be involved in the formation of a ring. There are three possible structures that can accommodate this. In these structures

the multiplicity of the carbon to which the hydroxyl functional group is attached is different. The three possibilities involve a quaternary (phenolic) carbon (formation of a four membered ring), a methylene carbon (five membered ring) or a methine carbon (seven membered ring) - see *Figure 6.2.17* and *18*.

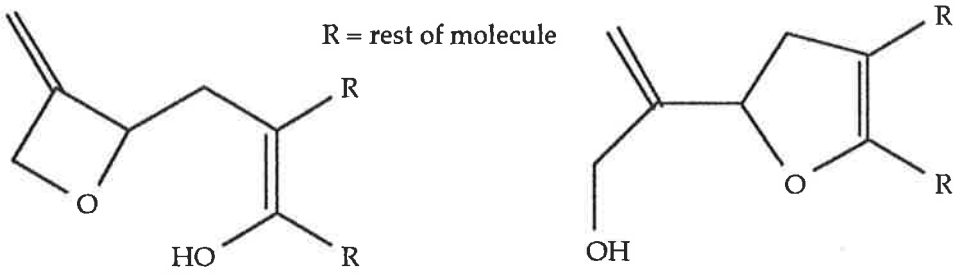


Figure 6.2.17: Possible structures A and B

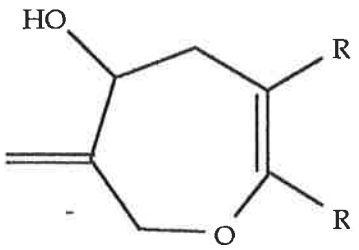
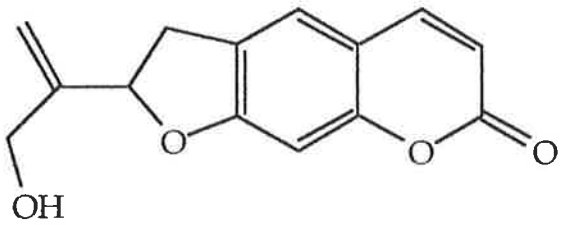


Figure 6.2.18: Possible structure C

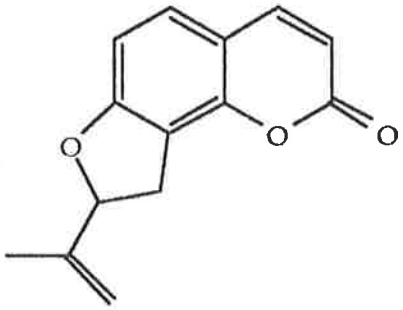
All three structures account for the molecular formula $C_{14}H_{12}O_4$ and all DBE. A ^1H NMR spectrum was run in dry d_6-DMSO. The hydroxyl proton was observed as a triplet, indicating that the hydroxyl functional group was attached to a methylene carbon (Structure B).

Therefore Compound F has the structure (**98**).

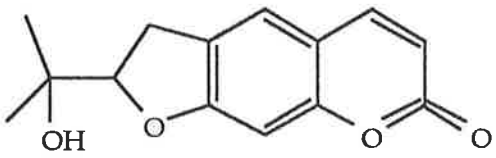


(98)

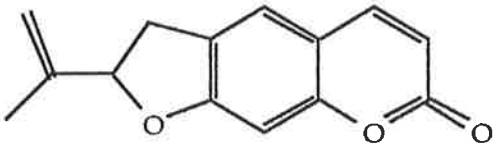
There have been coumarins reported from plants of the *Scaevola* genera that bear a close relationship to emmarin (**98**). Angelomanin (**44**), nodakenetin / marmesin (**45**) and isoangelomanin (**46**) have all been isolated previously from plants of the *Scaevola* genera.[35, 54, 55]



(44)



(45)



(46)

6.3 Compound G

Compound G (4 mg) was isolated as a white amorphous solid (See Chapter 6.1, **Diagram 6.1.2**, pg 76) with a MP of 211 - 214°C.

HRMS (EI) analysis revealed a molecular mass of 168.0425 to give a molecular formula of $C_8H_8O_4$ (calculated = 168.0422, +/- 0.0003). This gave five DBEs and with such a small molecular formula, indicated an aromatic ring.

The IR indicated a carbonyl carbon (1730 cm^{-1}), sp^2 hybridised carbons (1660 cm^{-1}) and a large number of peaks in the aromatic fingerprint region as well as an hydroxyl group (3100 cm^{-1}). Thus, it was expected that Compound G would be a trisubstituted benzene ring.

Routine ^1H, ROESY and COSY NMR experiments were run. It was necessary to run an HMQC experiment to determine the ^{13}C chemical shift values for the protonated carbons and an HMBC experiment to determine the ^{13}C chemical shift values for the quarternary carbons as there was insufficient sample or time on the NMR instrument to complete a routine ^{13}C NMR experiment.

Compound G was found to be vanillic acid (**99**) by spectroscopic analysis and comparison with recorded ^1H and ^{13}C chemical shift values[81a] (see *Figure 6.3.1*) and comparison with the recorded MP values.[80] Its systematic name is 4-hydroxy-3-methoxy benzoic acid. The data from the NMR experiments is given in **Table 6.3.1**, pg 89. The numbering system is that commonly used in the literature for substituted benzoic acid derivatives.

The MP for (**99**) also matched the literature value for vanillic acid (**99**).[80]

Vanillic acid (**99**) has not been recorded from *Scaevola spinescens* or any plant of the *Scaevola* genera before.

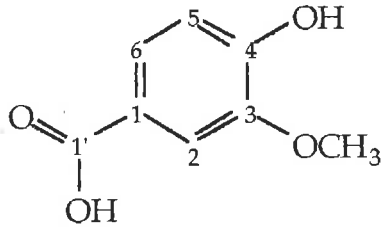


Figure 6.3.1: Structure of vanillic acid (99).

Table 6.3.1: NMR data for (99)

C	δ C	H	δ H	Int.	Mult.	J (Hz)	COSY	ROESY	HMBC
C_1	121.5								
C_2	112.0	H_2	7.507	1	d	1.2	H_6	OCH_3	C_1, C_3, C_4
C_3	146.3								
C_4	151.2								
C_5	114.6	H_5	6.905	1	d	8.4	H_6	4-OH	C_3, C_4, C_6
C_6	125.2	H_6	7.638	1	dd	1.2, 8.4	H_2, H_5		C_2, C_4, C_1'
C_1'	170.5								
C_3'	56.0	OCH_3	3.893	3	s			H_2	C_3
		4-OH	5.986	1	s			H_5	

600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$

Int. = Integration Mult. = Multiplicity

CHAPTER 7 FRACTION 5

7.1 Initial treatment

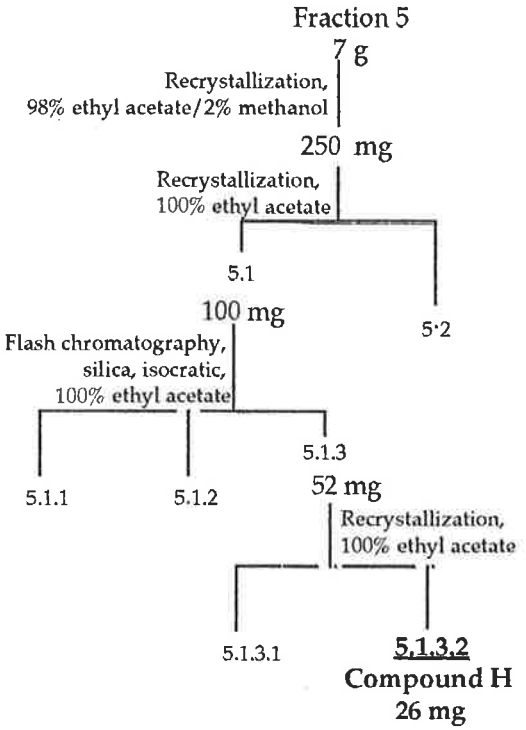
Treatment of Fraction 5 yielded one compound, as outlined in **Diagram 7.1.**

Fraction 5 (4 g) and Fraction 6 (3 g) were separated from the original methanol extract (828 g) using 99% ethyl acetate /1% methanol and 98% ethyl acetate/2% methanol as the eluting solvent, respectively. When run on TLC, these two fractions appeared very similar and so they were combined, labelled Fraction 5 (7 g) and then dissolved in 98% ethyl acetate/2% methanol.

A white crystalline solid (250 mg) precipitated from Fraction 5 (98% ethyl acetate/2% methanol). It was then recrystallized in 100% ethyl acetate. These crystals were collected and labelled 5.1 (100 mg). The rest of the fraction was labelled 5.2.

Fraction 5.1 was then subjected to flash chromatography (silica, isocratic, 100 % ethyl acetate) with three fractions being separated. The third fraction - 5.1.3 (52 mg) - was then recrystallized in 100% ethyl acetate and the crystals collected and labelled 5.1.3.2. This fraction was one spot on TLC, and was labelled Compound H (26 mg) (the structure determination is in Chapter 5.2, pg 91).

Diagram 7.1: Isolation of Compound H



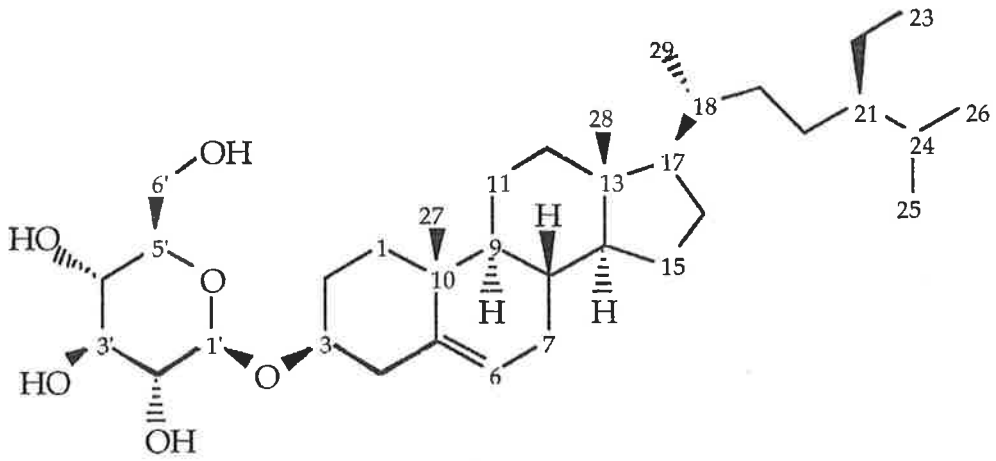
7.2 Compound H

Compound H (26 mg) was isolated as a white crystalline solid (see Chapter 7.1, **Diagram 7.1**, pg 90) with a MP of 280 - 286°C and an $[\alpha]_D$ of - 44° (c 0.1, $CHCl_3$).

HRMS gave a molecular ion ($M+NH_4^+$) of 594.4737 experimentally which gave a molecular formula of $C_{35}H_{60}O_6$ (calculated = 594.4686, +/- 0.0051). There were therefore six DBE to be accounted for. IR indicated the presence of a double bond (1660 cm^{-1}), which accounted for one DBE, and hydroxyl functional groups (3340 cm^{-1}). The other five DBE must be associated with rings.

A routine ^1H and ^{13}C NMR spectrum was run, as well as COSY, HSQC and HMBC experiments that allowed for the full determination of Compound H. This data is presented in **Table 7.2.1**. The numbering system is that commonly used in the literature for phytosterols.

A comparison of the ^1H and ^{13}C NMR data with the known values of β-sitosterol (**94**)[81b] as well as the recorded MP and $[\alpha]_D$[78] data for β-sitosterol glucopyranoside, confirmed that Compound H is the glycosylated form of β-sitosterol (**94**). β-Sitosterol glucopyranoside (**100**) has the common name daucesterol (**100**).



(100)

Daucesterol (**100**) has not been isolated previously from *Scaevola spinescens* or from any plant from the *Scaevola* genera. It is, however, a common plant metabolite, and β-sitosterol (**94**) (the non-glycosylated form) has been reported from *Scaevola spinescens*.[36]

(In **Table 7.2.1**, I stands for integration, while M stands for multiplicity; spectra recorded at 600 MHz (^1H), 150 MHz (^{13}C): δ (ppm) from TMS in d_6-DMSO).

Table 7.2.1: NMR data for (100)

C	δ C	H	δ H	I	M	J (Hz)	COSY	HMBC
C_1	40.0	H_{1a}	1.922	1	m		H_{1b}, H_{2a}, H_{2b}	C_{10}
		H_{1b}	1.114	1	m		H_{1a}, H_{2a}, H_{2b}	
C_2	29.2	H_{2a}	1.794	1	m		H_{1a},H_{1b},H_{2b},H_3	
		H_{2b}	1.417	1	m		H_{1a},H_{1b},H_{2a},H_3	
C_3	76.9	$H_{3\alpha}$	3.444	1	m		H_{2a}, H_{2b}, H_{4a}, H_{4b}	$C_{1'}$
C_4	38.3	H_{4a}	2.350	1	dd	3.0, 13.8	H_3, H_{4b}	C_5, C_{10}
		H_{4b}	2.116	1	m		H_3, H_{4b}	C_5
C_5	140.4							
C_6	121.1	H_6	5.310	1	br t	3.0	H_{7a}, H_{7b}	C_4, C_{10}
C_7	31.3	H_{7a}	1.893	1	m		H_6, $H_{8\beta}$	
		H_{7b}	1.439	1	m		H_6, $H_{8\beta}$	
C_8	50.5	$H_{8\beta}$	1.496	1	m		H_{7a}, $H_{9\alpha}$, $H_{14\alpha}$	
C_9	55.4	$H_{9\alpha}$	1.105	1	m		$H_{8\beta}$, H_{11a}, H_{11b}	C_{10}
C_{10}	36.4							
C_{11}	23.8	H_{11a}	1.496	1	m		$H_{9\alpha}$,H_{11b}, H_{12a},H_{12b}	
		H_{11b}	1.035	1	m		$H_{9\alpha}$,H_{11a}, H_{12a},H_{12b}	
C_{12}	27.7	H_{12a}	1.770	1	m		H_{11a}, H_{11b}, H_{12b}	
		H_{12b}	1.215	1	m		H_{11a}, H_{11b}, H_{12a}	
C_{13}	41.8							
C_{14}	49.5	$H_{14\alpha}$	0.861	1	m		$H_{8\beta}$	
C_{15}	20.5	H_{15a}	1.417	1	m			C_{14}, C_{16}
		H_{15b}	1.131	1	m			C_{14}, C_{16}
C_{16}	33.3	H_{16a}	1.286	1	m			C_{15}, C_{17}
		H_{16b}	0.979	1	m			C_{15}, C_{17}
C_{17}	45.7	$H_{17\alpha}$	0.918	1	m		H_{18}	C_{13}, C_{16}
C_{18}	35.4	H_{18}	1.308	1	m		$H_{17\alpha}$ H_{19a} H_{19b} Me_{29}	
C_{19}	36.7	H_{19a}	1.770	1	m		H_{18}, H_{19b}	C_{20}
		H_{19b}	0.979	1	m		H_{18}, H_{19a}	
C_{20}	22.4	H_{20a}	1.262	1	m		H_{20b}, H_{21}	
		H_{20b}	1.105	1	m		H_{20a}, H_{21}	
C_{21}	31.3	H_{21}	1.381	1	m		$H_{20a}H_{20b}H_{22a}H_{22b}H_{24}$	
C_{22}	28.6	H_{22a}	1.635	1	m		H_{21}, H_{22b}, Me_{23}	
		H_{22b}	1.224	1	m		H_{21}, H_{22a}, Me_{23}	
C_{23}	18.5	Me_{23}	0.890	3	m		H_{22a}, H_{22b}	
C_{24}	25.4	H_{24}	1.131	1	m		H_{21}, Me_{25}, Me_{26}	
C_{25}	21.0	Me_{25}	0.990	3	d	6.6	H_{21}	
C_{26}	14.2	Me_{26}	0.642	3	d	6.6	H_{21}	
C_{27}	18.8	$Me_{27\beta}$	0.780	3	s			C_{10}
C_{28}	20.8	Me_{28b}	0.820	3	s			C_{12}, C_{13}, C_{14}, C_{17}
C_{29}	19.0	Me_{29}	0.949	3	d	6.6	H_{18}	
$C_{1'}$	100.7	$H_{1'}$	4.210	1	d	7.8	$H_{2'}$	C_3, $C_{2'}$, $C_{5'}$
$C_{2'}$	73.4	$H_{2'}$	2.880	1	dd	7.8, 9.0	$H_{1'}$, $H_{3'}$, 2'-OH	$C_{1'}$, $C_{3'}$
$C_{3'}$	76.7	$H_{3'}$	3.114	1	t	9.0	$H_{2'}$, $H_{4'}$, 3'-OH	$C_{2'}$, $C_{4'}$
$C_{4'}$	70.0	$H_{4'}$	3.008	1	t	9.0	$H_{3'}$, $H_{5'}$, 4'-OH	$C_{5'}$
$C_{5'}$	76.7	$H_{5'}$	3.050	1	ddd	3.0, 5.4, 9.0	$H_{4'}$, $H_{6'a}$, $H_{6'b}$	$C_{1'}$, $C_{4'}$, $C_{6'}$
$C_{6'}$	61.0	$H_{6'a}$	3.630	1	dd	3.0, 11.4	$H_{5'}$, $H_{6'b}$, 6'-OH	$C_{5'}$
		$H_{6'b}$	3.399	1	dd	5.4, 11.4	$H_{5'}$, $H_{6'a}$, 6'-OH	$C_{5'}$
		2'-OH	4.842	1	br s		$H_{2'}$	$C_{1'}$, $C_{2'}$
		3'-OH	4.884	1	br s		$H_{3'}$	
		4'-OH	4.846	1	br s		$H_{4'}$	$C_{4'}$, $C_{5'}$
		6'-OH	4.409	1	br s		$H_{6'a}$, $H_{6'b}$	$C_{5'}$, $C_{6'}$

CHAPTER 8 FRACTION 7

8.1 Initial treatment

Treatment of Fraction 7 yielded four compounds, as outlined in **Diagram 8.1**.

Fraction 7 (16 g) was separated from the original methanol extract (828 g) using 95% ethyl acetate/5 % methanol as the eluting solvent. Fraction 7 was then subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) to yield ten fractions, which were analysed by TLC.

Fraction 7.3 and 7.4 appeared to contain the same compounds and were added together (10.75 g) and labelled 7.(3). This fraction was analysed by analytical reverse phase HPLC (gradient, 100% water - 100% methanol, 254 nm, 1 mL/min., 30 min.) that indicated the presence of four compounds. Fraction 7.(3) (10.75 g) was then subjected to flash chromatography (silica, isocratic, 90% chloroform /10% methanol) to give six fractions, of which Fraction 7.(3).5 (192 mg) appeared to contain the four compounds identified by reverse phase HPLC.

Fraction 7.(3).5 was then subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) to give eight fractions.

Fraction 7.(3).5.4 was analysed by reverse phase analytical HPLC (gradient, 100% water to 100% methanol, 254 nm, 1 mL/min., 30 min.) and was found to be pure. It was labelled Compound I (26 mg). The structure determination of Compound I is discussed in Chapter 8.2, pg 95.

Fraction 7.(3).5.5 (60 mg) was subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) to give ten fractions.

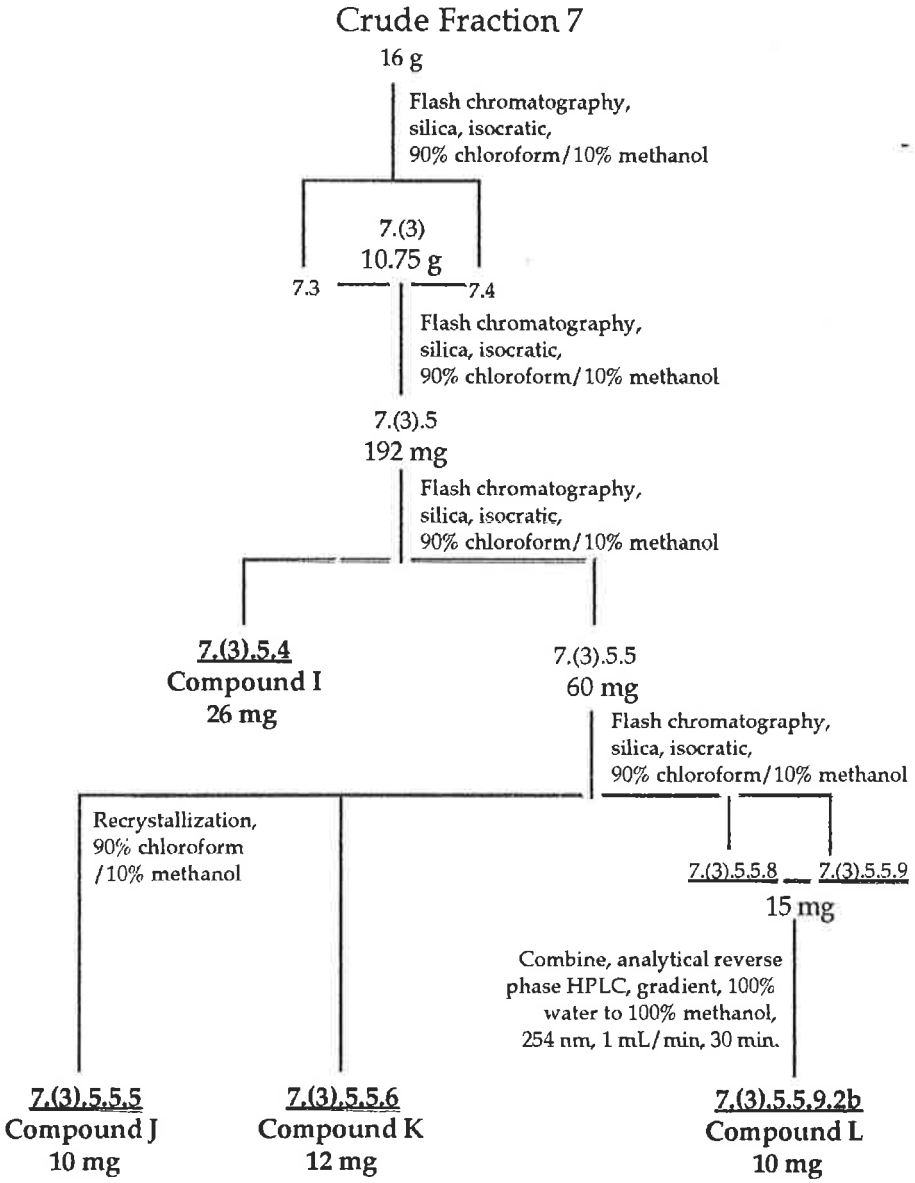
Fraction 7.(3).5.5.5 appeared to be pure and was recrystallized using 90% chloroform/10% methanol to give Compound J (10 mg). The structure determination of Compound J is discussed in Chapter 8.3, pg 118.

Fraction 7.(3).5.5.6 was pure by analytical reverse phase HPLC (gradient, 100% water - 100% methanol, 254 nm, 1 mL/min., 30 min.) to yield Compound K (12 mg) (see Chapter 8.4, pg 122 for structure determination).

Fractions 7.(3).5.5.8 and 7.(3).5.5.9 were combined to give 7.(3).5.5.9.2b (15 mg) which was then purified by analytical reverse phase HPLC (gradient, 100% water - 100% methanol, 254 nm, 1 mL/min., 30 min.) to yield Compound L (10 mg). The structure determination of Compound L is discussed in Chapter 8.5, pg 125.

The purity of these compounds was analysed by analytical reverse phase HPLC under two gradient systems and an isocratic system. The first gradient system was 100% water to 100% methanol (254 nm, 1 mL/min., 30 min.). The second gradient system was 70% water/30 % methanol to 30% water/70 % methanol (254 nm, 1 mL/min., 30 min.). The isocratic system was 50% water/50% methanol (254 nm, 1 mL/min., 30 min.). The compounds all appeared as one peak under all three systems.

Diagram 8.1: Isolation of four compounds from Fraction 7



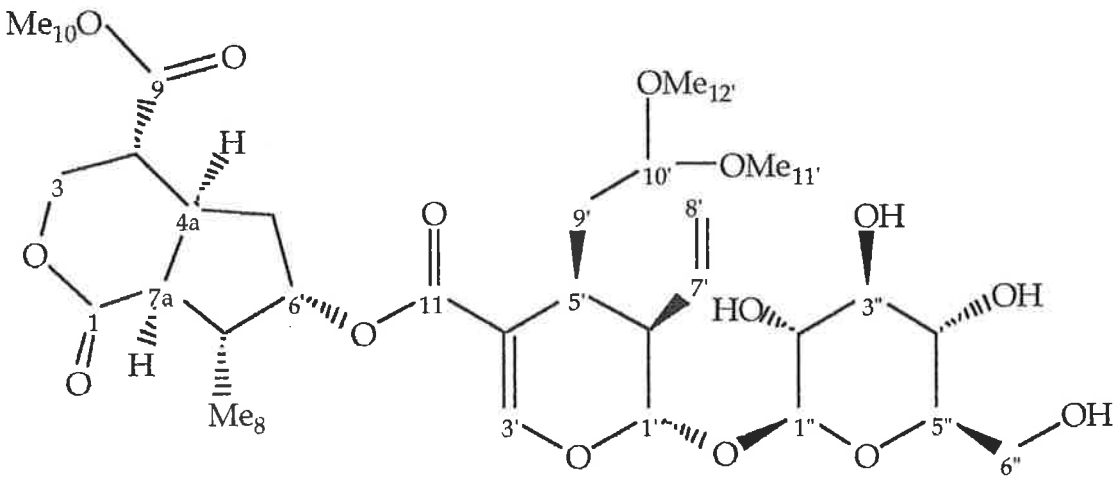
8.2 Compound I

Compound I (26 mg) was isolated as a white amorphous solid (see Chapter 8.1, **Diagram 8.1**, pg 94) with a MP of 94 - 96°C and an $[\alpha]_D$ of - 64° (c 0.1, MeOH).

Compound I was found to have a molecular ion of 653 (LSIMS, $M+Na^+$) and 648 (CI, $M+NH_4^+$) giving Compound I an overall mass of 630. HRMS gave a molecular mass of $M+Na^+$ of 653.2364 and therefore a molecular formula of $C_{29}H_{42}O_{15}Na$ (calculated = 653.2424, +/- 0.0060). This indicated nine DBE.

Standard ^1H and ^{13}C NMR spectra indicated the presence of three carbonyl carbons and two alkene double bonds (seven sp^2 hybridised carbons) accounting for five DBE, leaving four to be associated with ring systems. In addition, three methoxy signals (one ester and two ether) as well as carbon and proton signals consistent with a sugar unit were observed.

An analysis of all NMR data (^1H, COSY, ROESY, ^{13}C, DEPT, HMQC and HMBC) allowed for the assignment of Compound I as a novel iridoid that was named alydijosioside (**108**) (the numbering system is the one used for other iridoids in the literature) (see **Table 8.2.1**, pg 96).



(108)

The standard ^1H spectrum gave most spin-spin coupling constants, and a COSY experiment revealed three distinct and independent spin systems. The protons were then assigned to their respective directly bonded carbon atom using, initially, a DEPT experiment to establish the carbon multiplicity, followed by an HMQC experiment to unambiguously assign $^1J_{CH}$ correlations.

The three $^nJ_{HH}$ spin systems have been independently analysed.

Table 8.2.1: NMR data for (108)#

C	δ C	H	δ H	I.	M.	J (Hz)	COSY	HMBC
C_1	173.3							
C_3	68.0	$H_{3\beta}$	4.46	1	dd	3.6, 11.4	$H_{3\alpha}$, H_4	C_1, C_4, C_{4a}, C_9
		$H_{3\alpha}$	4.30	1	dd	9.6, 11.4	$H_{3\beta}$, H_4	C_1, C_4, C_9
C_4	46.5	H_4	2.55	1	td	3.6, 9.6	$H_{3\alpha}$, $H_{3\beta}$, H_{4a}	C_3, C_4, C_{4a}, C_9
C_{4a}	37.0	H_{4a}	3.04	1	dddd	7.8, 9.6, 9.6, 11.4	$H_{4a}H_{5\alpha}H_{5\beta}H_{7a}$	C_1, C_4, C_5, C_{7a}, C_9
C_5	38.5	$H_{5\beta}$	1.62	1	ddd	3.6, 11.4, 13.8	H_{4a}, $H_{5\alpha}$, H_6	C_4, C_{4a}
		$H_{5\alpha}$	2.20	1	dd	7.8, 13.8	H_{4a}, $H_{5\beta}$	C_5, C_7, C_{7a}
C_6	78.2	H_6	5.25	1	dd	3.6, 7.2	$H_{5\alpha}$, H_7	C_4, C_{4a}, C_7, C_{11}
C_7	43.7	H_7	2.48	1	ddq	7.2, 7.2, 11.4	H_6, H_{7a}, Me_8	C_1
C_{7a}	47.5	H_{7a}	2.77	1	dd	9.6, 11.4	H_{4a}, H_7	C_1 C_4 C_{4a} C_5 C_6 C_7
C_8	15.2	Me_8	1.21	3	d	7.2	H_7	C_6, C_7, C_{7a}
C_9	171.4							
C_{10}	53.8	Me_{10}	3.75	3	s			C_9
C_{11}	166.3							
$C_{1'}$	97.6	$H_{1'}$	5.36	1	d	5.4	$H_{6'}$	$C_{3'}$, $C_{5'}$, $C_{6'}$ $C_{1''}$
$C_{3'}$	154.9	$H_{3'}$	7.34	1	s			C_{11}, $C_{1'}$,$C_{4'}$,$C_{5'}$
$C_{4'}$	110.8							
$C_{5'}$	29.6	$H_{5'}$	2.90	1	ddd	5.4, 6.6, 7.2	$H_{6'}$, $H_{9'a}$, $H_{9'b}$	$C_{11}C_{1'}C_{3'}C_{4'}C_{6'}C_{7'}C_{9'}$
$C_{6'}$	44.5	$H_{6'}$	2.71	1	dt	5.4, 5.4, 14.4	$H_{1'}$, $H_{5'}$, $H_{7'}$	$C_{1'}$, $C_{5'}$, $C_{7'}$
$C_{7'}$	133.9	$H_{7'}$	5.68	1	m		$H_{6'}$, $H_{8'}$	$C_{1'}$, $C_{6'}$
$C_{8'}$	120.6	$H_{8'}$	5.32-.29	2	m		$H_{7'}$	$C_{6'}$, $C_{7'}$
$C_{9'}$	32.2	$H_{9'a}$	2.04	1	ddd	6.6, 7.2, 13.8	$H_{5'}$, $H_{9'b}$, $H_{10'}$	$C_{5'}$, $C_{10'}$
		$H_{9'b}$	1.67	1	ddd	4.8, 7.2, 13.8	$H_{5'}$, $H_{9'a}$, $H_{10'}$	$C_{5'}$, $C_{10'}$
$C_{10'}$	103.7	$H_{10'}$	4.49	1	dd	4.8, 7.2	$H_{9'a}$, $H_{9'b}$	$C_{11'}$, $C_{12'}$
$C_{11'}$	52.28	$Me_{11'}$	3.292	3	s			$C_{10'}$
$C_{12'}$	52.29	$Me_{12'}$	3.298	3	s			$C_{10'}$
$C_{1''}$	98.6	$H_{1''}$	4.72	1	d	7.2	$H_{2''}$	$C_{1'}$, $C_{5''}$
$C_{2''}$	72.9	$H_{2''}$	3.39	1	dd	7.2, 9.0	$H_{1''}$, $H_{3''}$	$C_{1'}$, $C_{5''}$
$C_{3''}$	76.7	$H_{3''}$	3.58	1	t	9.0	$H_{2''}$, $H_{4''}$	$C_{2''}$, $C_{4''}$
$C_{4''}$	70.5	$H_{4''}$	3.63	1	t	9.0	$H_{3''}$, $H_{5''}$	$C_{3''}$, $C_{5''}$
$C_{5''}$	75.9	$H_{5''}$	3.43	1	ddd	3.0, 8.4, 9.0	$H_{4''}$ $H_{6''a}$ $H_{6''b}$	$C_{1''}$, $C_{4''}$
$C_{6''}$	62.5	$H_{6''a}$	3.93	1	dd	3.0, 11.4	$H_{5''}$, $H_{6''b}$	$C_{5''}$
		$H_{6''b}$	3.86	1	dd	8.4, 11.4	$H_{5''}$, $H_{6''a}$	$C_{5''}$

#600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$

I. = Integration

M. = Multiplicity

Spin system A

The entry point for spin system A was by protons $H_{3\alpha}$ and $H_{3\beta}$ as their ^1H signals are clear of other signals. They are coupled to each other and each is also coupled to one other proton, H_4. The rest of the COSY correlations are outlined in *Figure 8.2.1*.

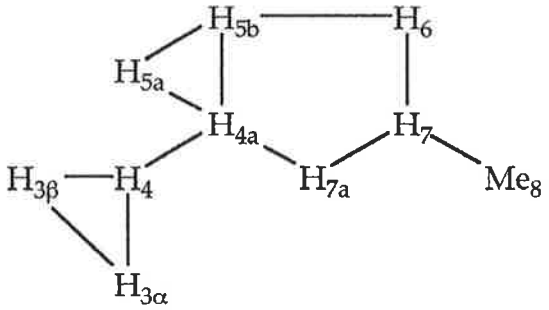


Figure 8.2.1: COSY correlations for spin system A

Protons $H_{3\alpha}$ and $H_{3\beta}$ are geminal protons attached to C_3. The ^1H and ^{13}C chemical shift values (^1H, δ 4.46, δ 4.30 Hz: ^{13}C, δ 68.1) are indicative of a carbon attached to oxygen and two protons. $H_{3\alpha}$ and $H_{3\beta}$ are also coupled to H_4 that is directly attached to C_4 (see *Figure 8.2.2*).

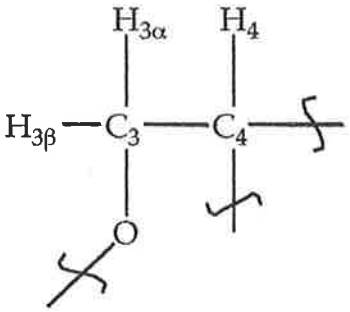
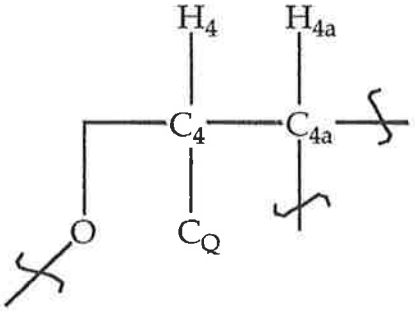


Figure 8.2.2: COSY correlations for $H_{3\alpha}$ and $H_{3\beta}$ to H_4

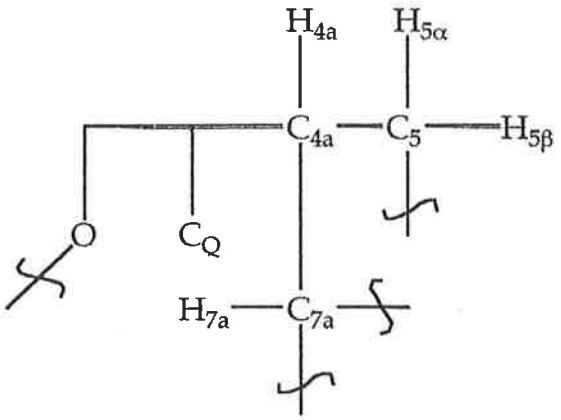
H_4 also shows COSY correlations to H_{4a} that is a methine signal. There must be one other carbon-carbon bond for C_4 to complete its valency, as its ^{13}C chemical shift value of δ 46.5 precludes being directly attached to oxygen. The last carbon-carbon bond to C_4 must therefore be to a quaternary carbon, as H_4 only correlates with $H_{3\alpha}$, $H_{3\beta}$ and H_{4a}. This is illustrated in *Figure 8.2.3*.



C_Q = quaternary

Figure 8.2.3: Further COSY correlations

H_{4a} is directly attached to C_{4a} (δ 37.0) and is a dddd, extensively coupled to H_4, $H_{5\alpha}$, $H_{5\beta}$ and H_{7a}. All ^1H and ^{13}C chemical shifts are in the range of alkanes. $H_{5\alpha}$ and $H_{5\beta}$ are geminal protons attached to C_5. It follows that the valency of C_{4a} is filled by being bonded to H_{4a}, C_4, C_5 and C_{7a}. This is shown in *Figure 8.2.4.*



8.2.4: COSY correlations for H_{4a}

$H_{5\beta}$ shows COSY correlations to H_6 as well as to H_{4a}. H_6 (δ 5.24) is directly bonded to C_6 (δ 78.2) with ^1H and ^{13}C chemical shifts values consistent with a carbon attached to oxygen. However the proton $H_{5\alpha}$ (geminal to $H_{5\beta}$) has no COSY correlation to H_6. One possible explanation for this is that $H_{5\alpha}$ and H_6 are approximately orthogonal to each other and so a coupling would not occur. The $^nJ_{HH}$ correlation between $H_{5\alpha}$, $H_{5\beta}$ and H_6 is illustrated in *Figure 8.2.5.*

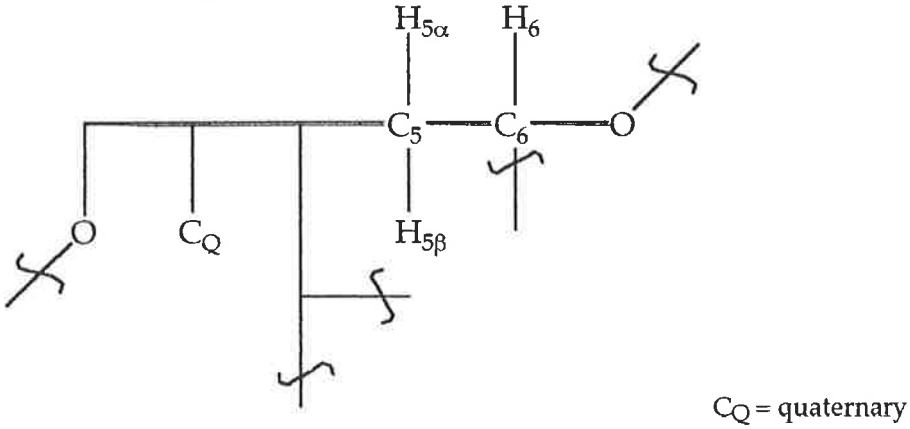


Figure 8.2.5: COSY correlations for part structure A

H_6 is also coupled to H_7 (δ 2.48) which is directly bonded to C_7 (δ 43.7). H_7 is coupled to Me_8 (δ 1.21) that is a doublet with an integration of three. The protons of Me_8 are bonded to a carbon with the ^{13}C chemical shift value of δ 15.2.

H_7 is coupled to H_{7a}, which has already been shown to be coupled to H_{4a}. C_{7a} is directly bonded to H_{7a}, which is coupled to H_{4a} and H_7. This indicates that C_{4a} is

attached to H_{7a}, C_{4a} and C_7 and therefore forms a ring. The valency of C_{7a} must be completed by attachment to a quaternary carbon as its ^{13}C chemical shift value (δ 47.5) is consistent with a carbon atom that is not attached to oxygen but is a ring carbon. This is illustrated in *Figure 8.2.6* and it also closes a cyclopentane ring system. Part structure A accounts for the sixth DBE (the cyclopentane ring), as well as having four positions where bonds need to be connected to satisfy the valency of all atoms.

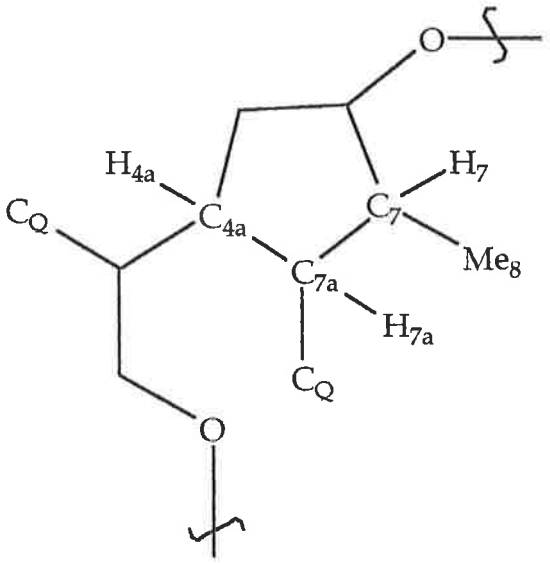


Figure 8.2.6: Part structure A

Spin system B

This COSY spin system (*Figure 8.2.7*) was analysed by starting at proton $H_{1'}$, as it was a clearly defined doublet at δ 5.36 and it correlated to one other proton. Most $^{n}J_{HH}$ coupling constants could be determined (see **Table 8.2.1**, pg 96).

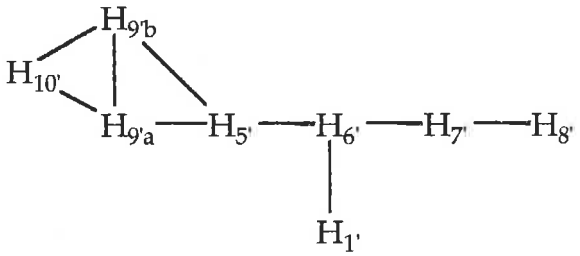


Figure 8.2.7: COSY correlations for spin system B

Proton $H_{1'}$ is a doublet with an ^{1}H chemical shift of δ 5.36, which indicates it is attached to a carbon that is attached to one or more oxygen atoms. The ^{13}C chemical shift of $C_{1'}$ (δ 97.6), its multiplicity (CH) and the fact that the proton signal is a doublet are consistent with it being the carbon of an acetal functional group.

Proton $H_{1'}$ has one COSY correlation to $H_{6'}$ (δ 2.71, m) which is directly attached to $C_{6'}$ (δ 44.5).These ^{1}H and ^{13}C chemical shift values are indicative of an alkane (see

Figure 8.2.8).

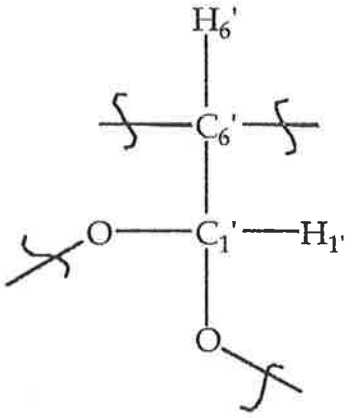


Figure 8.2.8: COSY correlations for part structure B

$H_{6'}$ is also COSY correlated to two other protons. $H_{6'}$ correlates to $H_{7'}$ that is a signal that does not overlap with any other proton signal. $H_{7'}$ has an integration of 1, a ^1H chemical shift of δ 5.68 and is directly attached to $C_{6'}$ (δ 133.9). This data indicates an alkene. $H_{6'}$ COSY correlates to $H_{8'}$ that are two protons with a ^1H chemical shift of between δ 5.32 - 5.29 and which are clearly terminal methylene protons attached to $C_{8'}$ (δ 120.6). Due to the complexity of the multiplet the coupling constants for $H_{7'}$ or $H_{8'}$ could not be determined (see *Figure 8.2.9*).

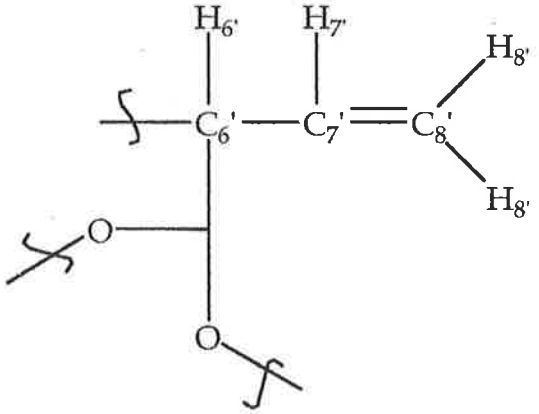
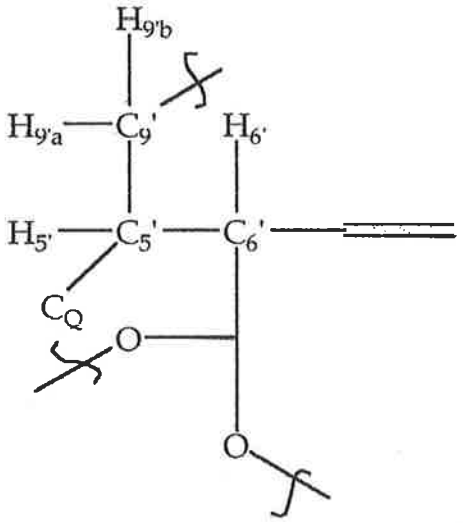


Figure 8.2.9: COSY correlations for part structure B

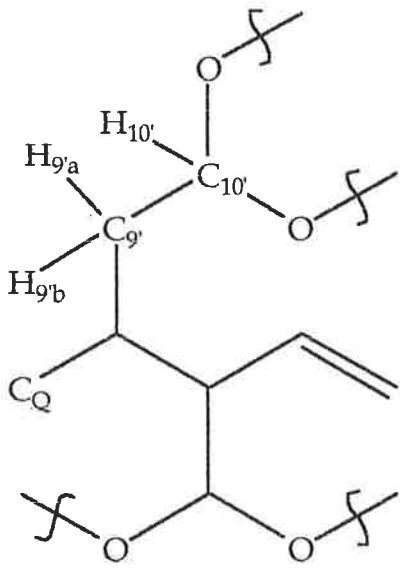
$H_{6'}$ has a COSY correlation to $H_{5'}$ that is a proton with a ^1H chemical shift of δ 2.90. $H_{5'}$ is directly attached to $C_{5'}$. $H_{5'}$ is also COSY correlated to $H_{9'a}$ and $H_{9'b}$ that are geminal protons attached to $C_{9'}$ (δ 32.2). $C_{5'}$ is directly bonded to $H_{5'}$, $C_{6'}$ and $C_{9'}$. There are no other COSY correlations for $H_{5'}$. This suggests that the valency for $C_{5'}$ will be completed by it being attached to a quaternary carbon (see *Figure 8.2.10*).



C_Q= quaternary

Figure 8.2.10: COSY correlations for part structure B

$H_{9'a}$ and $H_{9'b}$ correlate to each other and to $H_{10'}$. $H_{10'}$ is a proton with a chemical shift of δ 4.46 directly attached to $C_{10'}$ (δ 103.7). This indicates that $C_{10'}$ is an acetal carbon. This is illustrated in *Figure 8.2.11*.



C_Q = quaternary

Figure 8.2.11: COSY correlations for part structure B

This part structure accounts for one DBE associated with the double bond and has five positions where the valency of individual atoms needs to be completed. It also accounts for four oxygen atoms.

Spin system C

Spin system C (*Figure 8.2.12*) was entered via proton $H_{1''}$, as this was a clearly defined doublet correlated to one other proton. It was possible to determine all the coupling constants for this spin system (see **Table 8.2.1,** pg 96). The ^1H and ^{13}C chemical shift values, the COSY correlations and the $^1J_{CH}$ assignments are all

consistent with a sugar unit. $H_{1''}$ has a proton chemical shift of δ 4.72, is a doublet (J = 7.2 Hz) and is coupled to one other proton, $H_{2''}$.

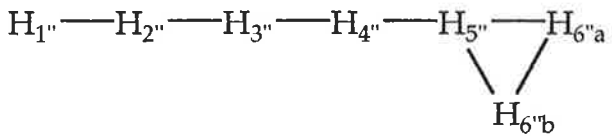


Figure 8.2.12: COSY spin system C

The ^{13}C chemical shift for $C_{1''}$ (δ 98.6) is indicative of an acetal carbon, considering that oxygen is the electronegative atom found in this compound (established by the M^+ and molecular formula). The ^{13}C chemical shift values (between δ 77 and 70) and multiplicities of the carbon atoms (established by a DEPT experiment) of four of the carbons are consistent with carbons attached to one oxygen as well as one hydrogen.

$C_{6''}$ is a $-CH_2-O$ carbon, with the geminal protons coupling to each other and to $H_{5''}$ on the adjacent carbon. This is consistent with a primary alcohol. The COSY correlations and HMQC data indicates the following structure, as shown in *Figure 8.2.13*.

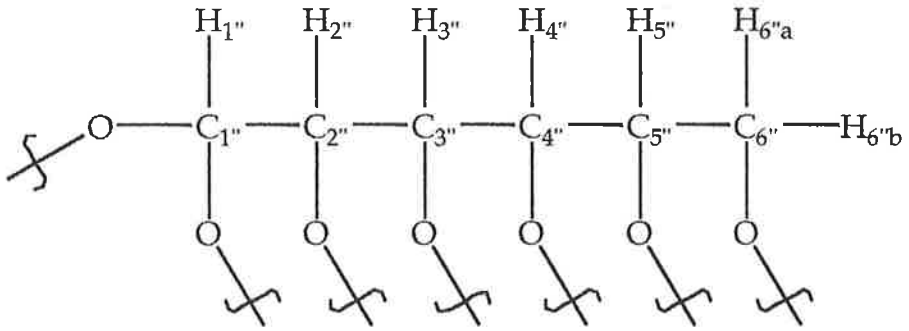


Figure 8.2.13: Part Structure C

Part structure C accounts for seven oxygen atoms and leaves seven positions where other bonds need to be assigned so that the valency of all atoms can be completed.

There were four ^1H proton signals that appear in the ^1H spectrum but which do not correlate to other protons in the three COSY spectra. They are correlated to specific carbons via the HMQC experiment. $H_{3'}$ and $C_{3'}$ have chemical shift values consistent with an enol ether functional group (^1H, δ 7.34: ^{13}C, δ 154.3). Protons Me_{10} (^1H, δ 3.75: ^{13}C, δ 53.8) are methoxy protons associated with an ester functional group while the protons $Me_{11'}$ (^1H, δ 3.298: ^{13}C, δ 52.28) and $Me_{12'}$ (^1H, δ 3.292: ^{13}C, δ 52.26) are ether methoxy protons.

There are four carbon signals that are not directly attached to a proton, and are therefore quaternary. Three of these signals correspond to carbonyl carbons (C_1 -

δ 173.3, C_9 - δ 171.4, C_{11} - δ 166.3) while the fourth is also sp^2 hybridised ($C_{4'}$ - δ 110.8) (see **Table 8.2.1**, pg 96).

Connectivity

An HMBC experiment was conducted to determine $^{2/3}J_{CH}$ correlations so that the three part structures and the non-COSY protons and non-protonated carbons could be connected to each other. This data is displayed in **Table 8.2.1**, pg 96.

Part structure A has four positions where connectivity needs to occur (Points A, B, C and D) as shown below in *Figure 8.2.14*.

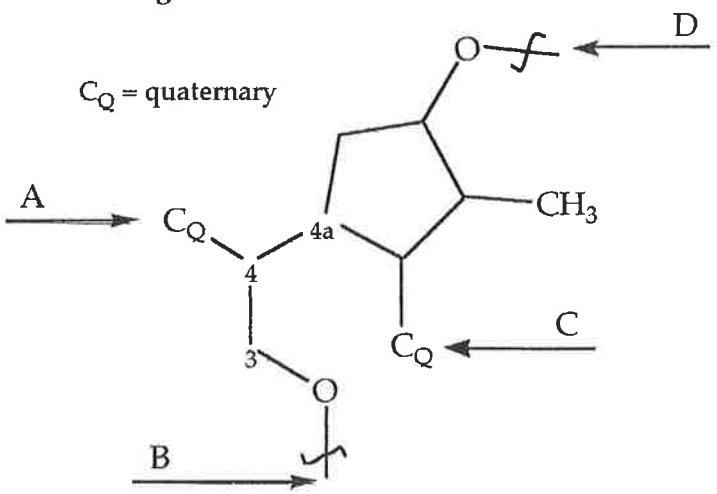


Figure 8.2.14: Points for connection in Part Structure A

Me_{10} protons are ester methoxy protons bonded to C_{10} and there is a $^{2/3}J_{CH}$ correlation from Me_{10} to C_9, a carbonyl signal at δ 171.4. There are HMBC correlations between $H_{3\alpha}$, $H_{3\beta}$, H_4 and H_{4a} to C_9. C_4 has one position where it has to be bonded to a quaternary carbon. Thus the valency of C_4 is completed by being bonded to C_9 (see *Figure 8.2.15*).

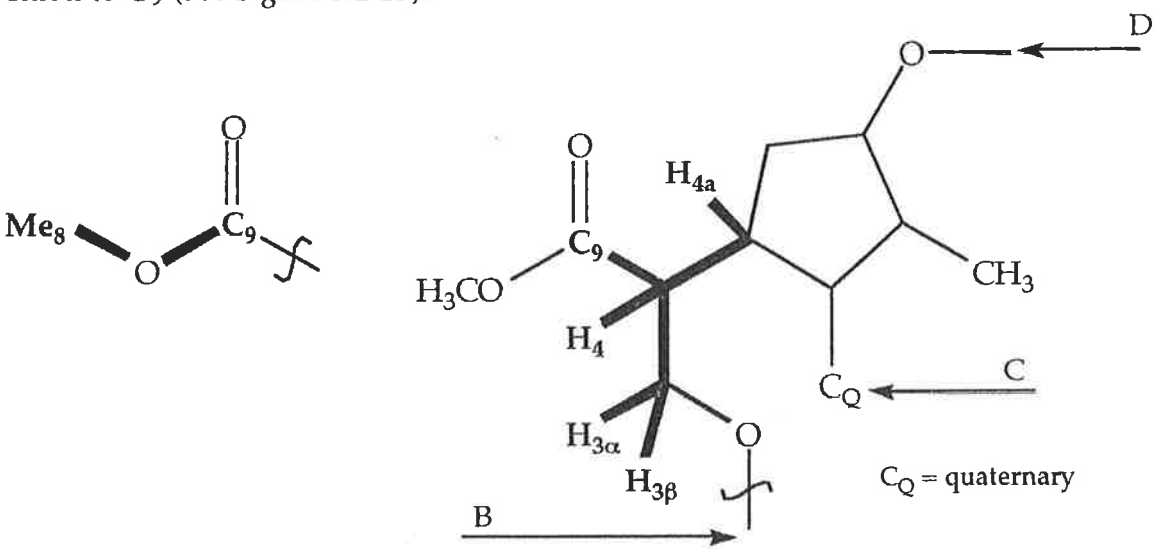


Figure 8.2.15: Connectivity of C_9

There are HMBC correlations between $H_{3\alpha}$, $H_{3\beta}$, H_{4a} and H_{7a} to C_1, another carbonyl carbon. $H_{3\alpha}$ and $H_{3\beta}$ are directly bonded to a carbon, C_3, with a ^{13}C chemical shift of δ 68.0, indicating that C_3 is bonded to an oxygen atom. Carbon C_{7a} has to be bonded to a quaternary carbon for its valency to be complete. Thus, the valency of the oxygen at position B is completed by being bonded to C_1 and the valency of C_{7a} at position C is also completed by being bonded to C_1 (see *Figure 8.2.16*).

Part structure A is complete except for the oxygen at position D. H_6 is bonded to the carbon bonded to the oxygen at position D and has a $^{2/3}J_{CH}$ correlation to a carbonyl, C_{11}. Therefore the oxygen at position D is bonded to C_{11}. This gives rise to the following part structure (*Figure 8.2.16*) and accounts for the seventh DBE. The valency of C_{11} still has to be completed.

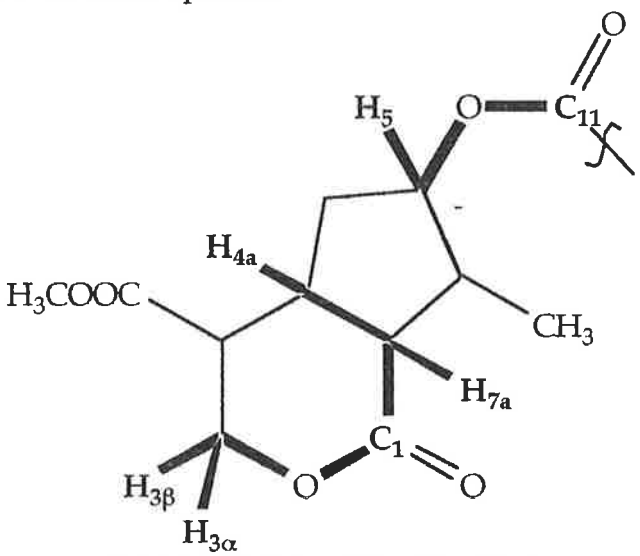


Figure 8.2.16: Connectivity of C_1 and C_{11}

Part structure B has five positions where connectivity has to be established as shown in *Figure 8.2.17*.

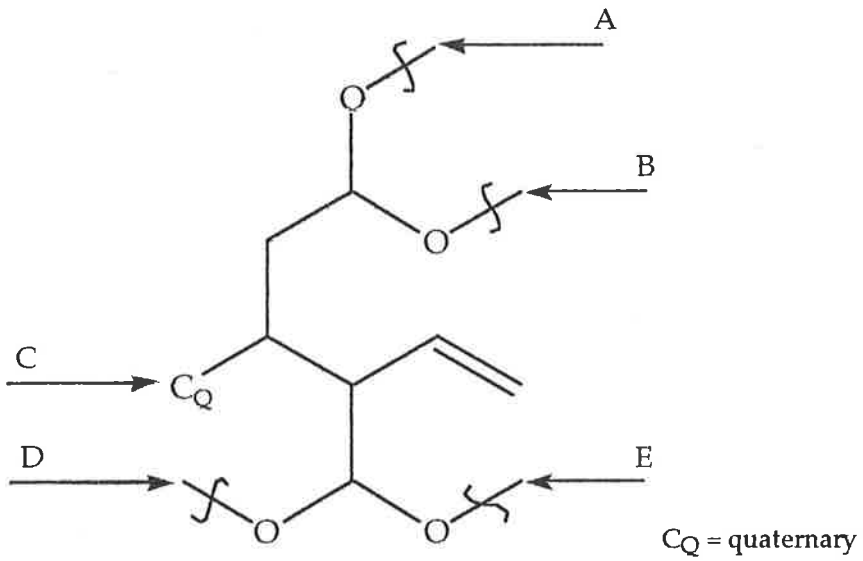


Figure 8.2.17: Connections for Part structure B

$Me_{11'}$ and $Me_{12'}$ are methoxy protons with ^1H chemical shift values consistent with those of an ether functional group. These protons both have a $^{2/3}J_{CH}$ correlation to $C_{10'}$ that has the ^{13}C chemical shift value of an acetal. As well as this, $H_{10'}$ (bonded to $C_{10'}$) shows HMBC correlations to $C_{11'}$ and $C_{12'}$. This is consistent with a dimethyl acetal functional group attached to $C_{10'}$ (see *Figure 8.2.18*).

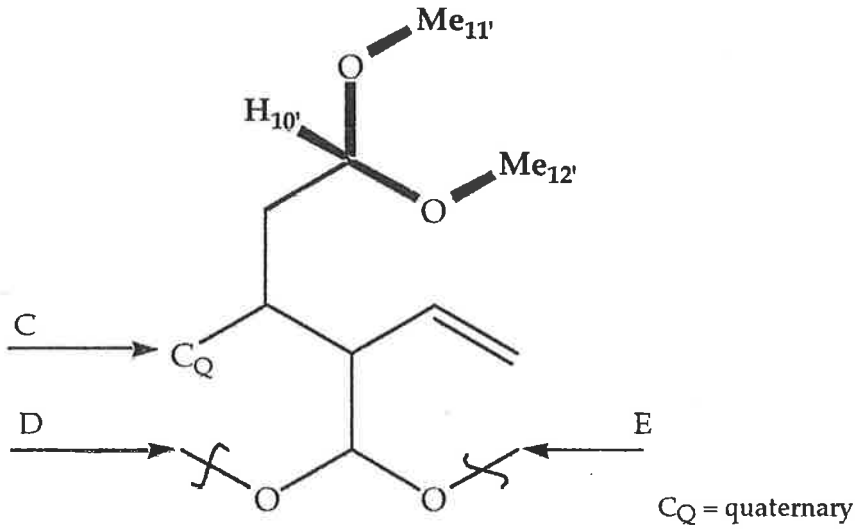


Figure 8.2.18: HMBC correlations for $Me_{11'}$ and $Me_{12'}$

There is an HMBC correlation from $H_{3'}$ to $C_{4'}$. $H_{3'}$ is the hydrogen of an enol ether and $C_{4'}$ (δ 110.8) is the only sp^2 hybridised carbon that has not yet been assigned, so must be bonded to $C_{3'}$ (δ 154.9). This is consistent with that of an alkene carbon adjacent to an enol ether proton (*Figure 8.2.19*).

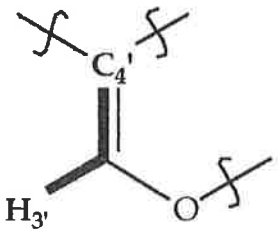


Figure 8.2.19: HMBC correlations from $H_{3'}$ to $C_{4'}$

The enol ether functional group can be placed in Part structure B because of the HMBC correlations from $H_{5'}$ to $C_{3'}$ and $C_{4'}$. and the correlations from $H_{3'}$ to $C_{5'}$. $C_{5'}$ has to be bonded to a quaternary carbon to complete its valency and $C_{4'}$ is the last quaternary carbon to be positioned in the molecule (*Figure 8.2.20*).

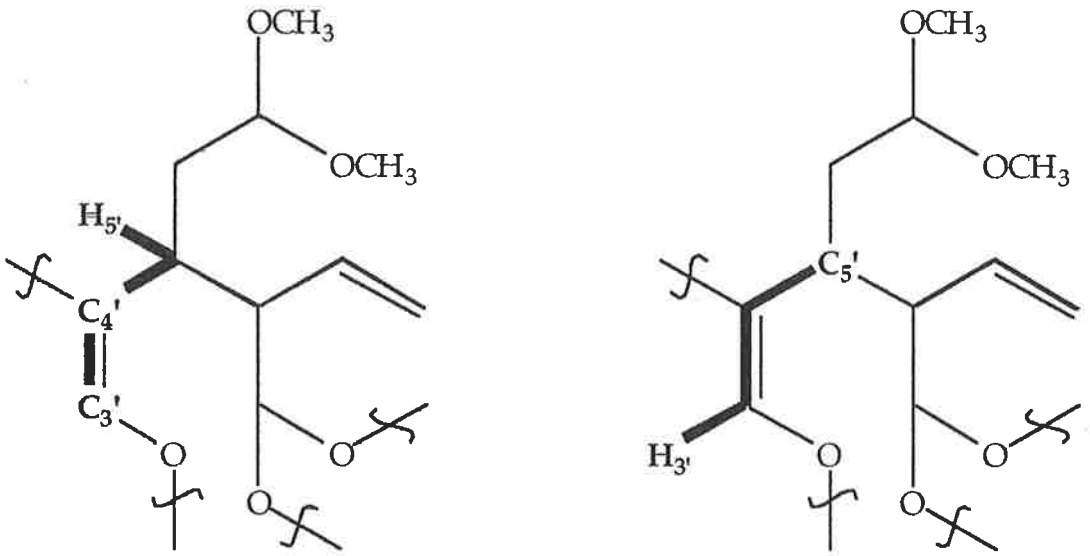


Figure 8.2.20: HMBC correlations for $H_{3'}$ and $H_{5'}$

A dihydropyran ring can be established, as there are HMBC correlations between $H_{3'}$ and $C_{1'}$ (the carbon of an acetal functional group) and between $H_{1'}$ and $C_{3'}$. This structure is also supported by the $^{2/3}J_{CH}$ correlations between $H_{5'}$ to $C_{1'}$ as well as the correlations between $H_{1'}$ to $C_{5'}$ (*Figure 8.2.21*).

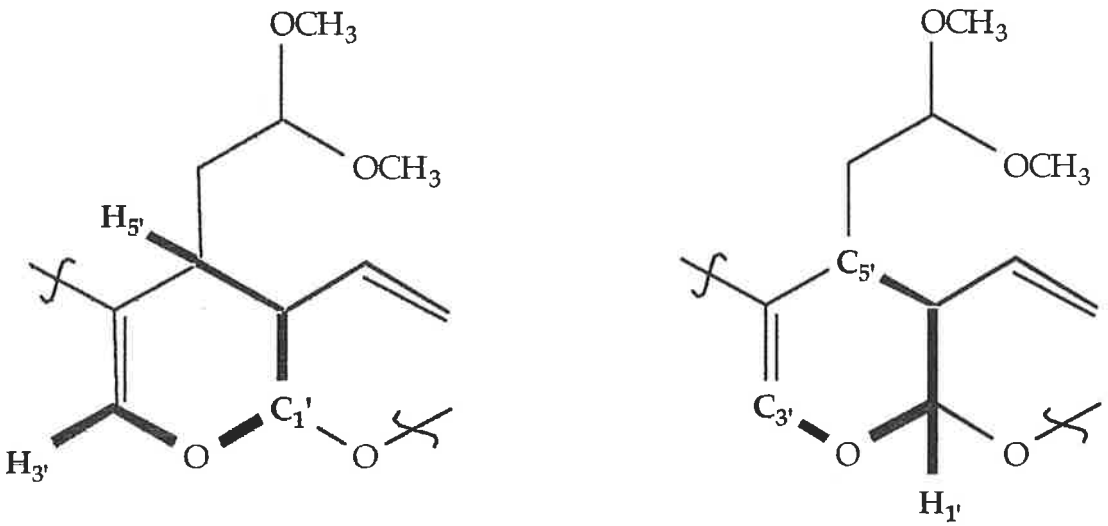


Figure 8.2.21: Closure of a dihydropyran ring

This is the third ring formed and accounts for the eighth DBE (The two double bonds that are part of the structure have already been accounted for).

Part structure A and B are joined by C_{11} (the carbonyl in Part structure A) bonding to $C_{4'}$ (the sp^2 hybridised carbon in Part structure B) because of an HMBC correlation between the protons $H_{3'}$ and $H_{5'}$ with $C_{11'}$ (see *Figure 8.2.22*). This part structure accounts for all non-protonated carbons, all protons and the respective carbons that

are not involved in COSY correlations and both part structures A and B. There is only one position for connectivity to part structure C, the sugar unit.

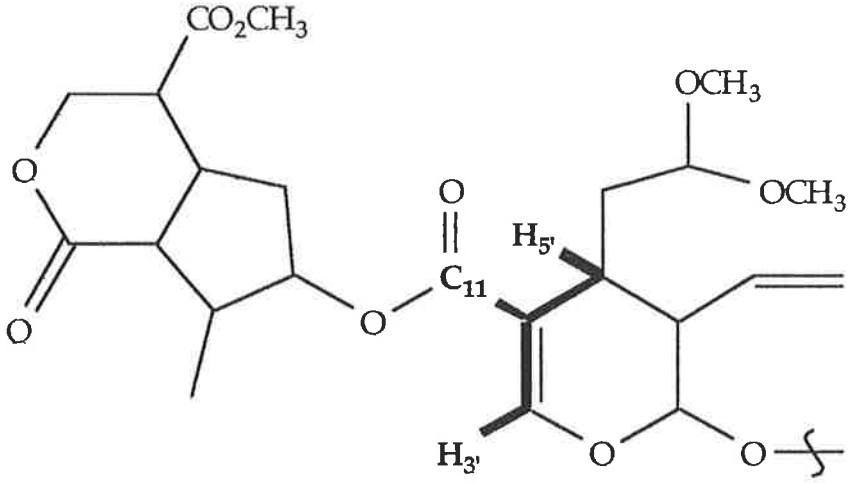


Figure 8.2.22: Connecting two part structures via C_{11}

The sugar unit is illustrated in *Figure 8.2.13* below.

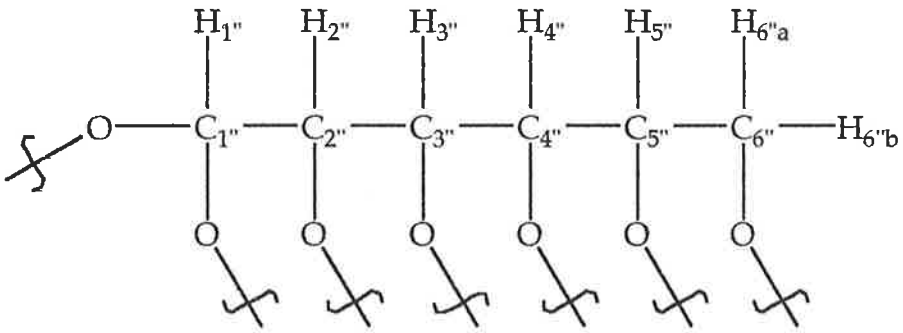


Figure 8.2.13: Part Structure C

There are HMBC correlations between $H_{1"}$ and $C_{5"}$ and between $H_{5"}$ and $C_{1"}$ so that a ring is formed. The sugar unit is a pyranose ring and accounts for the last DBE. $C_{1"}$ is an acetal carbon, while $C_{5"}$ is attached to a single oxygen atom (see *Figure 8.2.23*).

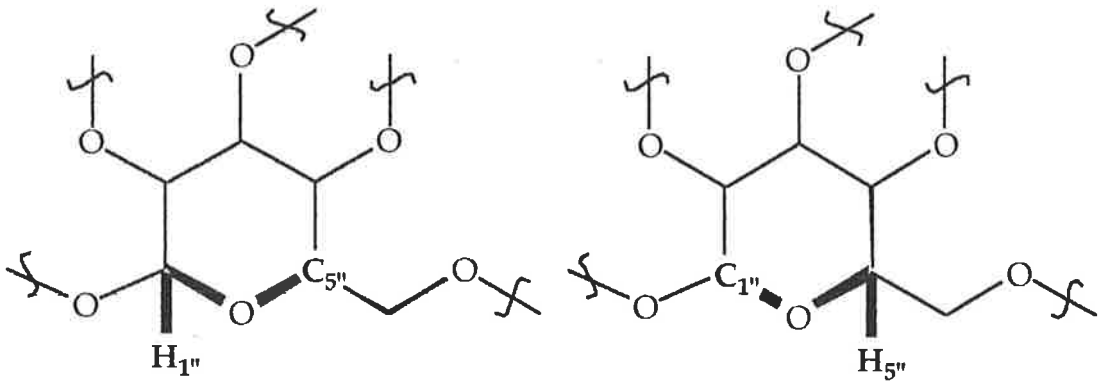


Figure 8.2.23: Formation of the sugar ring

The sugar unit and the rest of the molecule are connected by an oxygen bridge between $C_{1''}$ - the anomeric carbon of the sugar unit - and $C_{1'}$ - the acetal carbon of the dihydropyranose ring. This is established by the HMBC correlation between $H_{1''}$ and $C_{1'}$ and between $H_{1'}$ and $C_{1''}$ (*Figure 8.2.24*).

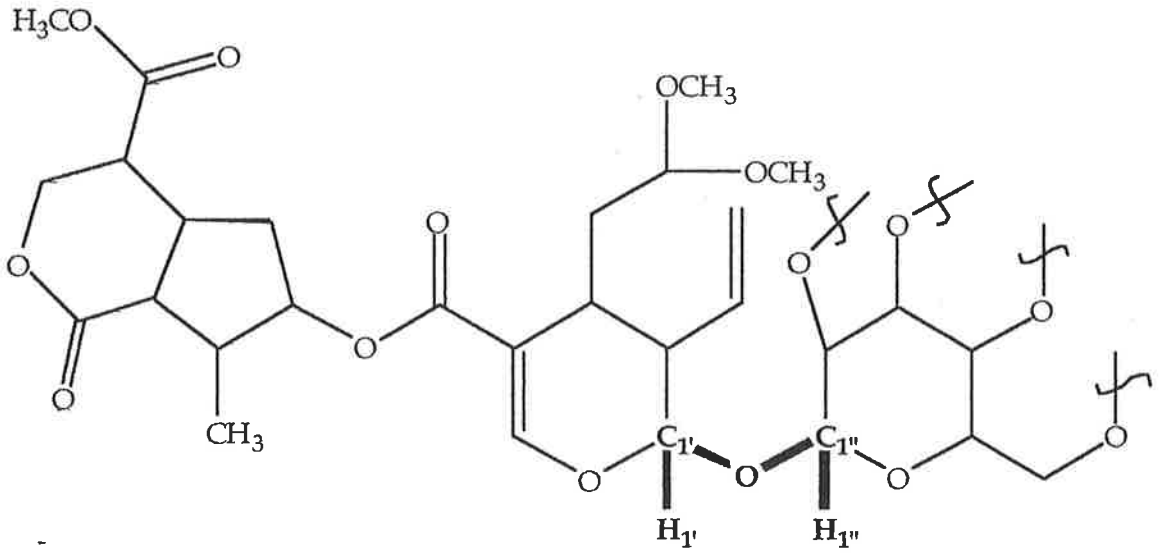


Figure 8.2.24: Connection of the sugar unit

There is a broad singlet in the ^1H spectrum that are hydroxyl protons as the peak decreased in intensity when D_2O was added to the sample tube. The molecular formula indicates that there are four hydroxyl protons.

The final structure (without stereochemistry) is illustrated in *Figure 8.2.25*. This structure accounts for the molecular formula $C_{29}H_{42}O_{15}$, and for the nine DBE (four rings, three carbonyls and two carbon - carbon double bonds).

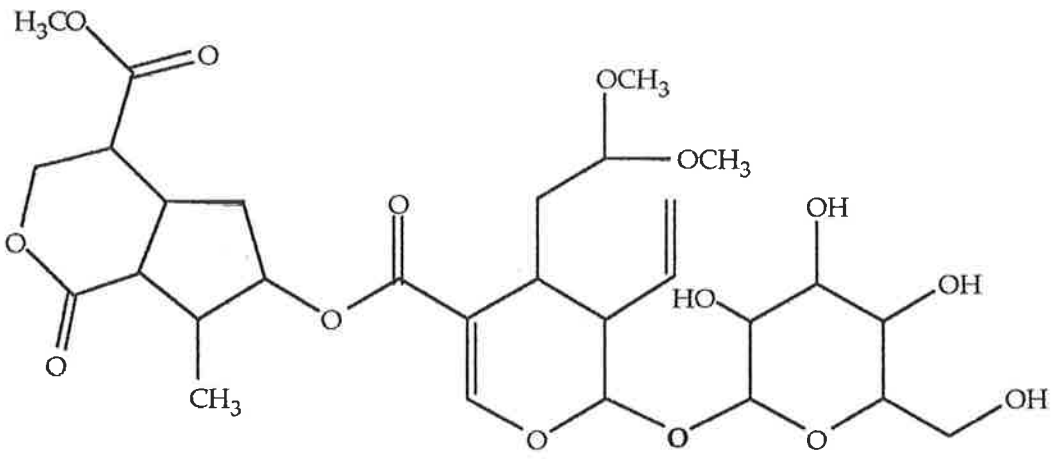


Figure 8.2.25: Full structure of Compound I without stereochemistry

Stereochemistry and conformation

NOESY and ROESY experiments and coupling constants (where applicable) were used to establish the stereochemistry of each part of the molecule. All stereochemical

data - including coupling constants, ROESY correlations and NOESY correlations for Compound I are given in **Table 8.2.2.**

Table 8.2.2: Stereochemical and conformational data for Compound I#

H	δH	Int	M.	J (Hz)	COSY	ROESY	NOESY
$H_{3\beta}$	4.46	1	dd	3.6, 11.4	$H_{3\alpha}$, H_4	$H_{5\alpha}$, H_7	$H_{5\alpha}$, H_7
$H_{3\alpha}$	4.30	1	dd	9.6, 11.4	$H_{3\beta}$, H_4	H_{4a}, H_{7a}	H_{4a}, H_{7a}
H_4	2.55	1	td	3.6, 9.6	$H_{3\alpha}$,$H_{3\beta}$,H_{4a}	$H_{5\alpha}$, H_7	$H_{5\alpha}$, H_7
H_{4a}	3.04	1	dddd	7.8, 9.6, 9.6, 11.4	$H_{4\alpha}H_{5\alpha}H_{5\beta}H_{7a}$	$H_{3\beta}$	$H_{3\beta}$, Me_8
$H_{5\beta}$	1.62	1	ddd	3.6, 11.4, 13.8	H_{4a}, $H_{5\alpha}$, H_6	H_6	H_6
$H_{5\alpha}$	2.20	1	dd	7.8, 13.8	H_{4a}, $H_{5\beta}$	$H_{3\alpha}$, H_4, H_7	$H_{3\alpha}$, H_4, H_7
H_6	5.25	1	dd	3.6, 7.2	$H_{5\beta}$, H_7	$H_{5\alpha}$, H_7, CH_3	$H_{5\alpha}$, Me_8
H_7	2.48	1	ddq	7.2, 7.2, 11.4	H_6, H_{7a}, Me_8	$H_{3\alpha}$, $H_{5\alpha}$	$H_{3\alpha}$, $H_{5\alpha}$
H_{7a}	2.77	1	dd	9.6, 11.4	H_{4a}, H_7	$H_{3\beta}$, CH_3	$H_{3\beta}$, Me_8
Me_8	1.21	3	d	7.2	H_7	H_6, H_7	
Me_{10}	3.75	3	s				
$H_{1'}$	5.36	1	d	5.4	$H_{6'}$	$H_{7'}$, $H_{9'a}$, $H_{9'b}$	$H_{5'}H_{7'}H_{9'a}H_{9'b}$
$H_{3'}$	7.34	1	s				
$H_{5'}$	2.90	1	ddd	5.4, 6.6, 7.2	$H_{6'}$,$H_{9'a}$,$H_{9'b}$	$H_{1'}$, $H_{7'}$, $H_{10'}$	$H_{1'}$, $H_{7'}$, $H_{10'}$
$H_{6'}$	2.71	1	dt	5.4, 5.4, 14.4	$H_{1'}$, $H_{5'}$, $H_{7'}$	$H_{8'}$, $H_{10'}$	$H_{8'}$, $H_{9'b}$, $H_{10'}$
$H_{7'}$	5.68	1	m		$H_{6'}$, $H_{8'}$	$H_{1'}H_{5'}H_{9'b}H_{10'}$	$H_{1'}H_{5'}H_{9'b}H_{10'}$
$H_{8'}$	5.32-5.29	2	m		$H_{7'}$	$H_{6'}$, $H_{10'}$	$H_{5'}$, $H_{6'}$, $H_{10'}$
$H_{9'a}$	2.04	1	ddd	6.6, 7.2, 13.8	$H_{5'}$, $H_{9'b}$, $H_{10'}$	$H_{1'}$	$H_{1'}$, $H_{6'}$, $H_{7'}$
$H_{9'b}$	1.67	1	ddd	4.8, 7.2, 13.8	$H_{5'}$, $H_{9'a}$, $H_{10'}$	$H_{1'}$, $H_{7'}$	$H_{1'}$, $H_{6'}$, $H_{7'}$
$H_{10'}$	4.49	1	dd	4.8, 7.2	$H_{9'a}$, $H_{9'b}$	$H_{5'}H_{6'}H_{7'}H_{8'}$	$H_{5'}H_{6'}H_{7'}H_{8'}$
$Me_{11'}$	3.292	3	s			all except $H_{6'}$	all except $H_{6'}$
$Me_{12'}$	3.298	3	s			all except $H_{6'}$	all except $H_{6'}$
$H_{1''}$	4.72	1	d	7.2	$H_{2''}$	$H_{3''}$, $H_{5''}$	$H_{3''}$, $H_{5''}$
$H_{2''}$	3.39	1	dd	7.2, 9.0	$H_{1''}$, $H_{3''}$	$H_{4''}$	$H_{4''}$
$H_{3''}$	3.58	1	t	9.0	$H_{2''}$, $H_{4''}$	$H_{1''}$, $H_{5''}$	$H_{1''}$, $H_{5''}$
$H_{4''}$	3.63	1	t	9.0	$H_{3''}$, $H_{5''}$	$H_{2''}$	$H_{2''}$
$H_{5''}$	3.43	1	ddd	3.0, 8.4, 9.0	$H_{4''}$ $H_{6''a}$ $H_{6''b}$	$H_{1''}$, $H_{3''}$	$H_{1''}$, $H_{3''}$
$H_{6''a}$	3.93	1	dd	3.0, 11.4	$H_{5''}$, $H_{6''b}$		
$H_{6''b}$	3.86	1	dd	8.4, 11.4	$H_{5''}$, $H_{6''a}$		

#600 MHz (^1H): δ (ppm) from internal TMS in $CDCl_3$

Int = Integration M. = Multiplicity

Sugar unit

The pyranose sugar could be positively identified as a β-glucoside from its coupling constants in the ^1H spectrum, with all $^nJ_{HH}$ values being between 7.8 - 9.0 Hz, indicating axial - axial coupling. The ROESY experiment confirmed this stereochemistry, as there was $H_{1''}$ - $H_{3''}$ - $H_{5''}$ ROE correlations for protons on one side of the ring (1,3-diaxial interactions) and $H_{2''}$ - $H_{4''}$ correlations for protons on the other side of the ring.

There are also ROE correlations between $H_{1''}$- $H_{3''}$ - $H_{5''}$ and protons on the dihydropyranose ring, such as $H_{1'}$, $H_{5'}$ and $H_{6'}$. This indicates that the glucose ring is fixed in a position underneath the dihydropyranose ring. There are no ROE correlations between protons $H_{2''}$ and $H_{4''}$ and protons on the dihydropyranose ring.

Part structure A

Part structure A with all protons labelled is displayed in *Figure 8.2.26*.

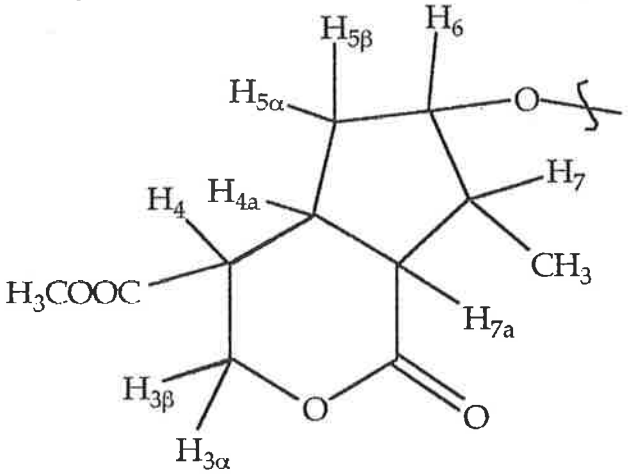


Figure 8.2.26: Position of the labelled protons in Part structure A

It is necessary to consider that the coupling constant data is an average of the conformers possible, while the ROE correlations are a reflection of the relaxation time scale and so it is possible that different conformers are observable in the ROESY data. (The absence of a ROE correlation does not mean that the correlation is not present, as changing the mixing time on the experiment might produce a correlation which wasn't observed previously).

It was possible to determine the stereochemistry of the ring junction using ROE correlations. Of particular interest were the ROE correlations of $H_{3\alpha}$ to H_{4a} and H_{7a}, while there were no ROE correlations between $H_{3\beta}$ and H_{4a} or H_{7a} (H_{4a} and H_{7a} COSY correlate to each other). This indicates that $H_{3\alpha}$ is on the same side of the molecule as the two protons (H_{4a} and H_{7a}) that are at the ring junction. These

two ring junction protons are therefore also on the same side of the ring junction.

This ROE relationship and the relative stereochemistry of the ring junction are outlined in *Figure 8.2.27*. Also displayed is a conformer, outlining the ROE relationships between $H_{3\alpha}$, H_{4a} and H_{7a} and also highlighting the absence of ROESY correlations between $H_{3\beta}$, H_{4a} and H_{7a}.

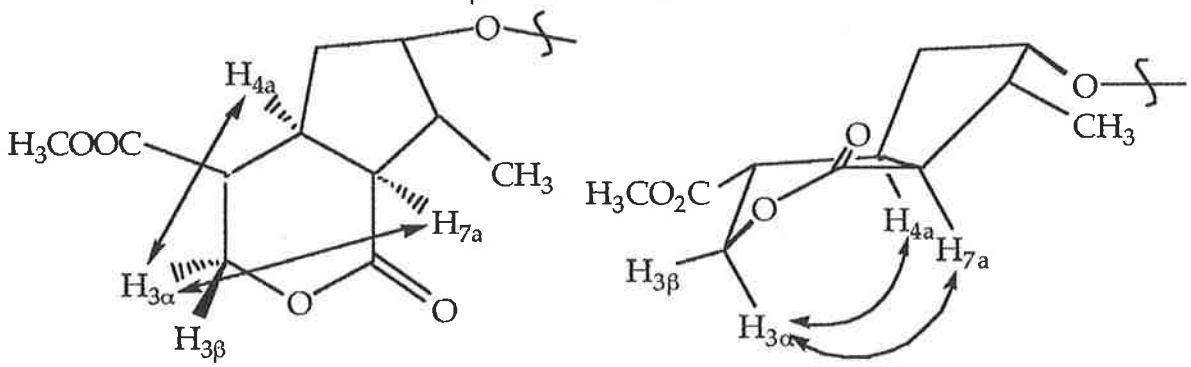


Figure 8.2.27: Stereochemistry of the ring junction

Also of interest is the ROE correlations of $H_{3\beta}$ to H_4 and H_4 to $H_{5\beta}$. These ROE correlations can only be explained if $H_{3\beta}$, H_4 and $H_{5\beta}$ are all on the same side of the molecule, but in a different conformation than the one displayed before. This stereochemistry with the relevant ROESY correlations is outlined in Figure *8.2.28*.

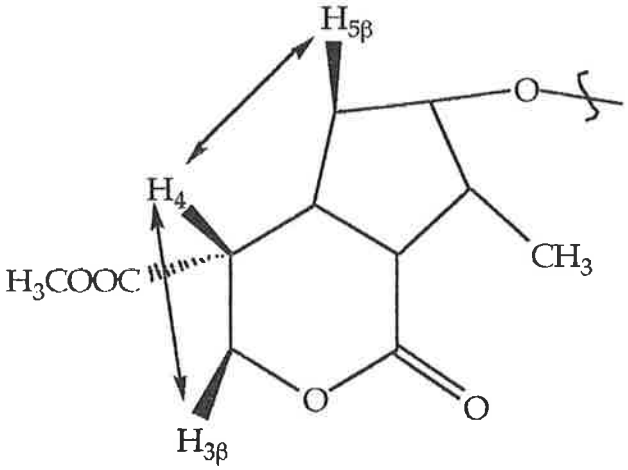


Figure 8.2.28: Stereochemistry of H_4 and $H_{5\beta}$

This data clearly indicates there must be two conformers. The structures of the two conformers, calculated using Chem3DPro (v3.2, CambridgeSoft Corporation, Cambridge, MA, US), are displayed in *Figure 8.3.29*. When both conformers were analysed, using Chem3DPro, the calculated energy difference between the two conformers was found to be 10.3 kJ. mol^{-1}.

These conformers of the structure (see *Figure 8.2.29*) explain the ROE correlations of $H_{3\beta}$ to $H_{5\beta}$ and H_4 to $H_{5\beta}$. These ROE correlations confirm the positioning of $H_{3\beta}$, H_4 and $H_{5\beta}$ on the other side of the ring to both H_{4a} and H_{7a}.

$H_{3\beta}$ and $H_{5\beta}$ also have ROE correlations to H_7. Therefore H_7 must also be on the same side of the molecule as $H_{3\beta}$ and $H_{5\beta}$. This ROE correlation is seen in both conformers and is displayed in *Figure 8.2.29* below.

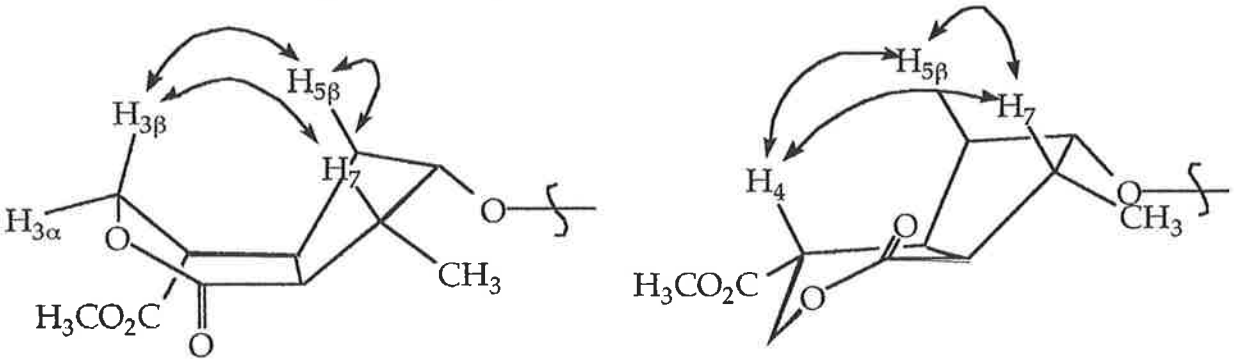


Figure 8.2.29: Stereochemistry of $H_{3\beta}$, H_4, $H_{5\beta}$ and H_7

The stereochemistry of H_6 still has to be determined. There is no ROE correlation to either of the ring junction protons, H_{4a} and H_{7a}, which you would expect from either conformer if these three protons were on the same side of the ring. Therefore H_6 is on the opposite side of the ring to the ring junction protons, H_{4a} and H_{7a}. Therefore, the stereochemistry of whole of this part structure is as outlined in *Figure 8.2.30*.

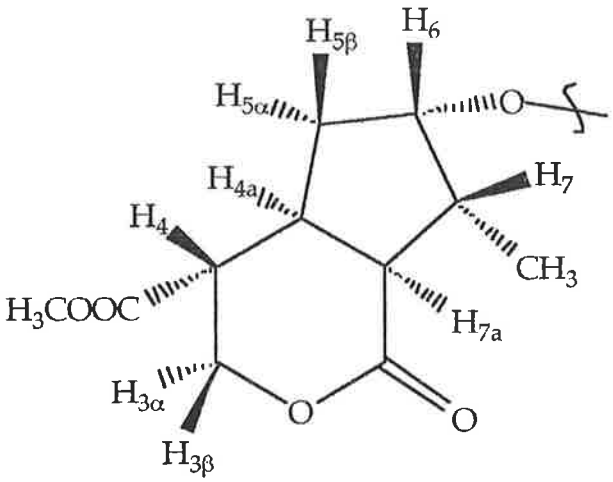
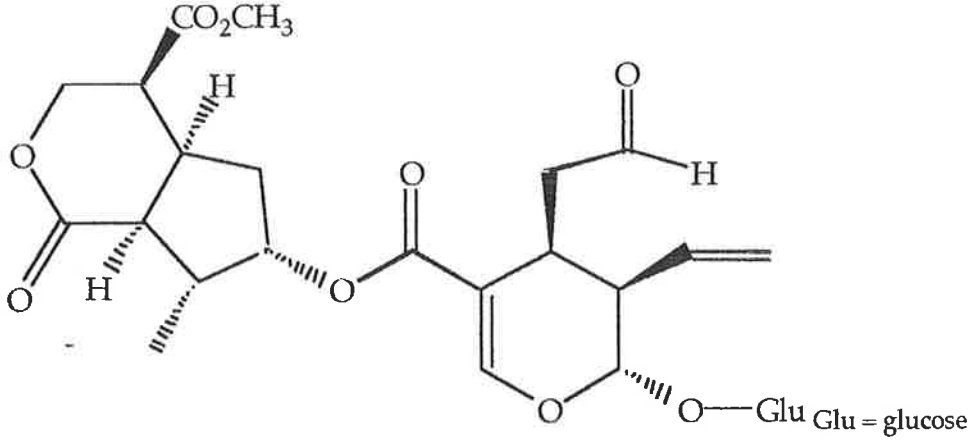


Figure 8.2.30: Stereochemistry of Part structure A

An analysis of the observed coupling constants supports the assignment of this part structure. $H_{3\alpha}$ and $H_{3\beta}$ are coupled to each other with a coupling constant of 13.8 Hz, which is typical of a geminal coupling on a six-membered ring. The coupling constant between $H_{3\beta}$ and H_4 is 3.6 Hz while the larger coupling constant of $H_{3\alpha}$ to H_4 is 9.6 Hz.

This supports the assignment of $H_{3\beta}$ on the same side of the ring to H_4 and with $H_{3\alpha}$ on the opposite side. H_{4a} and H_{7a} couple with a 9.6 Hz value that is consistent with two ring protons being on the same side of the ring junction. The coupling constants of the fused cyclopentane ring are not as useful in confirming the stereochemistry that has been determined using ROE correlations. Therefore part structure A has the stereochemistry as illustrated in *Figure 8.2.30.*

Skaltsounis[44] reported the isolation and structure determination of an iridoid, scaevoloside (**6**), in 1989 that bears a striking resemblance to part structure A.



(**6**)

The relevant part of scaevoloside (**6**) and part structure A are outlined in *Figure 8.2.31.* Most stereochemical centres in the two structures are identical, but there was a difference in the stereochemical assignment of the carboxymethyl group.

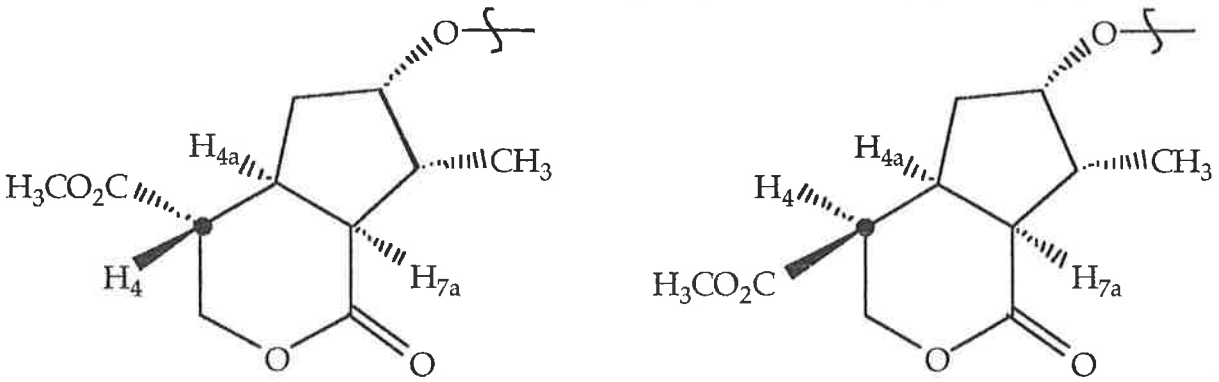
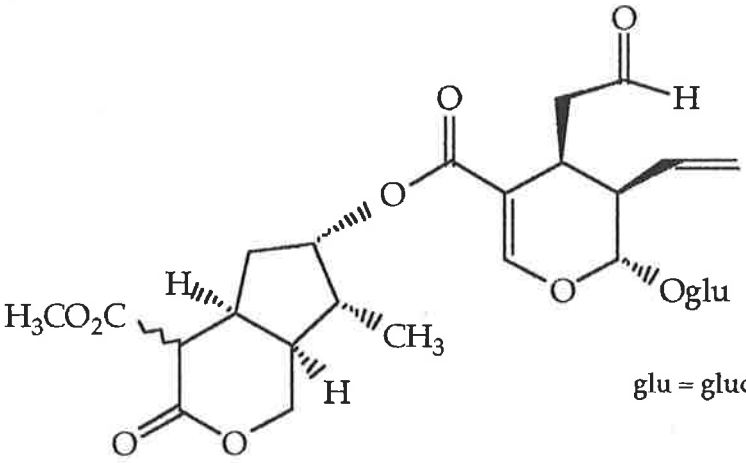


Figure 8.2.31: Comparison of Part structure A and scaevoloside

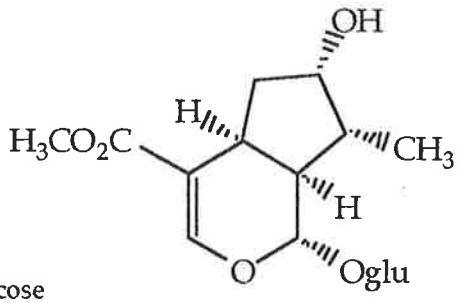
It was therefore necessary to review the method by which the original assignment of the stereochemistry of the carboxymethyl of scaevoloside (**6**) was determined. The ^1H chemical shift value for the relevant proton (H_{4a}) in scaevoloside (**6**) as reported by Skaltsounis is δ 2.55 (triplet of doublets, J = 4.0 and 10.0 Hz: ^1H NMR, $CDCl_3$, TMS).[44] H_4, in part structure A, is the equivalent proton and has a ^1H chemical shift value of δ 2.55 (triplet of doublets, J = 3.6 and 9.6 Hz: ^1H NMR,

$CDCl_3$, TMS). Skaltsounis[44] did not present any ROE data or any discussion of the ^1H data. He based his stereochemical assignment of all of this part of scaevoloside (**6**) on two prior references.



glu = glucose

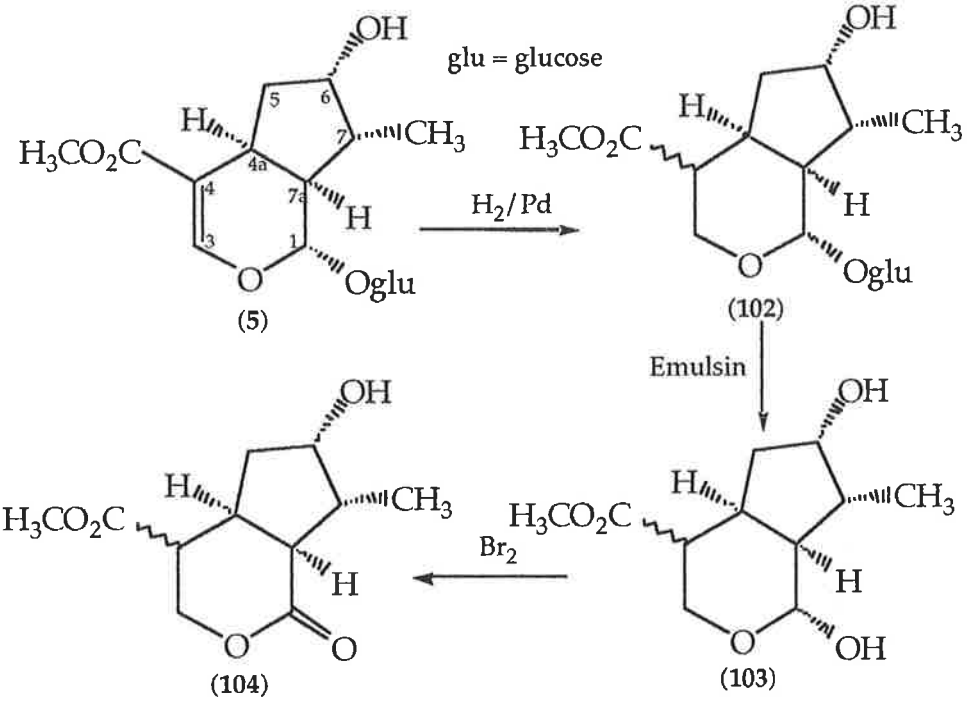
(101)



(5)

He refers, initially, to a paper by Jensen[82] who described a series of reactions, commencing with loganin (**5**) in an attempt to prove the structure of the related iridoid sylvestroside IV (**101**). Jensen treated loganin (**5**) with H_2/palladium catalyst to give (**102**), removed the sugar (**103**) and then oxidised the alcohol with Br_2 to form structure (**104**). This reaction scheme is outlined in **Scheme 8.1**.

Scheme 8.1: Hydrogenation of loganin (5)

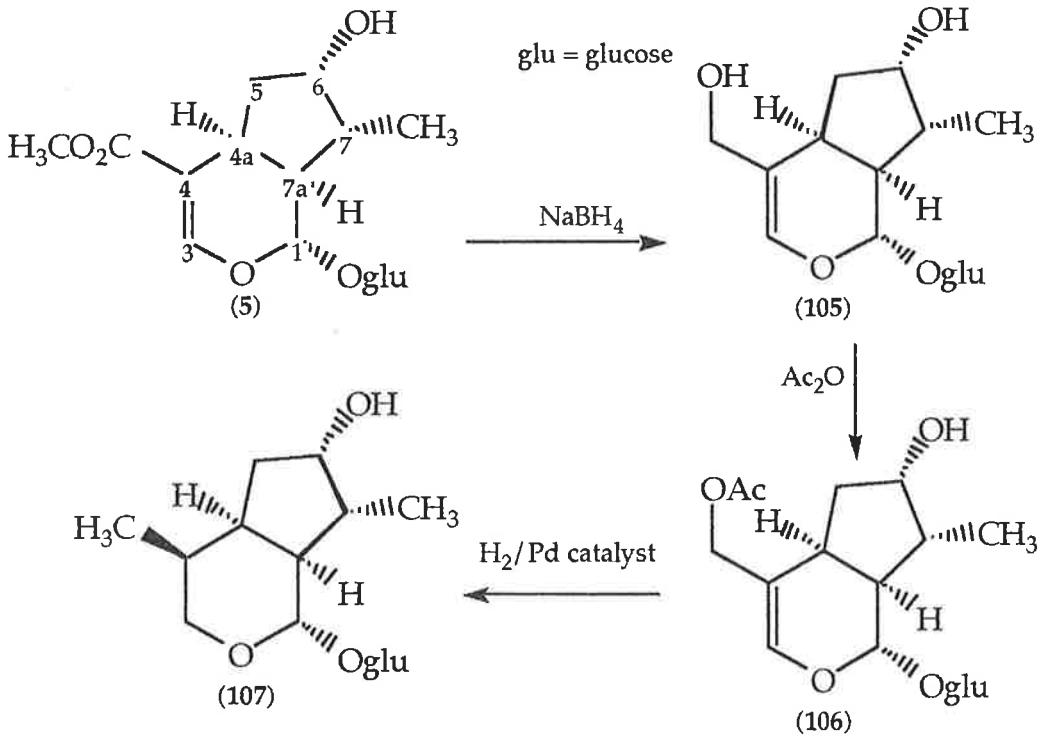


Based on the known stereochemistry of loganin (**5**) which has been established by X-ray crystallography and investigations of its biosynthesis,[83,84,85,86,87] Jensen assigned the absolute stereochemistry of C_{4a}, C_6, C_7 and C_{7a}. However, Jensen did

not specify the stereochemistry of the carboxymethyl group. The only NMR data given was for the protons at C_3 (2H, δ 4.42 m, AB of ABX: ^1H NMR, d_6-Me_2CO, TMS).

Skaltsounis then refers to a second paper, by Bianco[88], who investigated the reduction of loganin (**5**), using $NaBH_4$, to form the alcohol (**105**), which was then acetylated (Ac_2O) (**106**) and hydrogenated (H_2/Pd) to form the product (**107**). This reaction scheme is displayed in **Scheme 8.2**.

Scheme 8.2: Synthesis of (107)



Bianco states that catalytic hydrogenation of the double bond of loganin-derived iridoids takes place at the less hindered side of the molecule. However, the only NMR data published concerned the two methyl signals (δ 1.03, 3H, d, J = 6.5 Hz; δ 0.80, 3H, d, J = 6.5 Hz: ^1H NMR, $CDCl_3$, TMS). No justification was given for the indicated stereochemistry of the new methyl group at C_4. Certainly there was no stereochemical determination for the proton attached to C_4 and the final determination of the stereochemistry of (**107**) was therefore not definitive.

It would therefore appear that insufficient evidence has been reported to definitely establish the stereochemistry at C_4 (the carboxymethyl group) in

scaevoloside (**6**) as put forward by Skaltsounis.[44] and I propose that the stereochemistry of the carboxymethyl group reported for (**6**) be revised.

Part structure B

Part structure B has three chiral carbons, where stereochemistry has to be assigned. The COSY, ROESY, NOESY and coupling constants for part structure B are given in **Table 8.2.2**, pg 109, while the structure is given in *Figure 8.2.32*.

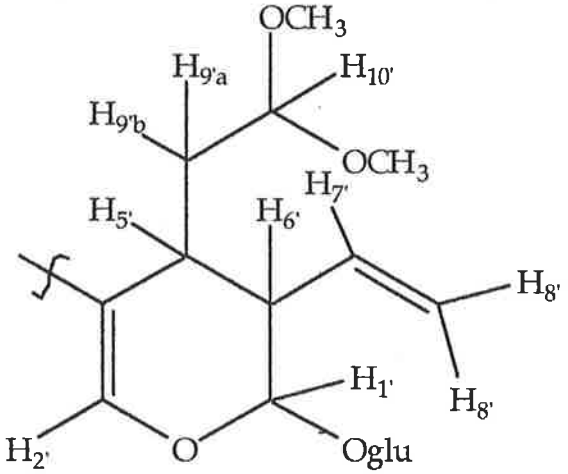


Figure 8.2.32: Part structure B

The important ROE correlations to consider are those associated with the protons attached to the chiral carbons that are $H_{1'}$, $H_{5'}$ and $H_{6'}$.

$H_{1'}$ ROESY correlates to $H_{6'}$, $H_{5'}$, $H_{9'a}$ and $H_{9'b}$. For $H_{1'}$ to correlate to the methylene protons, $H_{9'a}$ and $H_{9'b}$, they must be on the same side of the molecule (see *Figure 8.3.33*). $H_{7'}$ correlates to $H_{1'}$ as well as the methylene protons $H_{9'a}$ and $H_{9'b}$. This indicates that H_7 is also on the same side of the ring as $H_{9'a}$ and $H_{9'b}$ (see *Figure 8.3.34*).

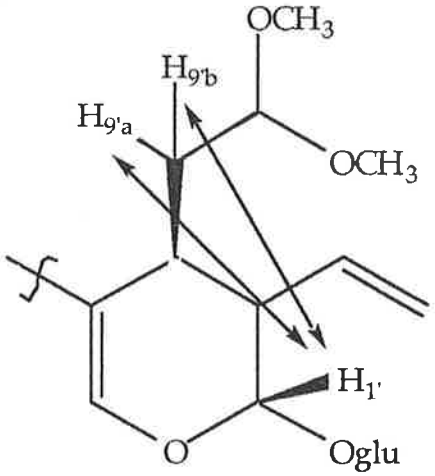


Figure 8.3.33: ROESY correlations for $H_{1'}$

glu = glucose

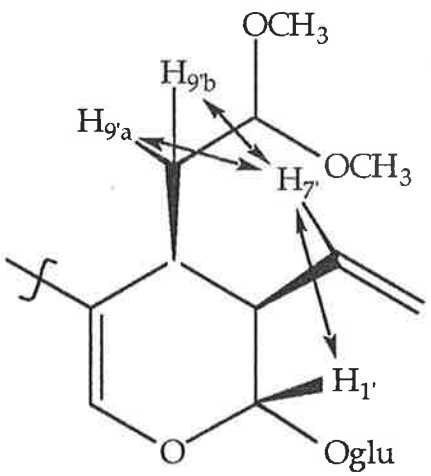
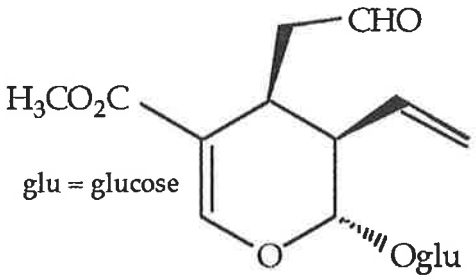


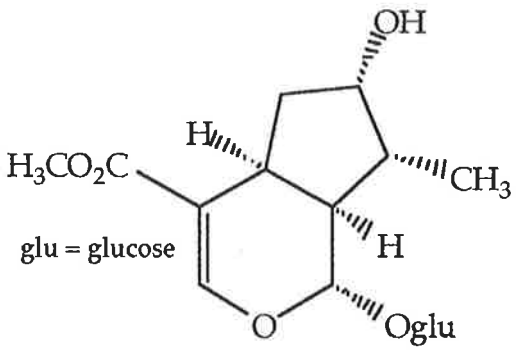
Figure 8.3.34: ROE correlations for $H_{7'}$

These ROE correlations can be used to establish the stereochemistry of each chiral carbon. This stereochemical assignment is identical to that of secologanin (**1**) the

structure of which has been established.[89] Loganin (**5**) has been shown to be derived from secologanin (**1**).[89]



(**1**)



(**5**)

A comparison of the equivalent ^{1}H and ^{13}C chemical shift data for part structure B and scaevoloside (**6**) indicate that these two part structures are identical in both chemical shift values and stereochemistry. However, part structure B contains a dimethyl acetal, while scaevoloside (**6**) has an aldehyde group.

Therefore, Compound I has the following overall structure, including stereochemistry (see *Figure 8.3.35*). It is almost identical to scaevoloside(**6**) (as previously reported[44]) apart from the stereochemistry at C_4, where the carboxymethyl group is on the same side of the ring as the ring junction protons.

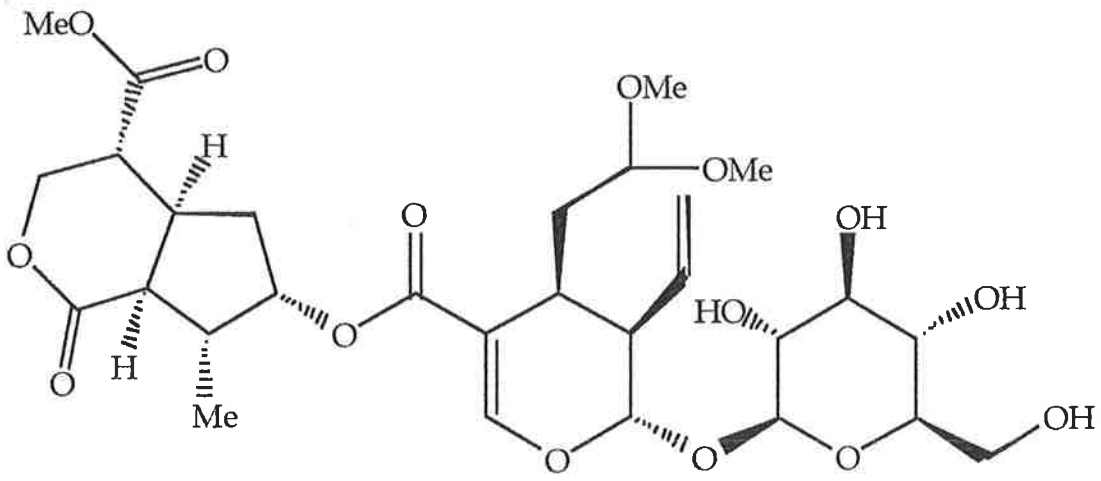


*Figure 8.3.35: Final structure of Compound I- alidyjosioside (**108**)*

This is a novel compound, although it is possible that it is an artefact of the isolation process.

It has been named alidyjosioside (**108**).

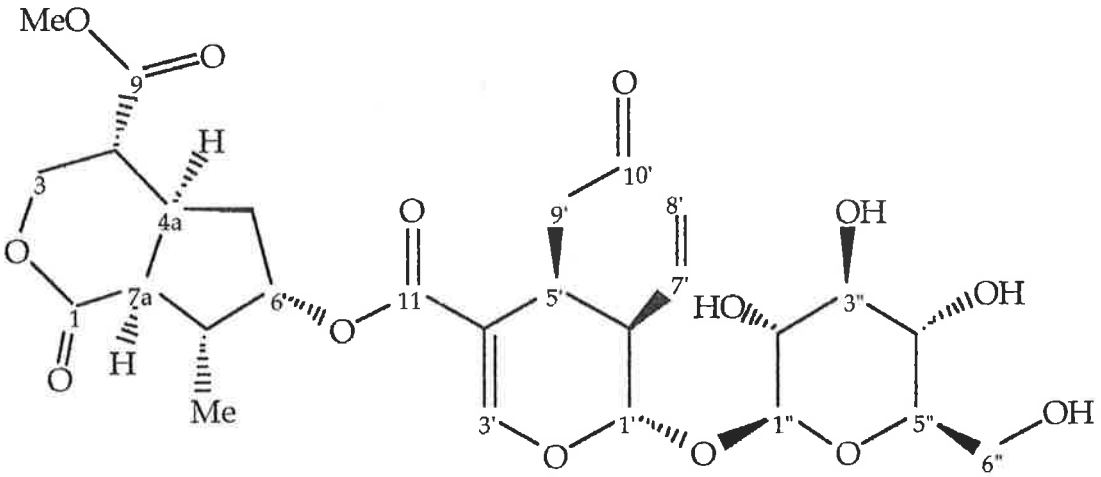
8.3 Compound J

Compound J (10 mg) was isolated as a white amorphous solid (see Chapter 8.1, **Diagram 8.1**, pg 94) with a MP of 120 - 124°C and an $[\alpha]_D$ of - 64° (c 0.1, MeOH).

Compound J was submitted for HRMS (LSIMS) analysis and the sodium adduct was found to have a molecular mass ($M+Na^+$) of 607.2013 that gave a molecular formula of $C_{27}H_{36}O_{14}Na$ (calculated = 607.2006, +/- 0.0007). There were therefore ten DBE to be accounted for as either rings or double bonds.

Standard ^1H and ^{13}C NMR spectra indicated signals consistent with four carbonyl carbons – one at δ 201 (an aldehyde) and three between δ 166 - 173. There were four other sp^2 hybridised carbons, which accounts for six DBE, leaving four to be associated with ring systems.

The following data (in **Table 8.3.1**, pg 119) was recorded from ^1H, COSY, ROESY, ^{13}C, HMQC and HMBC experiments on Compound J. The numbering system is that commonly used in the literature for iridoids (see *Figure 8.3.1*).



(6)

There is a great deal of similarity between alidyjosioside (**108**) and this compound in terms of chemical shift values and coupling constants. The only difference occurs in the presence of an aldehyde group in Compound J, and the absence of two methoxy carbons and an acetal carbon that are found in alidyjosioside (**108**). This suggests that Compound J is scaevoloside (**6**).

Table 8.3.1: NMR data for scaevoloside (6)

C	δ C	H	δ H	Int	COSY	ROESY	HMBC
C_1	173.6						
C_3	67.0	$H_{3\beta}$	4.34	1	$H_{3\alpha}$, H_4	$H_{5\beta}$, H_7	C_1, C_9
		$H_{3\alpha}$	4.27	1	$H_{3\beta}$, H_4	H_{4a}, H_{7a}	C_1, C_9
C_4	44.0	H_4	2.53	1	$H_{3\alpha}$,$H_{3\beta}$,H_{4a}	$H_{5\beta}$, H_7	C_9
C_{4a}	39.7	H_{4a}	3.00	1	H_3, $H_{5\alpha}$, $H_{5\alpha}$, H_{8a}	$H_{3\alpha}$	C_1, C_9
C_5	40.1	$H_{5\beta}$	1.54	1	H_{4a}, $H_{5\alpha}$, H_6	H_6	
		$H_{5\alpha}$	2.24	1	H_{4a}, $H_{5\beta}$	$H_{3\alpha}$, H_4, H_7	
C_6	76.3	H_6	5.21	1	$H_{5\alpha}$, H_7	$H_{5\beta}$, H_7, Me_8	C_{11}
C_7	45.6	H_7	2.44	1	H_6, H_{7a}, H_8	$H_{3\beta}$, $H_{5\beta}$	C_1
C_{7a}	46.0	H_{7a}	2.71	1	H_{4a}, H_7	$H_{3\alpha}$, Me_8	
C_8	16.0	Me_8	1.16	3	H_7	H_6	
C_9	172.4						
C_{10}	52.5	Me_{10}	3.72	3			C_9
C_{11}	166.7						
$C_{1'}$	96.2	$H_{1'}$	5.31	1	$H_{6'}$	$H_{7'}$, $H_{9'a}$, $H_{9'b}$	$C_{3'}$ $C_{5'}$ $C_{6'}$$C_{1''}$
$C_{3'}$	154.6	$H_{3'}$	7.42	1			C_{11}, $C_{1'}$, $C_{4'}$
$C_{4'}$	110.0						
$C_{5'}$	29.3	$H_{5'}$	3.39	1	$H_{6'}$, $H_{9'a}$, $H_{9'b}$	$H_{1'}$, $H_{7'}$, $H_{10'}$	C_{11}, $C_{1'}$, $C_{4'}$
$C_{6'}$	47.8	$H_{6'}$	2.78	1	$H_{1'}$, $H_{5'}$, $H_{7'}$	$H_{8'}$, $H_{10'}$	$C_{1'}$
$C_{7'}$	135.7	$H_{7'}$	5.53	1	$H_{6'}$, $H_{8'}$	$H_{1'}$ $H_{5'}$ $H_{9'b}$ $H_{10'}$	
$C_{8'}$	122.2	$H_{8'}$	5.22-5	2	$H_{7'}$	$H_{6'}$, $H_{10'}$	
$C_{9'}$	44.8	$H_{9'a}$	2.96	1	$H_{5'}$, $H_{9'b}$, $H_{10'}$	$H_{1'}$	$C_{4'}$
		$H_{9'b}$	2.41	1	$H_{5'}$, $H_{9'a}$, $H_{10'}$	$H_{1'}$, $H_{7'}$	
$C_{10'}$	201.2	$H_{10'}$	9.71	1	$H_{9'a}$, $H_{9'b}$	$H_{5'}$, $H_{6'}$, $H_{7'}$, $H_{8'}$	
$C_{1''}$	98.1	$H_{1''}$	4.69	1	$H_{2''}$	$H_{1'}$, $H_{3''}$, $H_{5''}$	$C_{1'}$, $C_{5''}$
$C_{2''}$	75.6	$H_{2''}$	3.42	1	$H_{1''}$, $H_{3''}$	$H_{4''}$	
$C_{3''}$	76.3	$H_{3''}$	3.56	1	$H_{2''}$, $H_{4''}$	$H_{1''}$, $H_{5''}$	
$C_{4''}$	70.0	$H_{4''}$	3.61	1	$H_{3''}$, $H_{5''}$	$H_{2''}$	
$C_{5''}$	73.8	$H_{5''}$	3.39	1	$H_{4''}$, $H_{6''a}$, $H_{6''b}$	$H_{1''}$, $H_{3''}$	$C_{1''}$
$C_{6''}$	62.0	$H_{6''a}$	3.86	1	$H_{5''}$, $H_{6''b}$		
		$H_{6''b}$	3.83	1	$H_{5''}$, $H_{6''a}$		

#600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$

Int = Integration

This ^1H and ^{13}C NMR data are identical to that in the literature for scaevoloside (**6**).[44] There are three COSY spin systems, two of which are identical to the spin systems analysed in alidyjosioside (**108**) and which, when the HMQC data was examined, supported the determination of the overall structure of Compound J being scaevoloside (**6**) (without analysis of the stereochemistry).

The first spin system was identical to that of the cyclopentane ring found in (**108**). The second was identified as a sugar unit that was β-glucose. The third spin system had the ^1H chemical shift values and COSY correlations identical to the protons and carbons in alidyjosioside (**108**) except that an aldehyde functional group was directly attached to $C_{9'}$ (instead of two methoxy functional groups being attached to an acetal carbon, $C_{10'}$).

The stereochemical analysis of Compound J was identical to that of (**108**). This has lead to a proposed revision of the stereochemistry of the carbomethoxy group attached at C_4 of the cyclopentane-fused dihydropyran ring.

The ROE correlations that were observed and used to determine the stereochemistry were those from $H_{3\beta}$ to $H_{5\beta}$ and H_7 as well as the one's from H_4 to $H_{5\beta}$ and H_7. These are illustrated in *Figure 8.3.2*, along with the two proposed conformers of this part of the molecule.

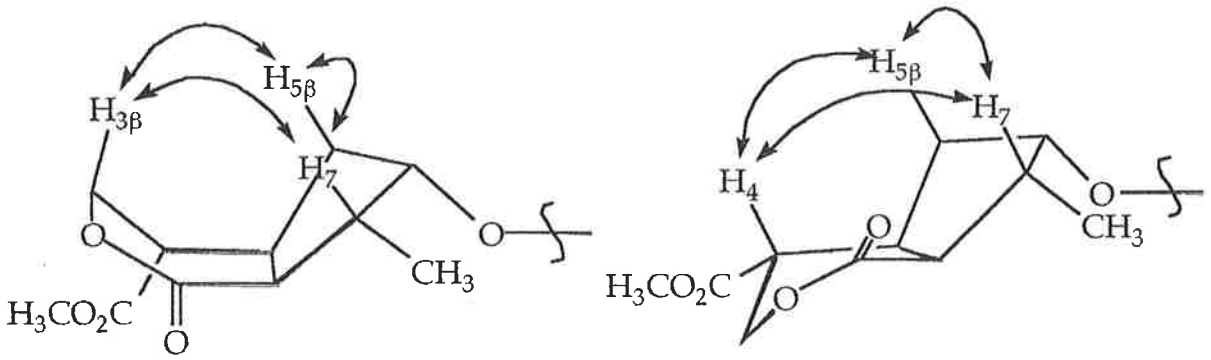


Figure 8.3.2: Two conformers illustrating the proposed stereochemistry of (6)

There were also ROE correlations from $H_{3\alpha}$ to the ring junction protons, H_{4a} and H_{7a}, so that these three protons are on the same side of the ring. This relationship is only observed in one of the conformers, as illustrated in *Figure 8.3.3*.

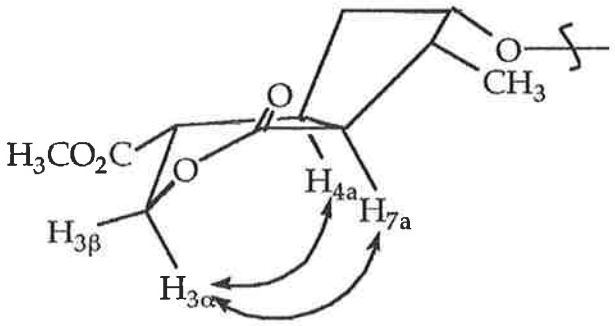


Figure 8.3.3: Conformer illustrating the stereochemistry of the ring junction protons.

This stereochemistry is identical for that established for alidyjosioside (**108**) (discussed in Chapter 8.2, pgs 108 - 117) but is different at the position of the carboxymethyl group as reported by Skaltsounis[44] for scaevoloside (**6**). The stereochemistry of the rest of the molecule (as shown in *Figure 8.3.1*) is identical in both (**108**) and (**6**). This structure is almost identical to the reported data for scaevoloside (**6**), as previously reported,[44] except for the stereochemistry of the carbon bearing the carboxymethyl group.

On the basis of the arguments presented in Chapter 8.2, pgs 108 - 117, a revision of the stereochemistry of scaevoloside (**6**) is proposed.

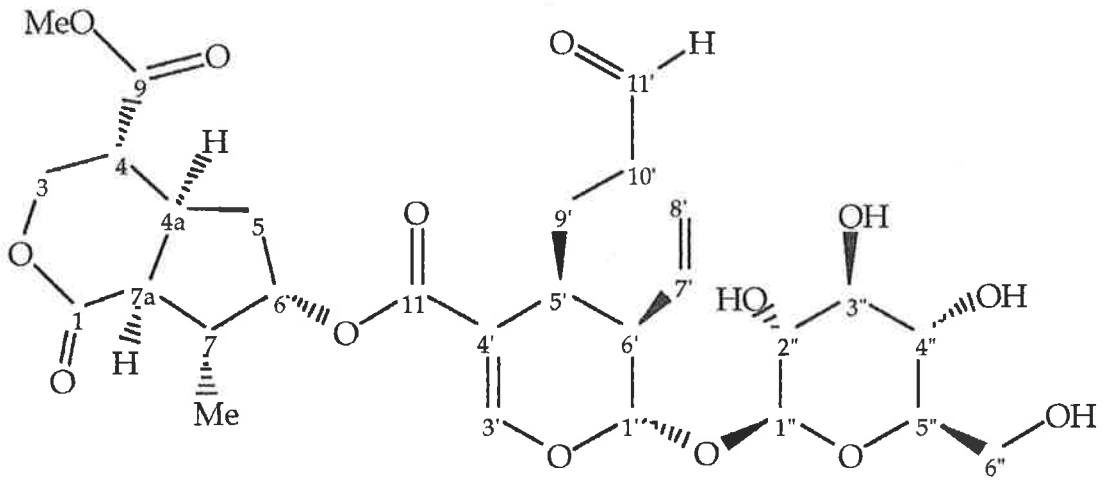
Scaevoloside (**6**) has been isolated previously from *Scaevola racemigera*[44] but not from *Scaevola spinescens*.

8.4 Compound K

Compound K (12 mg) was isolated as a white amorphous solid (see Chapter 8.1, **Diagram 8.1**, pg 94) with a MP of 148 - 150°C and an $[\alpha]_D$ - 64° (c 0.1, MeOH).

LSIMS in glycerol gave an $M+H^+$ of 599.3. Accurate mass (HRMS) gave an $M+H^+$ of 599.2361 and therefore the formula was $C_{28}H_{38}O_{14}$ (calculated = 599.2341, +/- 0.0020).

Routine ^1H and ^{13}C NMR spectra indicated a molecule very similar to both alidyjosioside (**108**) and scaevoloside (**6**) with one major difference and that was an extra methylene carbon. Compound K was identified as a new iridoid and named katecateroside (**109**). The numbering system is that commonly used in the literature for iridoids.



(109)

$^1J_{CH}$ correlations were determined by an HMQC experiment. The ^1H, ^{13}C and $^1J_{CH}$ correlations for this new compound, katecateroside (**109**), are displayed in **Table 8.4.1**, pg 123 with the different proton and carbon signals highlighted in bold as well as the equivalent proton and carbon signals in (**6**) and (**108**).

The NMR data for (**109**)#, displayed in **Table 8.4.1**, pg 123, was acquired at 600 MHz (^1H), 150 MHz (^{13}C): δ (ppm) from internal TMS in CD_3OD. The NMR data for (**6**)* and (**108**)* was acquired at 600 MHz (^1H), 150 MHz (^{13}C): δ (ppm) from internal TMS in $CDCl_3$.

Table 8.4.1: NMR data for (109)#

C	δ C#	H	δ H#	δ C- (6)*	δ H- (6)*	δ C- (108)*	δH- (108)*
C_1	176.2			173.6		173.3	
C_3	68.3	$H_{3\beta}$	4.40	67.0	4.34	68.0	4.46
		$H_{3\alpha}$	4.27		4.27		4.30
C_4	43.8	H_4	2.34	44.0	2.53	46.5	2.55
C_{4a}	37.8	H_{4a}	2.97	39.7	3.00	37.0	3.04
C_5	38.7	$H_{5\beta}$	1.60	40.1	1.54	38.5	1.62
		$H_{5\alpha}$	2.10		2.24		2.20
C_6	78.8	H_6	5.14	76.3	5.21	78.2	5.25
C_7	45.3	H_7	2.56	45.6	2.44	43.7	2.48
C_{7a}	46.3	H_{7a}	2.60	46.0	2.71	47.5	2.77
C_8	14.5	Me_8	1.06	16.0	1.16	15.2	1.21
C_9	173.2			172.4		171.4	
C_{10}	52.7	Me_{10}	3.62	52.5	3.72	53.8	3.75
C_{11}	167.7			166.7		166.3	
$C_{1'}$	97.7	$H_{1'}$	5.42	96.2	5.31	97.6	5.36
$C_{3'}$	153.3	$H_{3'}$	7.37	154.6	7.42	154.9	7.34
$C_{4'}$	111.9			110.0		110.8	
$C_{5'}$	29.8	$H_{5'}$	2.87	29.3	3.39	29.6	2.90
$C_{6'}$	47.8	$H_{6'}$	2.79	47.8	2.78	44.5	2.71
$C_{7'}$	134.8	$H_{7'}$	5.64	135.7	5.53	133.9	5.68
$C_{8'}$	120.5	$H_{8'}$	5.12-5.22	122.2	5.22-5.25	120.6	5.29-5.32
$C_{9'}$	37.4	$H_{9'a}$	1.86	44.8	2.96	32.2	2.04
		$H_{9'b}$	1.56		2.41		1.67
$C_{10'}$	**46.1**	**$H_{10'a}$**	**2.60**	**201.2**	**9.71**	**103.7**	**4.49**
		$H_{10'b}$	**2.40**				
$C_{11'}$	**203.0**	**$H_{11'}$**	**9.58**				
$C_{11'}$		**$Me_{11'}$**				**52.28**	**3.292**
$C_{12'}$		**$Me_{12'}$**				**52.29**	**3.298**
$C_{1''}$	98.0	$H_{1''}$	4.53	98.1	4.69	98.6	4.72
$C_{2''}$	77.9	$H_{2''}$	3.15	75.6	3.42	72.9	3.39
$C_{3''}$	78.3	$H_{3''}$	3.26	76.3	3.56	76.7	3.58
$C_{4''}$	71.5	$H_{4''}$	3.15	70.0	3.61	70.5	3.63
$C_{5''}$	74.5	$H_{5''}$	3.09	73.8	3.39	75.9	3.43
$C_{6''}$	63.1	$H_{6''a}$	3.79	62.0	3.86	62.5	3.93
		$H_{6''b}$	3.55		3.83		3.86

As can be observed:

- Both (**109**) and scaevoloside (**6**) have an aldehyde group whereas alidyjosioside (**108**) does not.

- (**109**) has a ^{13}C methylene signal at δ 46.1 and two methylene protons - $H_{10'a}$ and $H_{10'b}$ - which do not appear in either of the other two compounds.

- Alidyjosioside (**108**) has an acetal carbon at $C_{10'}$ and a proton at δ 4.49 which is not present in the other two compounds.

- Alidyjosioside (**108**) has two ether methoxy signals ($Me_{11'}$ and $Me_{12'}$) which are not present in either of the other two compounds.

- The COSY experiment confirmed the positioning of the extra methylene protons alpha to the aldehyde in (**109**) and the overall structure of a pyran ring.

- The other two spin systems were identical to those of alidyjosioside (**108**) and scaevoloside (**6**), establishing a cyclopentane ring fused to a dihydropyran ring and a sugar unit.

- The sugar unit in (**6**), (**108**) and (**109**) was identified as β-glucose.

There was an electron withdrawing effect of the carbonyl functional group on the adjacent carbon ^{13}C chemical shift values, making the carbon deshielded and therefore downfield (see ^{13}C chemical shifts of $C_{5'}$, $C_{9'}$ and $C_{10'}$, **Table 8.4.1**, pg 123). The protons ($H_{5'}$, $H_{9'a}$, $H_{9'b}$, $H_{10'a}$ and $H_{10'b}$) are also downfield, ranging from δ 2.96 - 2.41.

The stereochemistry of (**109**) at all chiral carbons was determined via coupling constant values and ROESY data and was found to be identical to that of (**6**) and (**108**) In particular (**109**) exhibited the same stereochemistry for the carboxymethyl group at C_4.

This compound is novel. It has been named katecateroside (109). It is an iridoid, based on scaevoloside (6) but with an extra methylene group.

8.5 Compound L

Compound L (10 mg) was isolated as a white amorphous solid (see Chapter 8.1, **Diagram 8.1**, pg 94) with a MP of 221 - 223°C and an $[\alpha]_D$ - 80° (c 0.1, MeOH).

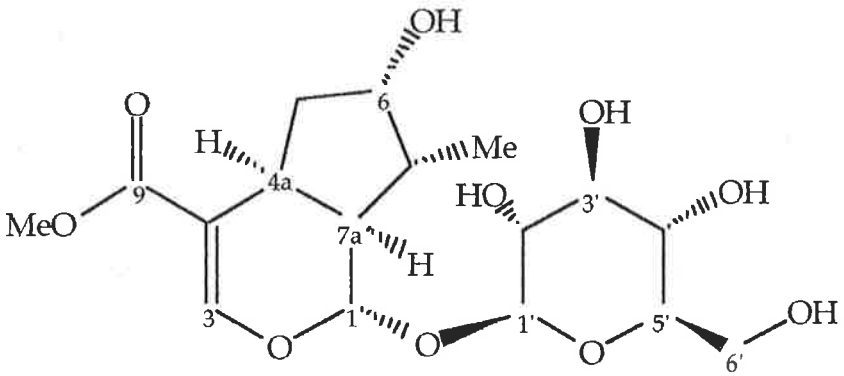
Compound L was found to have an $M+H^+$ of 391.1614 (LSIMS) giving a formula of $C_{17}H_{26}O_{10}$ (calculated = 391.1604, +/- 0.0010). This indicated there were five DBE to be accounted for, as either rings or double bonds.

The IR spectrum indicated one double bond (1660 cm^{-1}), a carbonyl (1722 cm^{-1}) and hydroxyl functional groups (3430 cm^{-1}). This accounts for two DBE.

The ^1H and ^{13}C spectra of Compound L were run under normal conditions and under D_2O exchange conditions. COSY, HMQC and HMBC experiments were also completed.

The ^1H, ^{13}C and stereochemical data for Compound L are identical to that of loganin (**5**).[83,84,85,86] This NMR data is displayed in **Table 8.5.1**, pg 126. The numbering system is that commonly used in the literature for iridoids.

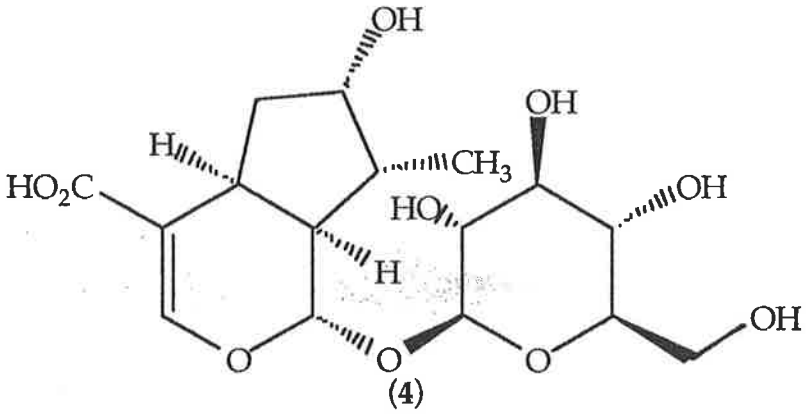
The MP[78,80] and $[\alpha]_D$[80] also matches the literature values for loganin (**5**).



(5)

Loganin (**5**) has been isolated from *Scaevola montana*[43] and *Scaevola racemigera*[44] but not from *Scaevola spinescens*.

Personnel communication with Dr Jensen[42], Technical University of Denmark, revealed that loganic acid (**4**) has been isolated from *Scaevola taccada*.



(4)

Table 8.5.1: NMR data for (5)#

C	δ C	H	δ H	Int	Mult.	J (Hz)	COSY	HMBC
C_1	96.1	H_1	5.11	1	d	4.8	H_{7a}	C_3, C_{4a} C_7 $C_{1'}$
C_3	150.5	H_3	7.35	1	d	1.2	H_{4a}	C_1, C_3, C_4, C_9
C_4	111.9							
C_{4a}	30.8	H_{4a}	2.96	1	dddd	1.2, 7.8, 9.6,11.4	H_3,$H_{5\alpha}$,$H_{5\beta}$,H_{7a}	C_3, C_4, C_9
C_5	42.0	$H_{5\beta}$	1.43	1	ddd	3.6, 11.4, 13.8	H_{4a}, $H_{5\alpha}$, H_6	C_{4a}, C_6
		$H_{5\alpha}$	2.05	1	dd	7.8, 13.8	H_{4a}, $H_{5\beta}$	C_{4a}, C_6
C_6	72.1	H_6	3.86	1	dd	3.6, 11.4	$H_{5\beta}$, 6-OH, H_7	C_5, C_7
		6-OH	4.54	1	br t		H_6	C_6
C_7	39.8	H_7	1.70	1	ddd	6.6, 9.6, 11.4	H_6, H_{7a}, Me_8	C_1 C_6 C_{7a} C_{10}
C_{7a}	44.7	H_{7a}	1.83	1	ddd	4.8, 9.6, 9.6	H_1, H_{4a}, H_7	C_1, C_{4a}, C_7
C_8	13.5	Me_8	0.97	3	d	6.6	H_7	C_6, C_7, C_{7a}
C_9	167.1							
C_{10}	50.7	Me_{10}	3.61	3	s			C_9
$C_{1'}$	98.5	$H_{1'}$	4.46	1	d	7.8	$H_{2'}$	C_1, $C_{5'}$
$C_{2'}$	73.1	$H_{2'}$	2.93	1	dd	7.8, 9.0	$H_{1'}$, $H_{3'}$	$C_{1'}$, $C_{3'}$
		2'-OH	5.04	1	br d		$H_{2'}$	$C_{2'}$
$C_{3'}$	76.7	$H_{3'}$	3.13	1	dd	9.0, 9.6	$H_{2'}$, $H_{4'}$	$C_{2'}$, $C_{3'}$
$C_{4'}$	70.1	$H_{4'}$	3.20	1	dd	9.0, 9.6	$H_{3'}$, $H_{5'}$	$C_{3'}$, $C_{5'}$
$C_{5'}$	77.3	$H_{5'}$	3.10	1	ddd	3.6, 5.4, 9.0	$H_{4'}$, $H_{6a'}$, H_{6b}	$C_{1'}$, $C_{4'}$, $C_{6'}$
$C_{6'}$	61.1	$H_{6'a}$	3.64	1	dd	3.6, 11.4	$H_{5'}$, $H_{6b'}$	$C_{5'}$
		$H_{6'b}$	3.41	1	dd	5.4, 11.4	$H_{5'}$, $H_{6a'}$	$C_{5'}$
		OH	1.22	2	br s			
		OH	4.40	1	br d			

#600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in d_6-DMSO

Int = Integration Mult. = Multiplicity

CHAPTER 9 FRACTION 8

9.1 Initial treatment

Treatment of Fraction 9 yielded three compounds, as outlined in **Diagram 9.1.**

23 g of Fraction 8 was separated from the original methanol extract (828 g) using 90% ethyl acetate/10% methanol as the eluting solvent.

5 g of Fraction 8 was subjected to flash chromatography (silica, isocratic, 90% ethyl acetate/10% methanol) with twelve fractions being separated. Fractions 8.3 and 8.4 were combined and labelled 8.(3).

Fraction 8.(3) (1.44 g) was dissolved in 100 mL chloroform/100 mL water and the two layers separated. The water layer was labelled 8.(3).H_1 (250 mg). The water was removed (freeze dried) and the residue was then placed in two vials and labelled 8.(3).H_1.MV.[X] (100 mg) and 8.(3).H_1.MV (150 mg).

Fraction 8.(3).H_1.MV.[X] was subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) with two fractions being collected.

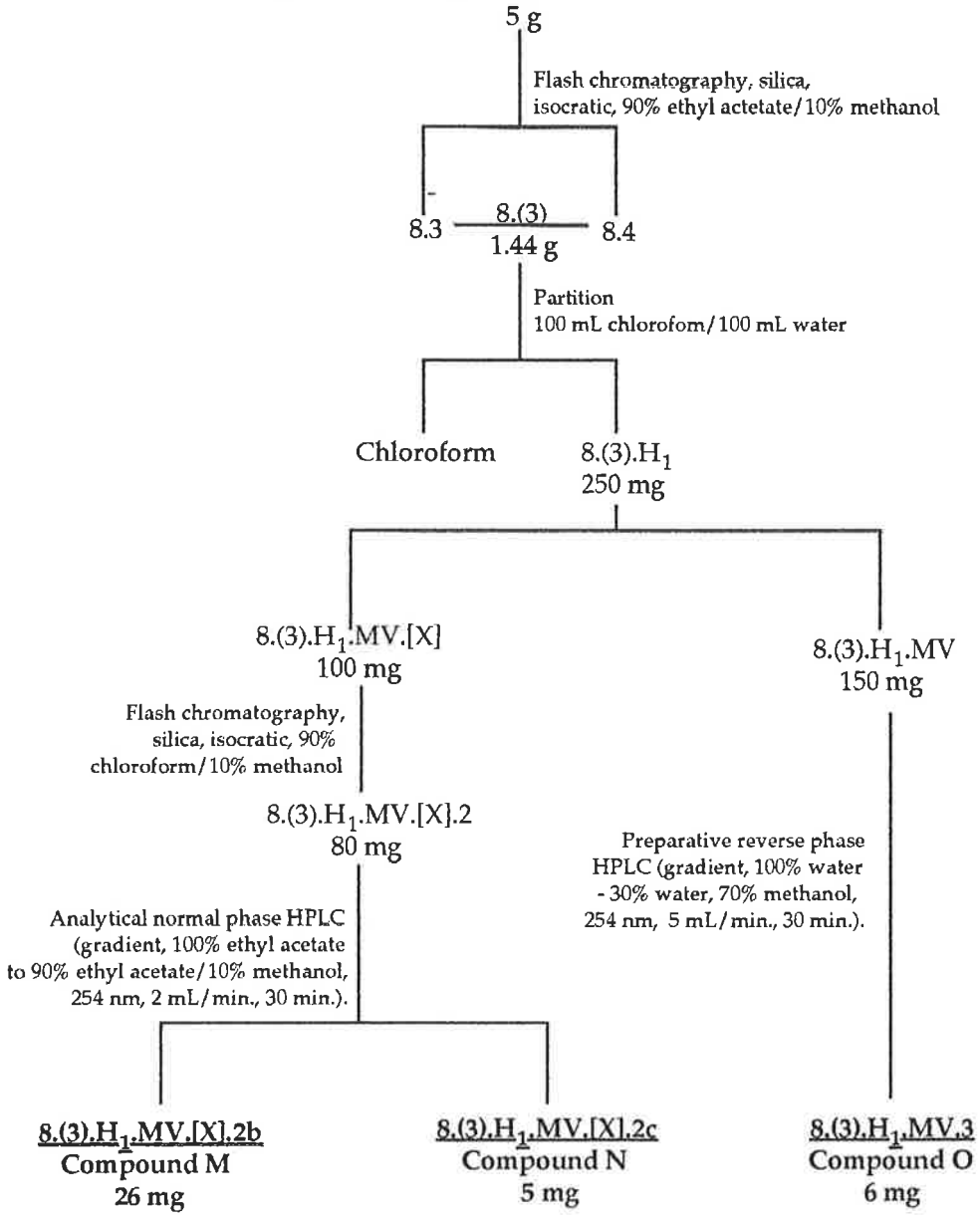
Fraction 8.(3).H_1.MV.[X].2 was then analysed by analytical normal phase HPLC (gradient, 100% ethyl acetate to 90% ethyl acetate/10% methanol, 254 nm, 2 mL/min., 30 min.) where two clearly defined, UV active components could be observed. The sample was then subjected to analytical normal phase HPLC (gradient, 100% ethyl acetate to 90% ethyl acetate/10% methanol, 254 nm, 2 mL/min., 30 min.) where two fractions were collected and shown to be pure.

Fraction 8.(3).H_1.MV.[X].2b was labelled Compound M (26 mg) and its purity determined using normal phase HPLC (R_f = 6.75 min., gradient, 100% ethyl acetate to 90% ethyl acetate/10% methanol, 254 nm, 2 mL/min., 30 min.) The structure determination of Compound M occurs in Chapter 9.2, pg 129.

Fraction 8.(3).H_1.MV.[X].2c was labelled Compound N (5 mg) and its purity determined by normal phase HPLC (R_f = 8.56 min., gradient, 100% ethyl acetate to 90% ethyl acetate/10% methanol, 254 nm, 2 mL/min., 30 min.). Its structure determination can be found in Chapter 9.3, pg 131.

Fraction 8.(3).H_1.MV (150 mg) was analysed by reverse phase analytical HPLC (gradient, 100% water to 30% water/70% methanol, 254 nm, 2 mL/min., 30 min.), where two peaks were observed. Preparative reverse phase HPLC (gradient, 100% water - 30% water/70% methanol, 254 nm, 5 mL/min., 30 min.) was then used to purify these compounds with the result that 6 mg of each compound was collected. One of these compounds was Compound O (6 mg) (see Chapter 9.3, pg 133 for the structure determination). The compound collected from the second peak was unstable and could not be identified. The compounds isolated by reverse phase HPLC were different to those isolated by normal phase HPLC. These conditions were used to selectively isolate different compounds.

Diagram 9.1: Separation of Fraction 8



9.2 Compound M

Compound M (26 mg) was isolated as yellow crystals (see Chapter 9.1, **Diagram 9.1**, pg 128) with a MP of 278 - 280°C and an $[\alpha]_D$ of + 1.3° (c 0.1, MeOH).

HRMS of the sample gave a $M+H^+$ of 477.1016 and therefore a molecular formula of $C_{22}H_{20}O_{12}$ (calculated = 477.1032, +/- 0.0016). This gave thirteen DBE to be associated with sp^2 hybridised carbons and ring systems. IR indicated the presence of carbonyls (1720 cm^{-1}), other sp^2 hybridised carbons (1660 cm^{-1}) and hydroxyl functional groups (3400 cm^{-1}).

Routine ^1H, COSY and ROESY NMR experiments were run. An HSQC experiment was used to determine all $^1J_{CH}$ correlations and an HMBC experiment was used to establish the connectivity of the compound.

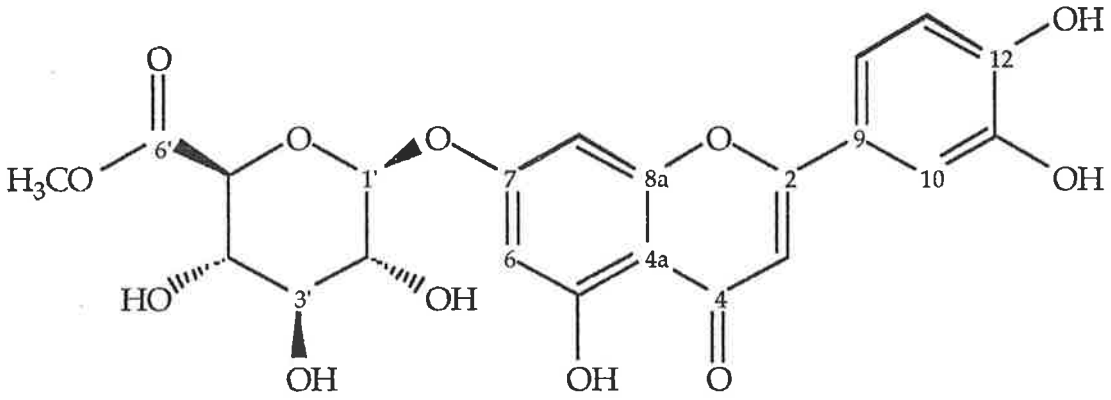
The protonated carbon signals account for twelve carbons and fourteen protons, indicating there are six protons associated with hydroxyl signals and ten quaternary carbons (as indicated by the molecular formula).

There were two carbonyl carbons and eight quaternary carbons that have ^{13}C chemical shift values that indicate they are sp^2 hybridised with some also directly attached to oxygen. The COSY spectrum indicated three separate spin systems, one of which was clearly β-glucuronic acid (coupling constants: 7.8, 9.0, 9.0, 9.6 Hz). The second COSY spin system involved a catechol type aromatic ring, while the third involved long range correlations in an aromatic ring.

The ^1H, ^{13}C, COSY, ROESY, HMQC and HMBC (see **Table 9.2.1**, pg 130) experiments confirmed Compound M to be luteolin-7-*O*-glucuronide methyl ester (**110**).[90] The numbering system is that used in the literature for flavonoids. It was not possible to find any MP or $[\alpha]_D$ literature values.

One article[91] discussed the isolation of methyl esters of flavone glycosides in *Digitalis lanata* that were methanol free extracted. The compounds were extracted with water, acetone and ethyl acetate. This supports the isolation of (**110**) as a true metabolite of *Scaevola spinescens*.

Luteolin-7-*O*-glucuronide methyl ester (**110**) has never been reported from *Scaevola spinescens* before or from any plants of the *Scaevola* genus.



(110)

Table 9.2.1: NMR data for (110)#

C	δ C	H	δ H	I.	M.	J (Hz)	COSY	ROESY*	HMBC
C_2	164.7								
C_3	103.2	H_3	6.72	1	s			H_{10}, H_{14}	C_2, C_4, C_{4a}, C_9
C_4	181.4								
C_{4a}	105.9								
C_5	161.8								
C_6	99.7	H_6	6.45	1	d	2.4	H_8	$H_{1'}$, $H_{2'}$, $H_{5'}$	C_{4a}, C_5, C_7
C_7	162.2								
C_8	94.2	H_8	6.80	1	d	2.4	H_6	$H_{1'}$, $H_{2'}$, $H_{5'}$	C_{4a}, C_6, C_7, C_{8a}
C_{8a}	156.0								
C_9	121.6								
C_{10}	113.2	H_{10}	7.39	1	d	1.2	H_{14}	H_3	C_2, C_9, C_{11}, C_{14}
C_{11}	145.5								
C_{12}	149.3								
C_{13}	115.5	H_{13}	6.87	1	d	8.4	H_{14}		C_9, C_{11}, C_{12}, C_{14}
C_{14}	119.2	H_{14}	7.42	1	dd	1.2, 8.4	H_{10}, H_{13}	H_3	C_2, C_{10}, C_{12}, C_{13}
$C_{1'}$	98.6	$H_{1'}$	5.31	1	d	7.8	$H_{2'}$	H_6, H_8	C_7, $C_{2'}$, $C_{5'}$
$C_{2'}$	73.4	$H_{2'}$	3.26	1	dd	7.8, 9.0	$H_{1'}$, $H_{3'}$	H_6, H_8	$C_{1'}$, $C_{3'}$
$C_{3'}$	74.7	$H_{3'}$	3.37	1	t	9.0	$H_{2'}$, $H_{4'}$		$C_{2'}$, $C_{4'}$, $C_{5'}$
$C_{4'}$	71.9	$H_{4'}$	3.40	1	dd	9.0, 9.6	$H_{3'}$, $H_{5'}$		$C_{3'}$, $C_{5'}$, $C_{6'}$
$C_{5'}$	75.2	$H_{5'}$	4.23	1	d	9.6	$H_{4'}$	H_6, H_8, OCH_3	$C_{1'}$, $C_{6'}$
$C_{6'}$	169.6								
$C_{7'}$	52.4	OCH_3	3.59	3	s			$H_{5'}$	$C_{6'}$

#600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in d_6-DMSO/D_2O

ROESY* = ROESY correlations not in the COSY spectrum

I. = Intergration M. = Multiplicity

9.3 Compound N

Compound N was isolated as an amorphous solid (see Chapter 9.1, **Diagram 9.1**, pg 128) with a MP of 82 - 89°C and an $[\alpha]_D$ of - 76° (c 0.1, MeOH).

It appeared as a yellow solid and was submitted for HRMS, where it gave a molecular weight of 179.0538 and therefore a molecular formula of $M+H^+$ of $C_6H_{11}O_6$ (calculated = 179.0555, +/- 0.0017). The molecular formula was therefore $C_6H_{10}O_6$. This gave two DBE.

Routine ^1H and ^{13}C NMR experiments were run. An HSQC experiment was used to establish the $^1J_{CH}$ relationships within the molecule. This data accounted for six carbons and six protons. One DBE was accounted for by the carbonyl carbon, C_1, with a ^{13}C chemical shift value of δ 175.9. The ^{13}C chemical shift values for the other carbons indicated a high degree of oxygenation, also indicated by the molecular formula. There was also a very broad singlet at δ 5.0 that was removed under D_2O exchange conditions, indicating the presence of hydroxyl protons.

A COSY experiment was conducted and it revealed two separate independent spin systems, with the coupling constants quite clearly defined. An HMQC experiment was used to establish $^1J_{CH}$ correlations, while an HMBC experiment was used to determine the connectivity of the compound.

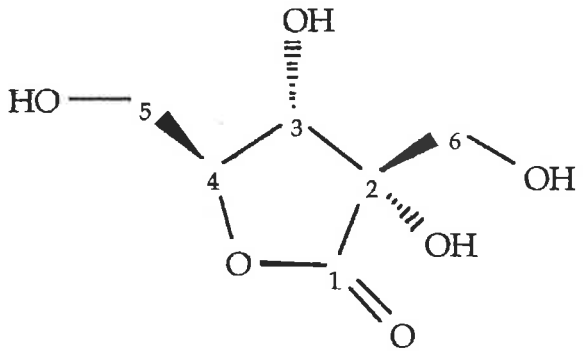
The NMR data is displayed in **Table 9.2.2**, pg 132. The structure is numbered according to that used in the literature for sugars.

Analysis of the NMR data revealed that Compound N was 2-C-(hydroxymethyl)-D-ribonic acid-γ-lactone (**111**) and this was supported by an extensive literature search.[92]

The MP and $[\alpha]_D$ values of 2-C-(hydroxymethyl)-D-ribonic acid-γ-lactone (**111**) also matched the literature values.[93]

There were four possible stereoisomers of the basic structure and a close analysis of the ^1H and ^{13}C chemical shift values and comparison with recorded literature values[92] of the possible isomers gave the structure with the relative stereochemistry as outlined in *Figure 9.2.1*

2-C-(Hydroxymethyl)-D-ribonic acid-γ-lactone (**111**) has not been isolated from *Scaevola spinescens* or any of the plant species found in the *Scaevola* genus.



(111)

Table 9.2.2: NMR data for (111)#

C	δ C	H	δ H	Int.	Mult.	J (Hz)	COSY	HMBC
C_1	175.9							
C_2	73.8							
C_3	67.9	H_3	4.07	1	d	8.4	H_4	C_4, C_5
C_4	83.4	H_4	4.11	1	ddd	2.4, 5.4, 8.4	H_3, H_{5a}, H_{5b}	C_3
C_5	60.2	H_{5a}	3.72	1	dd	2.4, 10.6	H_4, H_{5b}	C_3
		H_{5b}	3.44	1	dd	5.4, 10.6	H_4, H_{5a}	C_3, C_4
C_6	60.5	H_{6a}	3.40	1	d	10.2	H_{6b}	C_1, C_2, C_3
		H_{6b}	3.30	1	d	10.2	H_{6a}	C_1, C_2, C_3

#600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in d_6-DMSO/D_2O

Int. = Integration Mult. = Multiplicity

9.4 Compound Q

Compound Q (6 mg) was isolated as a white amorphous solid (see Chapter 9.1, **Diagram 9.1**, pg 128) with a MP of 131 - 134°C and an $[\alpha]_D$ + 20.0° (c 0.1, MeOH).

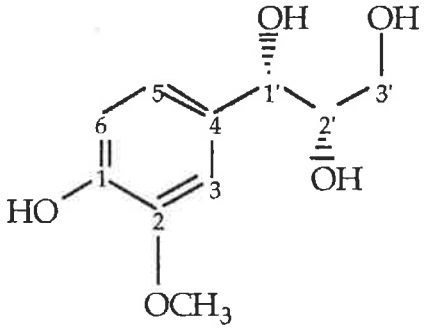
The molecular weight was determined by HRMS to be 214.0080 giving a molecular formula of $C_{10}H_{14}O_5$ (calculated = 214.0084,+/- 0.0004). This molecular formula reveals four DBE associated with either sp^2 hybridised carbons or a ring. The IR spectrum indicated hydroxyl functional groups (3450 cm^{-1}), as well as carbon-carbon bonds (1660 cm^{-1}).

A routine ^1H experiment was run and this revealed nine proton signals. The proton signals were assigned to their respective carbon by an HMQC experiment. There was one proton signal that did not have a $^1J_{CH}$ correlation, OH_1, with a ^1H chemical shift value of δ 8.503. This signal disappeared under D_2O exchange conditions. It was therefore identified as a phenolic proton.

There were two clear COSY spin systems observed in the COSY experiment. One was indicative of a 1,2,4 trisubstituted benzene ring, while the second was identified as a 1,2,3 propantriol substituent.

An HMQC experiment was completed to determine the ^{13}C chemical shift values of the protonated carbons and an HMBC experiment was used to establish the connectivity between COSY spin systems as well as to establish the ^{13}C chemical shift values for the non-protonated carbons. This was necessary as there was insufficient material to conduct a routine ^{13}C experiment. This data is displayed in **Table 9.4.1**, pg 134.

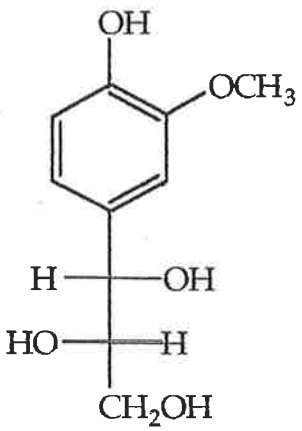
A ROESY experiment was also used to confirm the connectivity of Compound Q. Analysis of the NMR data (**Table 9.4.1**, pg 134) revealed the structure to be L-*threo*-guaiacyl glycerol (**112**).



(113)

There are four possible isomers with the general structure of guaiacyl glycerol. A comparison of the MP and $[\alpha]_D$ of Compound Q with all four isomers indicated that (**112**) was L-*threo*-guaiacyl glycerol.[94-96]

The Fischer Projection below is of L-*threo*-guaiacyl glycerol.[94]



(112)

L-*threo*-Guaiacyl glycerol has not been reported from *Scaevola spinescens* or from any plant of the *Scaevola* genera.

Table 9.4.1: NMR data for (112)#

C	δ C	H	δ H	I	M.	J (Hz)	COSY	ROESY	HMBC
C_1	147.3								
C_2	149.7								
C_3	110.4	H_3	6.919	1	d	1.8		OCH_3	C_1, C_2,C_4, C_5,$C_{1'}$
C_4	134.5								
C_5	121.0	H_5	6.760	1	dd	1.8, 7.8	H_2, H_6		C_1, C_3, $C_{1'}$
C_6	116.7	H_6	6.724	1	d	7.8	H_5		C_2, C_4
$C_{1'}$	75.7	$H_{1'}$	4.468	1	d	6.6	$H_{2'}$	H_5, OCH_3	C_3 C_4 C_5 $C_{2'}$ $C_{3'}$
$C_{2'}$	78.5	$H_{2'}$	3.613	1	ddd	3.0, 4.8, 6.6	$H_{1'}$, $H_{3'a}$,$H_{3'b}$		$C_{1'}$
$C_{3'}$	64.9	$H_{3'a}$	3.440	1	dd	3.0, 10.2	$H_{2'}$, $H_{3'b}$		$C_{1'}$
		$H_{3'b}$	3.314	1	dd	4.8, 10.2	$H_{2'}$, $H_{3'a}$		$C_{1'}$, $C_{2'}$
$C_{2''}$	56.0	OCH_3	3.850	3	s			H_2	C_2
		OH_1	8.503	1	br s				

#600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in d_6-DMSO, CD_3OD

I = Integration M. = Multiplicity

CHAPTER 10 FRACTION 9

10.1 Initial treatment

Treatment of Fraction 9 yielded two compounds, as outlined in **Diagram 10.1.**

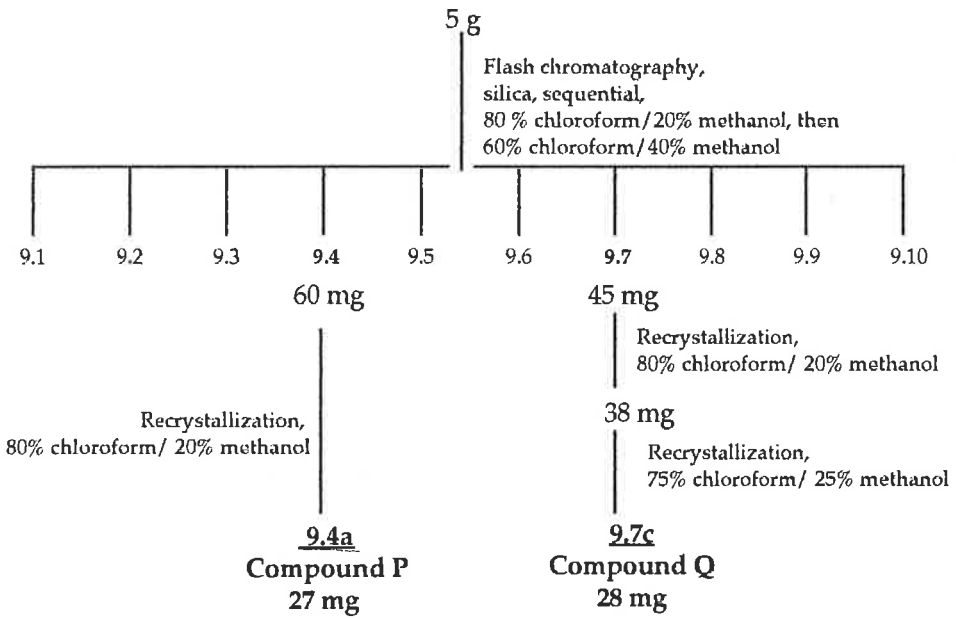
Fraction 9 (75 g) was separated from the original methanol fraction (828 g) using 75% ethyl acetate/25% methanol as the eluting solvent.

5 g of Fraction 9 was subjected to flash chromatography (silica, sequential, 80% chloroform/20 % methanol then 60% chloroform/40% methanol). Ten fractions in total were collected. TLC analysis indicated that both Fractions 9.4 (60 mg) and 9.7 (45 mg) were relatively pure. Both these fractions were independently dissolved in 80% chloroform/20% methanol and left overnight.

Fraction 9.4a crystallized out and was labelled Compound P (27 mg). Its purity was confirmed on analytical reverse phase HPLC (gradient, 100% water to 100% methanol, 254 nm, 1 mL/min., 30 min., R_f = 13.45 min.). Its structure determination is outlined in Chapter 10.2, pg 136.

Fraction 9.7c formed a white solid (38 mg) and was recrystallized using 75% chloroform /25% methanol. These crystals were labelled Compound Q (28 mg). The structure determination for Compound Q is given in Chapter 10.3, pg 138.

Diagram 10.1: Separation of Compounds P and Q



10.2 Compound P

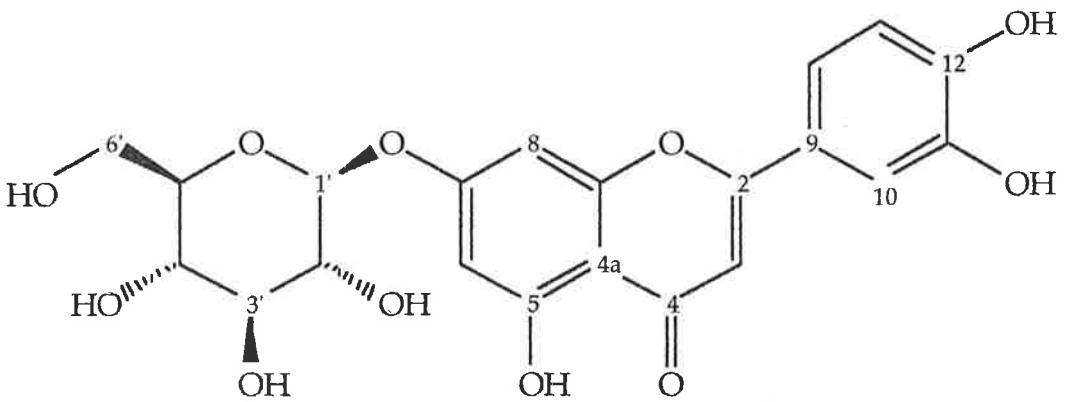
Compound P (27 mg) was isolated as yellow crystals (see Chapter 10.1, **Diagram 10.1**, pg 135) with a MP of 251 - 253°C and an $[\alpha]_D$ + 1.3° (c 0.1, MeOH).

Compound P had a molecular weight of 448, by negative ion electrospray mass spectrometry. Analysis of the ^1H and ^{13}C NMR data gave a molecular formula of $C_{21}H_{20}O_{11}$ and therefore seven DBE.

^1H; COSY, ROESY, ^{13}C, HMQC and HMBC NMR experiments were run and this data confirmed that Compound P was luteolin-7-*O*-glucoside (**113**). This NMR data is given in **Table 10.2.1**, pg 137. The ^1H and ^{13}C NMR data was compared to the literature values[74,90] and found to match. The MP and $[\alpha]_D$ values also matched those in the literature for luteolin-7-*O*-glucoside.[78,80]

The numbering system is that used in the literature for flavonoids.

This compound has not previously been isolated from *Scaevola spinescens* or any plant from the *Scaevola* genus. Luteolin-7-*O*-glucuronide methyl ester (**110**) was isolated from *Scaevola spinescens* during the course of this study and its isolation and structure determination is discussed in Chapter 9.2, pg 129. Other flavonoids have been isolated from species of *Scaevola* and these are discussed in Chapter 1.3.5, pg 27.



(113)

Table 10.2.1: NMR data for (113)#

C	δ C	H	δ H	I	M	J (Hz)	COSY	ROESY*	HMBC
C_2	164.4								
C_3	103.1	H_3	6.73	1	s			H_{10}, H_{14}	C_2, C_4, C_{4a}, C_9
C_4	181.8								
C_{4a}	105.2								
C_5	161.0								
		OH_5	12.97	1	s				
C_6	99.4	H_6	6.43	1	d	2.4	H_8	$H_{1'}$, $H_{2'}$, $H_{5'}$	C_{4a}, C_5, C_7
C_7	162.9								
C_8	94.6	H_8	6.77	1	d	2.4	H_6	$H_{1'}$, $H_{2'}$, $H_{5'}$	C_{4a}, C_6, C_7, C_{8a}
C_{8a}	156.8								
C_9	121.2								
C_{10}	113.5	H_{10}	7.40	1	d	2.4	H_{14}	H_3	C_2, C_9, C_{11}, C_{14}
C_{11}	145.7								
C_{12}	149.9								
C_{13}	115.9	H_{13}	6.88	1	d	8.4	H_{14}		C_9, C_{11}, C_{12}, C_{14}
C_{14}	119.1	H_{14}	7.43	1	dd	2.4, 8.4	H_{10}, H_{13}	H_3	C_2, C_{10}, C_{12}, C_{13}
$C_{1'}$	100.0	$H_{1'}$	5.06	1	d	7.2	$H_{2'}$	H_6, H_8	C_7, $C_{5'}$
$C_{2'}$	73.0	$H_{2'}$	3.25	1	dd	7.2, 9.6	$H_{1'}$, $H_{3'}$	H_6, H_8	$C_{1'}$, $C_{3'}$
$C_{3'}$	76.3	$H_{3'}$	3.29	1	dd	9.0, 9.6	$H_{2'}$, $H_{4'}$		$C_{2'}$, $C_{4'}$, $C_{5'}$
$C_{4'}$	69.5	$H_{4'}$	3.17	1	t	9.0	$H_{3'}$, $H_{5'}$		$C_{3'}$, $C_{5'}$, $C_{6'}$
$C_{5'}$	77.1	$H_{5'}$	3.44	1	ddd	5.4, 9.0, 9.6	$H_{4'}$, $H_{6'a}$, $H_{6'b}$	H_6, H_8	$C_{1'}$,$C_{3'}$,$C_{4'}$,$C_{6'}$
$C_{6'}$	60.5	$H_{6'a}$	3.71	1	dd	9.6, 11.4	$H_{5'}$, $H_{6'b}$		$C_{5'}$
		$H_{6'b}$	3.48	1	dd	5.4, 11.4	$H_{5'}$, $H_{6'a}$		$C_{4'}$

#600 MHz (^{1}H), 150 MHz (^{13}C): δ (ppm) from internal TMS in d_6-DMSO

I = Integration M = Multiplicity

ROESY* = ROE correlations not in the COSY spectrum

10.3 Compound Q

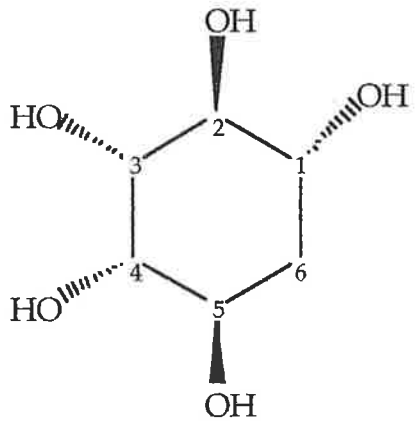
Compound Q (28 mg) was isolated as white crystals (see Chapter 10.1, **Diagram 10.1**, pg 135) with a MP of 234 - 239°C and an $[\alpha]_D$ + 28° (c 0.1, MeOH).

Positive ion electrospray gave a molecular weight of 164, and hence a molecular formula of $C_6H_{12}O_5$. This indicated that the molecule had one DBE. The IR indicated the presence of hydroxyl groups (3340 cm^{-1}).

Analysis of the ^1H, ^{13}C, COSY, HMQC and HMBC spectra, in particular the coupling constant data, as well as comparison with the ^{13}C NMR literature values,[97] allowed for the complete stereochemical assignment of Compound Q as 2-deoxy-D-*chiro*-inositol (**114**) (see **Table 10.3.1**, pg 139). This compound has never been reported from *Scaevola spinescens* or any plant from the *Scaevola* genera.

The MP and $[\alpha]_D$ values also matched the literature values for 2-deoxy-D-*chiro*-inositol.[78,98]

The numbering system is that commonly used in the literature for inositol.



(114)

The ^1H NMR spectrum indicated twelve proton signals, ten of which occurred with a ^1H chemical shift between δ 3.2 – 4.8, while the other two signals occurred at δ 1.69 and δ 1.59 (see **Table 10.3.1**, pg 139).

Protons OH_1 to OH_5 disappeared when a D_2O exchange experiment was conducted and are hydroxyl protons, considering their ^1H chemical shift values, the fact they are doublets with small $^nJ_{HH}$ coupling constants, and that they disappeared under D_2O exchange conditions. A COSY experiment was

undertaken, under normal conditions, and it was possible to observe the hydroxyl protons – OH_1 to OH_5 – correlating with other protons. The D_2O exchange spectrum allowed for the determination of all other $^1J_{HH}$ coupling constants.

Table 10.3.1: NMR data for (114)#

C	δ C	H	δ H	Int	Mult	J (Hz)	COSY	HMBC
C_1	68.3	H_1	3.65	1	**ddd**	4.3, 8.6, 10.7	H_2,$H_{6\alpha}$,$H_{6\beta}$, 1-OH	C_6
C_2	72.7	H_2	3.56	1	**dd**	8.6, 9.0	H_1, H_3, 2-OH	C_1
C_3	71.3	H_3	3.37	1	**dd**	3.0, 9.0	H_2, H_4, 3-OH	
C_4	74.9	H_4	3.26	1	**dd**	3.0, 3.6	H_3, H_5, 4-OH	C_3, C_5
C_5	68.8	H_5	3.41	1	**ddd**	2.6, 3.6, 4.3	H_4,$H_{6\alpha}$,$H_{6\beta}$, 5-OH	
C_6	34.8	$H_{6\beta}$	1.69	1	**ddd**	4.3, 4.3, 13.3	H_1, H_5, $H_{6\alpha}$	C_2, C_4
		$H_{6\alpha}$	1.55	1	**ddd**	2.6,10.7,13.3	H_1, H_5, $H_{6\beta}$	
		1-OH	4.68	1	d	3.0	H_1	C_1, C_2
		2-OH	4.45	1	d	3.6	H_2	C_1, C_2, C_3
		3-OH	4.20	1	d	6.0	H_3	C_2, C_3, C_4
		4-OH	4.37	1	d	4.2	H_4	C_3, C_4, C_5
		5-OH	4.31	1	d	4.8	H_5	C_4, C_5, C_6

ddd* = multiplet resolved to indicated multiplicity under D_2O exchange conditions

#600 MHz (^1H), 150 MHz (^{13}C): δ (ppm) from internal TMS in d_6-DMSO

Int = Integration Mult = Multiplicity

The full COSY spin system, including the hydroxyl protons, is illustrated in *Figure 10.3.1.*

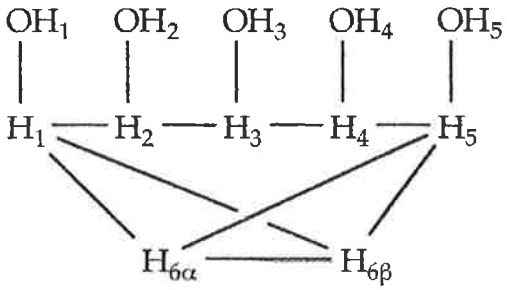


Figure 10.3.1: Complete COSY spin system for Compound Q

An HSQC experiment under normal conditions revealed six protonated carbons as outlined in **Table 10.3.1,** pg 139 (accounting for all carbon atoms indicated by the molecular formula). Five of the ^{13}C chemical shifts (δ 75 – δ 68) are consistent with carbon atoms directly attached to oxygen (in this case an hydroxyl functional group) while the sixth, with a ^{13}C chemical shift value of δ 34, was that of a methylene carbon. The HMQC correlations as well as the COSY spin system data

indicate the structure of an inositol (*Figure 10.3.2*), without the stereochemistry being assigned.

This accounts for the molecular formula of $C_6H_{12}O_5$, a molecular mass of 164, as well as the one DBE indicated by the molecular formula, associated with a ring.

An HMBC experiment was conducted and these correlations, displayed in **Table 10.3.1**, pg 139 confirm the general structure of (**114**) as shown in *Figure 10.3.2*.

Figure 10.3.3 illustrate the HMBC correlations that confirm the connectivity of all protons to their adjacent carbon atoms.

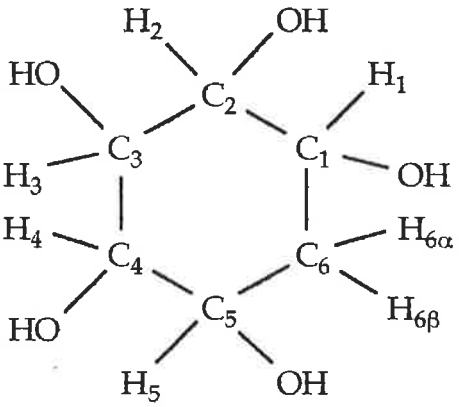


Figure 10.3.2: General structure of (**114**)

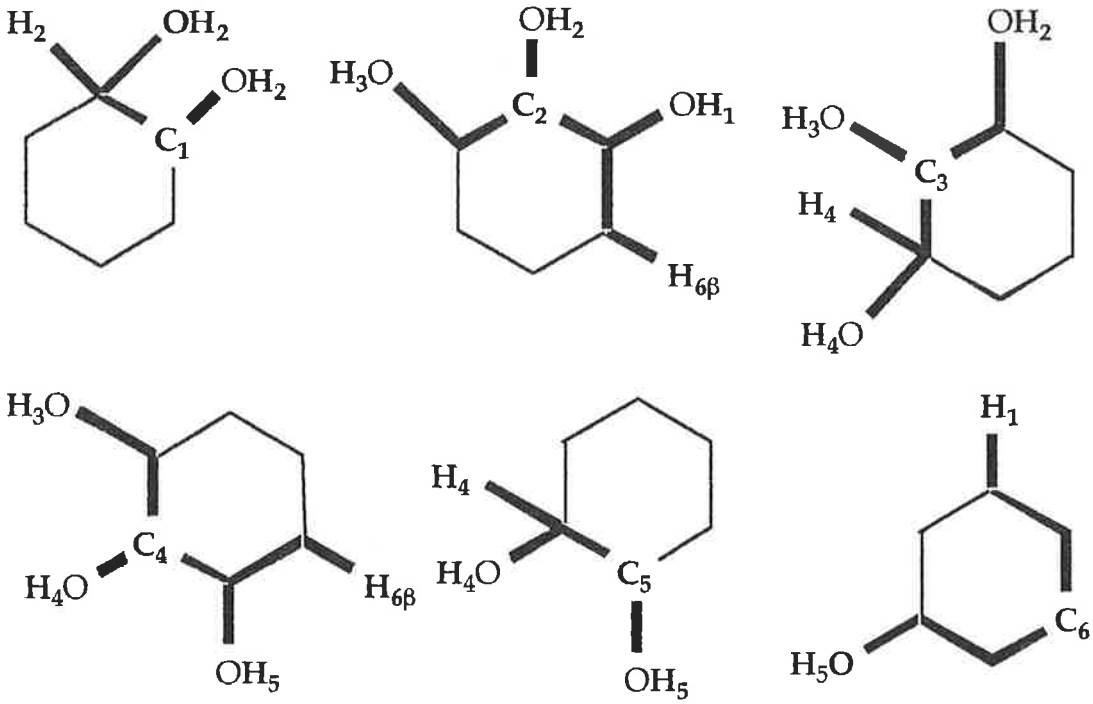


Figure 10.3.3: HMBC correlations

Stereochemistry

The coupling constants for the protons of (**114**) can be used to determine the relative stereochemistry.

Under normal conditions the multiplicities of the non-hydroxyl protons were difficult to determine. However, when run under D_2O exchange conditions and with the removal of the coupling between the hydroxyl protons and their adjacent protons, the multiplicities could be determined. They are given in **Table 10.3.1**, pg 139 and illustrated in *Figure 10.3.4*.

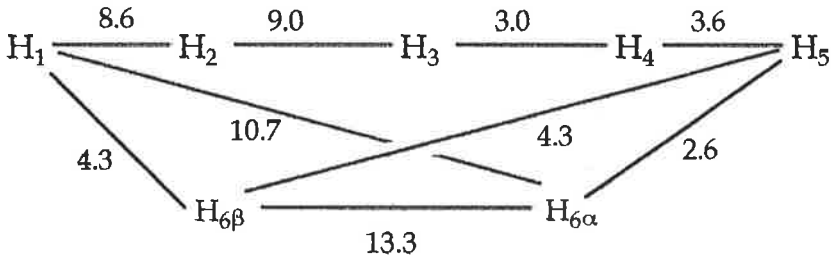


Figure 10.3.4: Coupling constants for (114)

The starting point for analysis of (**114**) is the most obvious coupling constant between $H_{6\alpha}$ and $H_{6\beta}$ of 13.3 Hz for this pair of geminal protons. It was then necessary to distinguish any axial-axial relationships.

H_1 has a 10.7 Hz coupling constant to $H_{6\alpha}$ while H_1 has one of 4.3 Hz to $H_{6\beta}$. Therefore $H_{6\alpha}$ is trans diaxial to H_1 and $H_{6\beta}$ must therefore be equatorial (*Figure 10.3.5*). It then follows that H_1 is trans diaxial with H_2 (8.6 Hz) while H_2 is trans diaxial with H_3 (9.0 Hz), as they possess large coupling constants (*Figure 10.3.6*).

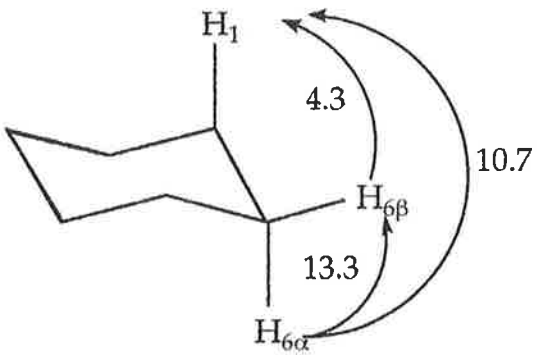


Figure 10.3.5: Coupling for $H_{6\alpha}$ and $H_{6\beta}$

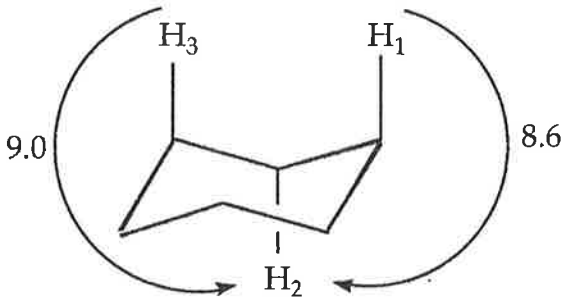


Figure 10.3.6: Coupling for H_2 H_3 and H_4

H_3 has a coupling constant of 3.0 Hz to H_4, indicating they are cis to each other (*Figure 10.3.7*). H_5 is the last proton to be assigned, either axial or equatorial. H_5 has a coupling of 2.6 Hz to $H_{6\alpha}$ and a coupling of 4.3 Hz to $H_{6\beta}$. This indicates that H_5 and $H_{6\alpha}$ are in an axial - equatorial relationship (the smaller of the two

coupling constants) with H_5 equatorial. This conformation is supported by the coupling constant between H_4 and H_5 that is 3.6 Hz (see *Figure 10.3.8*).

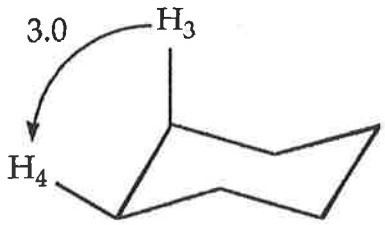


Figure 10.3.7: Coupling of H_4

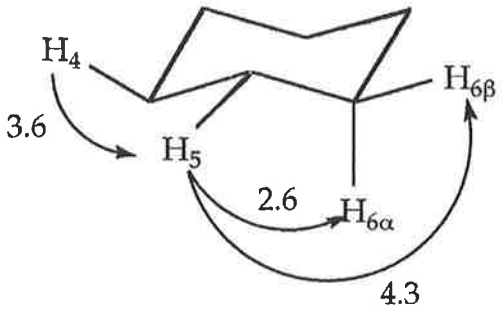


Figure 10.3.8: Coupling of H_5

Thus, the overall structure of (**114**) can be illustrated in two different representations that are shown in *Figure 10.3.9*.

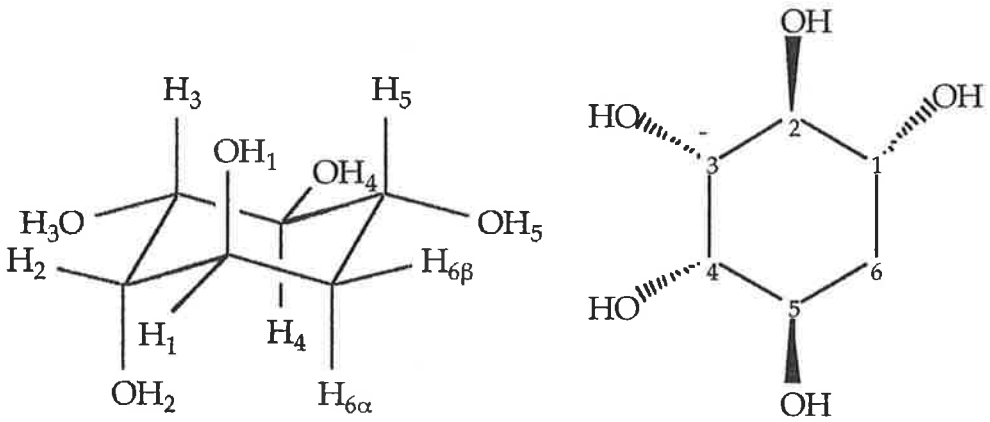


*Figure 10.3.9: Different representations of (**114**), indicating stereochemistry*

The relative stereochemistry has been unambiguously assigned using the available coupling constants. Therefore (**114**) is 134/25 cyclohexanepentol, also known as 2-deoxy-D-*chiro*-inositol.

This assignment was confirmed by an analysis of the ^{13}C chemical shift values for all ten possible isomers of inositol that have been reported.[97] These assignments were recorded in D_2O and were established using both isotopic labelling and, where the ^1H NMR signals were well separated, by single-frequency proton decoupling. The recorded ^{13}C chemical shift values for 134/25 cyclohexanepentol most closely fit those of (**114**). All other isomers have at least one ^{13}C chemical shift value that does not fit the recorded values for (**114**). As well, the MP and [α]D of (**114**) also match the values found in the literature.[78,98]

These confirmed that (**114**) can be identified as 134/25 cyclohexanepentol, also known as 2-deoxy-D-*chiro*-inositol).

CHAPTER 11 BIOLOGICAL TESTING OF *SCAEVOLA SPINESCENS*

A range of biological tests were conducted on crude extracts of *Scaevola spinescens* as well as on semi purified and purified compounds. These tests included anti bacterial, cytotoxic and anti viral testing. The experimental methods used are outlined in more detail in Chapter 13 - Experimental.

11.1 Anti bacterial testing

Anti bacterial testing was conducted on all crude fractions of *Scaevola spinescens*.

The crude fractions were tested against the following bacterial strains - *Staphylococcus aureus* (*S. aur*), *Staphylococcus epididemus* (*S. epi*), *Escherichia coli* (*E. coli*) and *Candida albicans* (*C. alb*), all of which were supplied by the School of Pharmacy, University of South Australia.

The methanol extract (828 g) was initially separated into ten fractions (see Chapter 3, Diagram 3.1.1). These crude fractions were labelled Fraction 1 to Fraction 10.

60 mg of Fraction 1 was dissolved in 1 mL of hexane. 15 μL of the solution was then pipetted separately onto twenty prepared filter discs and the solvent allowed to evaporate. The four bacterial strains were inoculated onto four separate plates and five of the prepared filter discs placed on the agar plate, as well as one disc that had been inoculated with water. The plates were incubated for two days and then analysed. Bacterial cultures grew on all plates, and the control discs had no inhibiting effect.

This procedure was repeated for all of the crude extracts, from Fraction 1 to Fraction 10, with the only difference being the type of solvent used to prepare the initial concentrations. 60 mg of Fraction 2 and Fraction 3 were dissolved in 90% hexane/10% ethyl acetate. 60 mg of Fractions 4 and 5 were dissolved in 90% chloroform/10% methanol while Fractions 6 to 10 were dissolved in methanol.

Only on the discs inoculated with Fractions 4, 8 and 9 were zones of inhibition observed and an average zone determined. None of the other fractions exhibited any inhibitory effect on the four bacterial strains. The above experiments for

Fractions 4, 8 and 9 were conducted in duplicate, with very similar results each time.

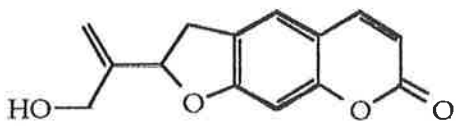
- Fraction 4 had an average zone of inhibition of 13 mm against *S. aur*, 13 mm against *S. epi* and 16 mm against *C. alb.*

- Fraction 8 also inhibited the growth of *S. aur* (with a zone of inhibition of 10 mm) as well as *S. epi* (7 mm).

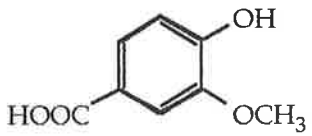
- Fraction 9 was effective against all bacterial cultures. The zones of inhibition were 7 mm (*S. aur*), 12 mm (*S. epi*), 10 mm (*E. coli*) and 6 mm (*C. alb*).

Fractions 4, 8 and 9 were subjected to a range of separation techniques (partition, flash chromatography, crystallization and HPLC as outlined in Chapters 6, 9 and 10).

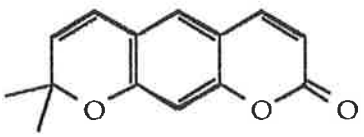
Two compounds were isolated from Fraction 4 - a novel coumarin, emmarin (**98**) as well as vanillic acid (**99**) (discussed in Chapter 6.2-6.3). The purified compounds would need to be tested to see if the anti bacterial activity is associated with these compounds. Coumarins were discussed in Chapter 1.3.3 and a number of coumarins previously reported from *Scaevola* species have shown anti bacterial activity, such as xanthyletin (**43**).[53b]



(**98**)



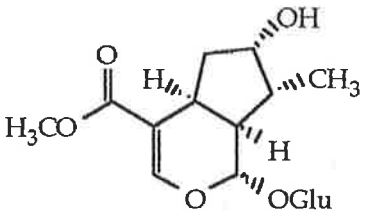
(**99**)



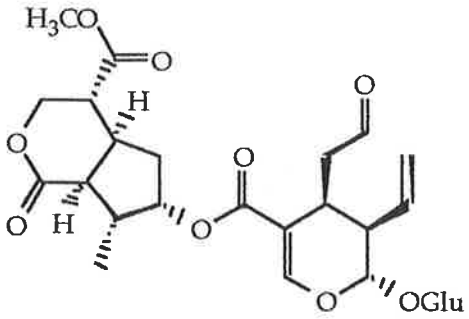
(**43**)

Four iridoids - loganin (**5**), scaevoloside (**6**), alidyjosioside (**108**) and katecateroside (**109**) were isolated from Fraction 8 (this is discussed in Chapter 8.2 - 8.5). The iridoid family was discussed in detail in Chapter 1.3.1.2. Some iridoids, such as aucubin (**11**) do exhibit remarkable anti bacterial activities, associated with the hemi-acetal structure of the iridoid aglycone. Isiguro (1986)[46] treated aucubin (**11**) with beta-glycosidase and found that the resultant aglycone was very active

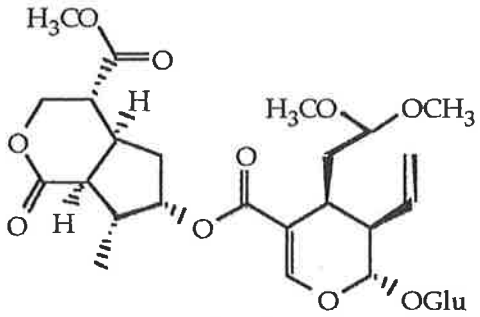
against *S. aur*. Elich (1962)[39] also found aucubin (**11**) to be very effective against *S. aur*.



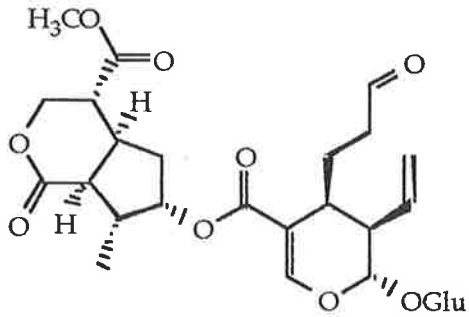
(**5**)



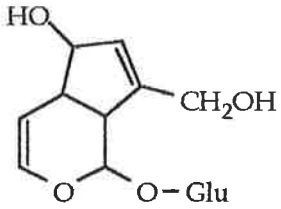
(**6**)



(**108**)



(**109**)



(**11**)

Glu = glucose

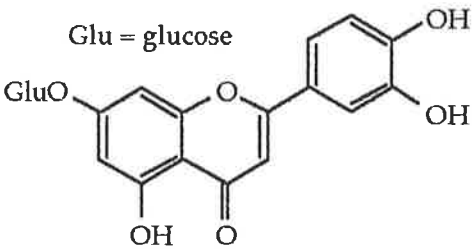
At this time of writing, the purified compounds from Fraction 8 have not been tested. It may also be worthwhile to test the iridoid aglycones.

Fraction 9 was separated by flash chromatography, into ten Fractions, labelled 9.1 - 9.10, and these were tested. Fraction 9.2 was very active against *S. aur* (zone of inhibition of 10 mm) and slightly active against *S. epi* (zone of inhibition of 2 mm). Fraction 9.3 and 9.10 were slightly active against *S. aur* (zone of inhibition of 2 mm).

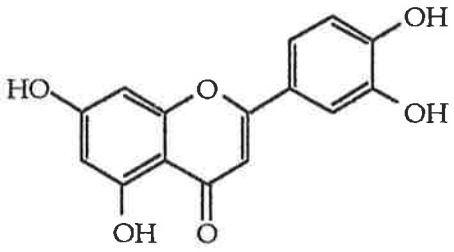
Fraction 9.4, from which luteolin-7-*O*-glucoside (**113**) crystallised out (See Chapter 9.2), had a zone of inhibition of 11 mm against *S. epi*. Luteolin (**115**) has been reported as having anti inflammatory and anti bacterial activities, although the

glycoside (**113**) does not appear to be active, except as a feeding attractant to beetles.[53b]

It was planned that all compounds that were purified and their structure determined would be individually tested using this technique, but that has not occurred to date.



(113)



(115)

11.2 Anti tumour activity

Four separate anti tumour testing procedures were conducted on various crude extracts of *Scaevola spinescens* with the assistance of Dr Robert Fowler and Dr Sue Semple of the School of Pharmacy and Medical Sciences, University of South Australia. The methods used are outlined in Chapter 13.

11.2.1 Cytotoxicity tests - Procedure A

The aim of this experiment was to provide a preliminary screen of the effect of a number of crude and partially purified extracts against three different cancer cell lines, supplied through the Pharmacy Department, University of South Australia.

The three tumour cell lines were:

A An adherent human melanoma cell line - MM418E, which secretes melanin (obtained from the Department of Pharmacy, University of South Australia).

B An adherent human bladder carcinoma cell line - 5637 (obtained from Dr D Rathjen, Department of Immunology, Women's & Children's Hospital, Adelaide).

C An adherent human breast adenocarcinoma cell line - MDA MB 231 (obtained from the Department of Pharmacy, University of South Australia).

All cell lines were incubated at 37°C and 5% CO_2. Cell lines A and B were kept growing in RMP1 1640 medium supplemented with 10% heat inactivated foetal calf serum, 2 mM/L-glutamine, 50 mg/mL penicillin/streptomyocin and 10^{-5} M 2-mercaptoethanol. Cell line C was kept growing in DMEM medium, supplemented with all of the above plus 50 mg/mL insulin.

All extracts were solubilised in either water, ethanol, methanol or DMSO, then the solvent removed and the material left was then weighed so as to produce an initial concentration of 100 mg/mL. This initial concentration then underwent serial dilution to determine the presence of any potential cytotoxicity.

Extracts 1 -7 were the crude extracts from the partition of the 12.7 kg of plant matter into hexane, ethyl acetate, methanol and water (see Chapter 3). Methanol extracts - Fractions 7 - 10 were obtained from the flash chromatography of 828 g of the methanol extract. The extracts tested were:

Extract 1	Crude H_2O extract
Extract 2	Methanol extract
Extract 3	Water extract
Extract 4	Crude ethyl acetate extract
Extract 5	Crude Hexane extract
Extract 6	Water extract (after partition with chloroform)
Extract 7	Chloroform
Extract 2.4	Methanol extract - Fraction 7
Extract 2.8	Methanol extract - Fraction 8
Extract 2.9	Methanol extract - Fraction 9
Extract 2.10	Methanol extract - Fraction 10

Controls for methanol, ethanol and DMSO were included to ensure any cytotoxicity was not due to the presence of these solvents.

Methods for preparation of cells, the cytotoxicity assay and plate preparation are outlined in Chapter 13.

Originally, three cell lines were to be investigated, but due to the slow growth of the MDA cells only limited results were available for that cell line.

Instead of the desired inhibitory/cytotoxic effect of the extracts, there was a massive stimulatory effect at higher dilutions (1/100 - 1/2700) for most of the

extracts against the 5637 bladder carcinoma cell line. This stimulation at high concentration was quite consistent throughout the initial seven extracts and so indicates that more than one compound is responsible. However, it is also possible that the stimulatory effect at high concentrations may be due to the fractions acting as nutrients.

This high stimulation of growth would appear to be masking any cytotoxic effects, especially as the more purified extracts (Fractions 7 to 10) show both cytotoxicity at low concentrations and stimulation at high concentrations. There was a clear stimulatory effect on MM418E (the melanoma cell line) which was only apparent in the aqueous extracts (extracts 1 and 6) and so any compounds that are causing the stimulation are only present in the polar fractions.

There was a stimulatory effect for the breast adenocarcinoma cell line (MDA MB 231) in extract 1 (the aqueous extract) and cytotoxicity associated with extract 2 - the methanol fraction. However, there was limited data for this cell line.

These results are difficult to explain and subsequent re-testing, using similar procedures, was not able to replicate these results.

11.2.2 Cytotoxicity tests - Procedure B

Two cell lines were obtained:

A Primary adherent bladder carcinoma cell line 5637 (ATCC HTB 9) (obtained from Dr D. Rathjen, Department of Immunology, Women's and Children's Hospital, Adelaide).

B Diploid human fibroblast cell line, human embryonic lung (HEL) cells (obtained from CSL, Parkville, Victoria). The HEL cells are normal, non-cancerous cells and are used as a control, to determine whether the extracts are cytotoxic to normal cells.

The cell lines were routinely cultured at 37°C, in 5% CO_2. Cell line 5637 was grown on RPMI 1640 medium supplemented with FCS, HEPES buffer (20 mM), L-glutamine (2 mM), 2-mercaptoethanol (10^{-5} M), gentamicin (16 μg/mL) and penicillin (12 μg/mL), while the HEL cells were grown in Dulbecco's modified eagle buffer (DMEM) supplemented with sodium bicarbonate (3.7 g/L); glucose (4.5 g/L); HEPES buffer (15 mM, CSL, Parkville, Victoria, Australia); FCS (10% v/v); glutamine (2 mM); gentamicin (16 μg/mL) and penicillin (12 μg/mL).

Three extracts were tested against the two tumour cell lines, the crude water extract and Fraction 4 and 9, due to their strong stimulatory effect, as indicated by the results of procedure A. A positive result in a cytotoxicity test would be indicated by death of cells at low concentration, not just at high concentrations. It is also necessary for the cytotoxicity to be associated with the cancerous cell line, not normal cells.

No clear cytotoxic effect could be observed at relevant concentrations of the crude extracts, nor was the stimulatory effect seen in Procedure A observed. The crude water fraction gave weak proliferation (131.9% at a concentration of 1372 μg/mL) of the 5637 cells. Crude Fraction 4 gave weak proliferation (121%, concentration of 4.116 μg/mL) against the HEL cells but otherwise the cytotoxicity of these fractions appeared to be small.

11.2.3 Cytotoxicity tests - Procedure C

The crude hexane, crude ethyl acetate extracts and Fractions 8, 9, 10 and 11 were retested against two cell lines:

A HEL cells - human embryonic lung cells, obtained from CSL (Parkville, Victoria).

B Caco-2 cells - colon cancer cell line (obtained through the Department of Pharmacy, University of South Australia).

There was no visible cytotoxic effect in any of the extracts tested.

3.2.4 Cytotoxicity tests - Procedure D

One of the references[1] to the use of *Scaevola spinescens* indicated that it was used in conjunction with *Codonocarpus cotinifolius*, although no proportion of each plant was given.

200 g of each plant was collected. *Scaevola spinescens* was collected from a site 10 km west of Morgan on the Morgan-Eudunda road. A sample was sent to the SA State Herbarium and verified. *Codonocarpus contifolius* was collected from 58 km north of Mannahill, on Weekeroo Station. A sample was sent to the SA Herbarium and verified.

10 g of *Scaevola spinescens* was boiled for 10 minutes in distilled water. The aqueous extract was then freeze dried, weighed and lyophilised. A second aqueous extract was prepared from 9 g of *Scaevola spinescens*/1 g *Codonocarpus cotinifolius*, with further extracts prepared according to the **Table 11.2.4.1**, pg 150.

The water was removed and then the extracts were dissolved in cell culture media to give an extract concentration of 1 mg/mL. Serial 1: 2 dilutions of each extract were prepared in a volume of 100 μL/well in a 96 - well microtiter plate. 100 μL of a cell suspension containing Hel cell lines were added to each well, to give a final volume of 200 μL/well. This procedure was repeated for Caco-2 cells, in separate plates. These plates were then incubated at 37°C for seven days, then cytotoxicity was assessed using the neutral red cytotoxicity assay.

Table 11.2.4.1: Extracts prepared from proportions of *Scaevola spinescens* and *Codonocarpus cotinifolius*

Extract	g *Scaevola spinescens*	g *Codonocarpus cotinifolius*
1	10	0
2	9	1
3	8	2
4	7	3
5	6	4
6	5	5
7	4	6
8	3	7
9	2	8
10	1	9
11	0	10

The two cell lines that were used to measure cytotoxicity were:

A HEL - low passage human embryonic lung (HEL) cells (obtained form CSL, Parkville, Victoria).

B Caco-2 - ATCC, HTB37 (obtained from the Pharmacy Department of the University of South Australia).

The HEL cells are normal, non-cancerous cells and are used as a control. The cell lines were grown in DMEM (Gibco) containing HEPES (20 mM), gentamicin (16 μg/mL), penicillin (12 μg/mL), glutamine (2 mM) and 10% heat inactivated FCS. All extracts followed the same general trend, with inhibition decreasing as the concentration of the samples decreased. There was very little difference between the HEL and Caco-2 result.

Overall, these cytotoxicity tests were inconclusive. Procedure A appeared to indicate that the crude aqueous extracts had a stimulatory effect on the growth of cancerous cells at high concentrations and inhibition at lower concentrations, but these results could not be repeated.

The purified compounds isolated from *Scaevola spinescens* will be tested for cytotoxicity in the future.

11.3 Anti viral testing

Six purified compounds were submitted for anti viral testing with the assistance of Dr Robert Fowler and Dr Sue Semple of the School of Pharmacy and Medical Sciences, University of South Australia. These compounds were:

Compound C Taraxerol
Compound E Ursolic acid
Compound F Emmarin
Compound I Alidyjosioside
Compound J Scaevoloside
Compound L Loganin

A maximum concentration of 50 μg/L of Compounds C, E, F, I and J was prepared. Compound L (due to the small quantity isolated) was tested at a maximum concentration of 25 μg/L.

The compounds were tested for anti viral activity against three viruses, representing three different virus families:

A Herpes simplex type 1 (HSV1, Herpesviridae).
B Ross River virus strain T48 (RRV, Togaviridae).
C Poliovirus type 2 (Picornaviridae).

All viruses were obtained from the Infectious Diseases Laboratory, Institute of Medical and Veterinary Sciences, Adelaide.

HSV1 and RRV were grown in Vero cells (African green monkey kidney) and poliovirus was grown in BGM (Buffalo green monkey kidney) cells. The anti viral assays are outlined in Chapter 13.

None of the compounds exhibited activity against any of the three viruses, at non-cytotoxic concentrations. The compounds were tested at a maximum concentration of 50 $\mu g/L$ in cell culture media in the anti viral assays (except for Compound L).

CHAPTER 12 SUMMARY

The following fourteen species of *Scaevola* have previously been investigated.

Scaevola spinescens	36, 38
Scaevola suavolens	42
Scaevola taccada *	42, 54, 55, 56, 61
Scaevola montana	43
Scaevola floribuna	45
Scaevola racemigera	51, 52
Scaevola chamissoniana	63
Scaevola coriacea	63
Scaevola gaudichaudiana	63
Scaevola gaudichaudii	63
Scaevola kilaueau	63
Scaevola mollis	63
Scaevola procera	63
Scaevola gaudichaudiana x mollis	63

(* *Scaevola taccada* is the preferred name[12] for *Scaevola plumieri*[42], *Scaevola frutescens* [54,56] and *Scaevola lobelia*[55]).

A total of ten iridoids, thirteen alkaloids, eight coumarins, thirty terpenoids, five essential oils and seven flavonoids have been previously isolated from these species. They have been identified by a range of spectroscopic techniques, ranging from MS Peak matching, identification by UV absorbance, to full structure determination by NMR experiments. The compounds that have been isolated previously were discussed in Chapter 1.

In this study, seventeen compounds have been isolated, purified and their structure determined by a number of spectroscopic methods.

Of these seventeen compounds four are novel and fifteen have never been reported from *Scaevola spinescens*. As well as this, thirteen compounds have never been reported from any species of *Scaevola*.

The occurrence of these compounds in *Scaevola spinescens* and in the family Goodeniacae is outlined in **Table 12.1**, pg 154.

The structure of all but one of the compounds (**95**) was determined using NMR experiments, often on amounts of sample of less than 3 mg. The structure of compound (**95**) was determined using MS peak matching only. Where the compound was known, a comprehensive analysis of NMR data, MP, $[\alpha]_D$, IR and UV spectra were undertaken and compared to previously reported literature values.

Table 12.1 : Compounds isolated from *Scaevola spinescens*

Compound	Novel	New to *Scaevola spinescens*	New to *Scaevola* genera
Hexadecanoic acid, methyl ester (**95**)		*	*
Taraxerol acetate (**92**)			
Taraxerol (**73**)			
Judarrylol (**96**)	*	*	*
Ursolic acid (**97**)	-	*	*
Emmarin (**98**)	*	*	*
Vanillic acid (**99**)		*	*
Daucesterol (**100**)		*	*
Alidyjosioside (**108**)	*	*	*
Scaevoloside (**6**)		*	
Katecateroside (**109**)	*	*	*
Loganin (**5**)		*	
Luteolin-7-*O*-glucuronide methyl ester (**110**)		*	*
2-C-(Hydroxymethyl)-D-ribonic acid-γ-lactone (**111**)		*	*
L-*threo*-Guaiacyl glycerol (**112**)		*	*
Luteolin-7-*O*-glucoside (**113**)		*	*
2-Deoxy-D-*chiro*-inositol (**114**)		*	*

COMPOUND A : Hexadecanoic acid, methyl ester (95)

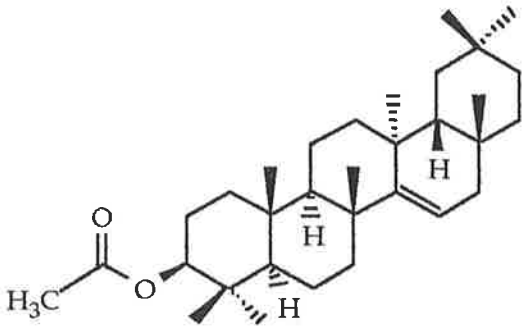
(**95**)

Hexadecanoic acid, methyl ester (**95**) is the methyl ester of palmitic acid (**50**). Palmitic acid (**50**), oleic acid (**51**) and linoleic acid (**52**) have previously been

reported from species of the *Scaevola* genus.[36,61] The occurrence of essential oils and hydrocarbons in *Scaevola* species is discussed in Chapter 1, 1.3.4, pg 26.

Hexadecanoic acid, methyl ester (**95**) was identified by comparison and analysis of its MS spectrum. The discussion of its structure determination can be found in Chapter 4, 4.2, pg 54. This compound has not been reported from *Scaevola spinescens* or any other species of *Scaevola* before.

COMPOUND B : Taraxerol acetate (92)

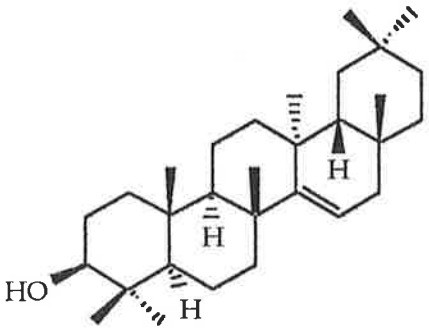


(**92**)

Taraxerol acetate (**92**) has been reported previously from *Scaevola spinescens*.[35] In total, thirty terpenoids have been isolated from *Scaevola spinescens, Scaevola taccada* and *Scaevola floribunda*.[35,36,38,45,55,56] The occurrence of terpenoid compounds isolated from *Scaevola* is discussed in Chapter 1, 1.3.6, pg 31.

The structure of taraxerol acetate (**92**) was determined by both MS comparisons and library searching of the MS data as well as by comparisons of the recorded ^{1}H and ^{13}C NMR data. The description of its structure determination can be found in Chapter 4, 4.3, pg 56.

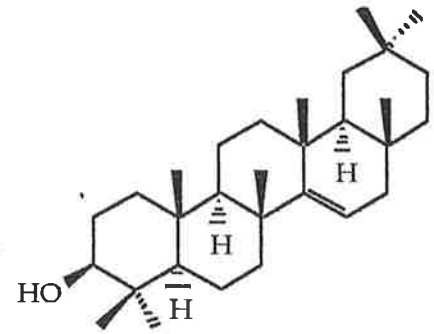
COMPOUND C : Taraxerol (73)



(**73**)

Taraxerol (73) has also been described previously from both *Scaevola spinecsens* and *Scaevola taccada*.[35,36,56] It is a very common plant metabolite. It was isolated from the non-polar fractions as a white crystalline solid. Its structure was elucidated from its MP, a very close library match of its MS spectrum and finally by analysis and comparison of its ^{1}H and ^{13}C NMR data (using routine ^{1}H and ^{13}C NMR experiments as well as COSY, HMQC and HMBC experiments) to literature values for taraxerol (73). The structure determination of taraxerol (73) is discussed in Chapter 4, 4.4, pg 59.

COMPOUND D : Judarrylol (96)



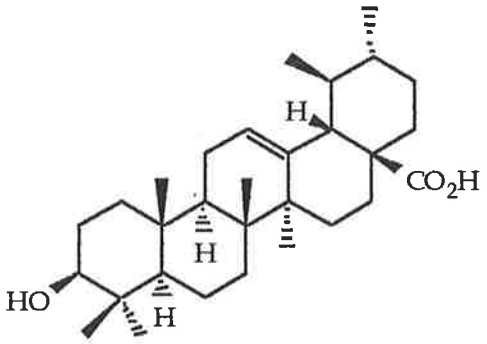
(96)

Judarrylol (96) is a novel compound.

It belongs in the terpenoid class (see Chapter 1.3.6, pg 31 for terpenoids isolated from *Scaevola* species).

It had a similar MS spectrum to taraxerol (73) (some differences were noted), but a significantly different MP (190 - 193°C for judarrylol against 282-283°C for taraxerol) and was chromatographically distinct when co-spotted with taraxerol (73) on TLC. It was therefore considered a stereoisomer of taraxerol (73). A complete proton and carbon assignment was possible using a wide range of NMR experiments to give the novel compound above. The complete relative stereochemistry was determined, using coupling constants and ROESY data. The structure determination of judarrylol (96) is discussed in Chapter 4, 4.5, pg 61.

COMPOUND E : Ursolic acid (97)



(97)

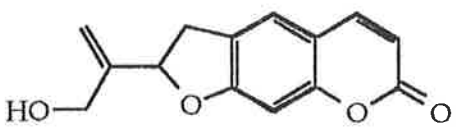
Ursolic acid (**97**) was isolated from Fraction 3.

Ursolic acid (**97**) is also known as α-amyrinic acid. Its structure determination was by comparison of its MP, $[\alpha]_D$ and MS spectrum as well as a comparison of both the ^1H and ^{13}C chemical shift values with those of the known compound. COSY, HMQC and HMBC data were very useful in determining multiplicity of carbons and to make connectivity.

The ROESY data was used to unambiguously assign the stereochemistry of all ring junctions. This compound has not been reported before from any species of *Scaevola*, although α-amyrin (**74**), γ-amyrin acetate (**75**) and γ-amyrone (**76**)[56] have been has been reported from *Scaevola taccada* while ursolic acid acetate (**78**) has been reported from *Scaevola floribunda*.[45]

The structure determination of ursolic acid (**97**) is discussed in Chapter 5, 5.2, pg 71.

COMPOUND F : Emmarin (98)



(98)

Emmarin is a novel coumarin.

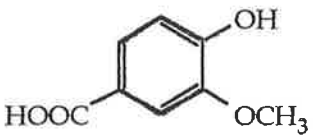
A total of eight coumarins have been reported previously from two *Scaevola*

species (*Scaevola taccada* and *Scaevola spinescens*).[36,38,54,55,56] The coumarins that have been isolated previously are discussed in Chapter 1, 1.3.3, pg 23.

The structure of emmarin (**98**) was elucidated using routine ^{1}H and ^{13}C NMR experiments as well as COSY, HMQC and HMBC experiments. One interesting problem was that initially it was possible from the data to formulate either a three, five or seven membered ring. A routine ^{1}H NMR experiment in dry d_6-DMSO gave the hydroxyl proton as a triplet. Therefore the hydroxyl functional group was attached to a methylene carbon. This enabled the construction of the dihydrofuran ring observed in the above structure.

The data and discussion for the assignment of emmarin (**98**) can be found in Chapter 6, 6.2, pg 77. The structure of emmarin is very similar to furanocoumarins previously isolated from species of the *Scaevola* genera.

COMPOUND G : Vanillic acid (99).

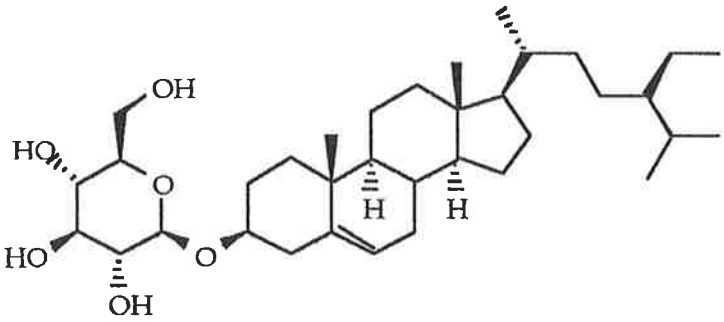


(**99**)

The common name for this compound is vanillic acid (**99**) and its systematic name is 3-methoxy-4-hydroxybenzoic acid.

This compound was isolated by normal phase HPLC. Its structure was determined using standard NMR techniques. Although a simple compound, it was difficult to fully characterise. The substitution pattern was established by a combination of analysis of the MS spectrum and nOe data. This compound has not been reported from either *Scaevola spinescens* or any other species of *Scaevola* before. The structure determination of this compound is discussed in Chapter 6, 6.3, pg 88.

COMPOUND H : Daucesterol (100)

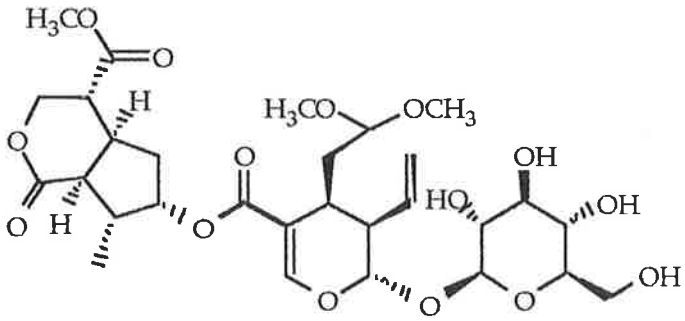


(100)

Daucesterol is glycosylated β-sitosterol. It was isolated as crystals. Its structure was determined by a close examination of routine ^{1}H and ^{13}C NMR spectra as well as COSY, ROESY, HMQC and HMBC correlations. The stereochemistry of all ring junctions was established using ROESY data, although the stereochemistry of the other chiral centres was not established. However, comparison of the MP and $[\alpha]_D$ of this sample to recorded values for daucesterol (**100**)[80] were identical.

Daucesterol has not been isolated previously from *Scaevola spinescens* or any other *Scaevola* species. β-Sitosterol (**94**)[36] itself has been reported from *Scaevola spinescens*.[36] This compound is discussed in Chapter 7, 7.2, pg 91.

COMPOUND I : Alidyjosioside (108)



(108)

Alidyjosioside (108) is a novel compound.

It is an iridoid. Ten iridoids have been reported from five species of *Scaevola*.[41,42,43,44,45] The occurrence of iridoids in *Scaevola* is outlined in Chapter 1, 1.3.1, pg 13.

It was isolated from the more polar fractions by extensive chromatography, including flash chromatography, normal phase HPLC and reverse phase HPLC. The full structure was determined using ^{1}H and ^{13}C experiments (including DEPT). A COSY experiment established three independent spin systems. $^{1}J_{CH}$ correlations were established using an HMQC experiment and three part structures were established. A $^{2/3}J_{CH}$ experiment was used to establish connectivity between the three part structures.

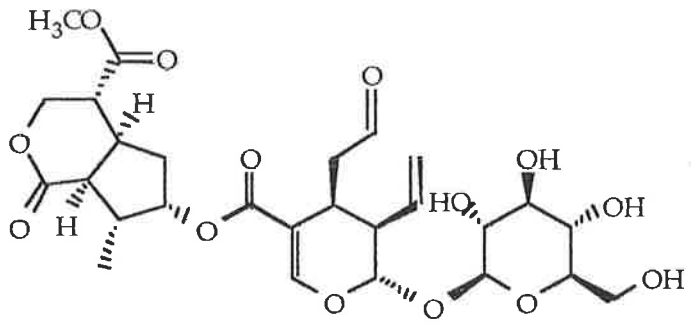
Stereochemistry was established using both NOESY and ROESY experiments. This data and arguments for the stereochemical assignments put forward are presented in Chapter 8, 8.2, pg 95.

Two conformers of the ring to which the carboxymethyl group is attached were observed in the ROESY and NOESY experiments.

This compound is very similar to scaevoloside (**6**), reported previously from *Scaevola racemigera*.[44] There are two differences. Firstly, the aldehyde present in scaevoloside (**6**) is here presented as the dimethyl acetal. Secondly, the stereochemistry around one chiral carbon (the attachment of the carboxymethyl group) is different to that reported.[44]

It is possible that alidyjosioside (**108**) is an artefact of the extraction process. It is a novel compound, nonetheless.

COMPOUND J : Scaevoloside (6)



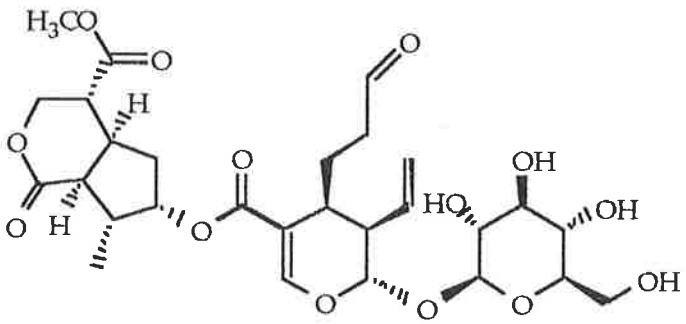
(**6**)

Scaevoloside (**6**) is an iridoid (see Chapter 1, 1.3.1, pg 13). It has been isolated from *Scaevola racemigera*[44] but not from *Scaevola spinescens*.

The structure of scaevoloside (**6**) was established using a range of NMR spectroscopic techniques as well as by comparison with the reported data.[44] The stereochemistry of all chiral carbons was assigned using nOe and ROESY correlations from ROESY and NOESY experiments. The data for scaevoloside (**6**) is presented in Chapter 8.3, pg 118.

Analysis of the ROESY data has lead to a revision of the stereochemistry of scaevoloside (**6**) as proposed by Skaltsounis[44] with the carboxymethyl group on the same side of the ring as the ring junction protons. The discussion of the revision of the stereochemistry is found in Chapter 8.2, pgs 108 - 117.

COMPOUND K : Katecateroside (109)



(109)

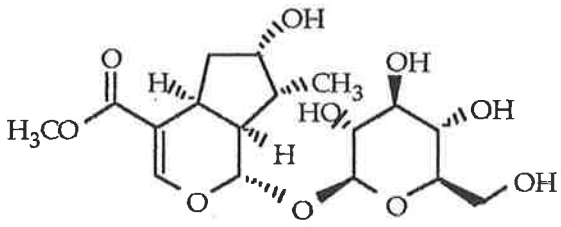
Katecateroside is a novel compound.

It is an iridoid that is very similar to alidyjosioside (**108**) and scaevoloside (**6**) (as outlined in Chapter 8, 8.2 and 8.3, pgs 95, 118). The routine ^{1}H and ^{13}C NMR experiments gave data that was almost identical to that of (**108**) and (**6**). The molecular formula indicated that it had one extra methylene group.

It had an aldehyde and only one methoxy functional group. The important COSY correlations established the extra methylene group adjacent to the aldehyde functional group. This positioning was supported by the data obtained by a ROESY experiment. The stereochemistry, established by ROE data, was identical to both alidyjosioside (**108**) and the revised scaevoloside (**6**).

Therefore this compound is novel, and has been named katecateroside (**109**). Its structure determination is outlined in Chapter 8, 8.4, 122.

COMPOUND L : Loganin (5)



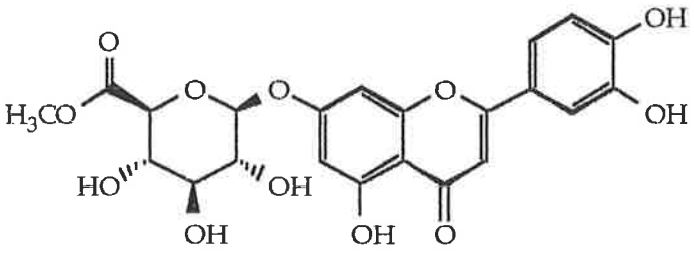
(5)

Loganin is an iridoid that has been found in many different species of plants and has been reported from *Scaevola montana*[43] and *Scaevola racemigera*[44]. Loganic acid (**4**), the demethylated form of loganin (**5**) has been reported by personal communication[42] as having been isolated from *Scaevola taccada* although this data has not been published.

The structure was determined by ^{1}H, ^{13}C, HSQC and HMBC NMR experiments and the data obtained closely matched the established ^{1}H and ^{13}C chemical shift values. The stereochemistry was determined using NOESY and ROESY experiments as well as observed coupling constants and was found to be identical to the recorded stereochemistry of loganin (**5**).

The discussion of the isolation, purification and structure determination of loganin (**5**) is given in Chapter 8, 8.5, pg 125.

COMPOUND M : Luteolin-7-*O*-glucuronide methyl ester (110)



(110)

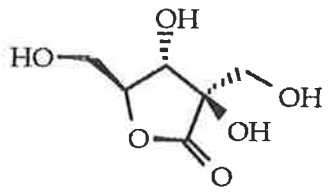
Luteolin-7-*O*-glucuronide methyl ester (**110**) was isolated from the more polar extracts and its structure determined using NMR experiments.

Seven flavonoids have previously been reported from eight species of *Scaevola* found on the Hawaiian islands. These flavonoids were identified by their characteristic UV chromophore (see Chapter 1, 1.3.5, pg 27)

Luteolin-7-*O*-glucuronide methyl ester (**110**) has not been isolated from any species of *Scaevola* before. There have been examples of flavonoid glucuronide methyl esters being isolated in the absence of methanol extraction,[91] so that (**110**) is a true metabolite of *Scaevola spinescens*.

Its structure was determined using NMR techniques. This is discussed in Chapter 9, 9.2, pg 129.

COMPOUND N : 2-C-(Hydroxymethyl)-D-ribonic acid-γ-lactone (111)



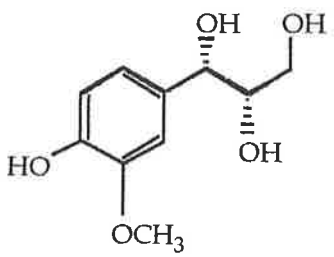
(111)

2-C-(Hydroxymethyl)-D-ribonic acid-γ-lactone (**111**) was isolated from the more polar extracts, using flash chromatography. It is an example of a simple sugar and its full structure, with relative and absolute stereochemistry, was determined using a range of NMR experiments (discussed in Chapter 9, 9.3, pg 131) as well as comparison of MP and $[\alpha]_D$ to literature values.

A detailed analysis of the ^1H and ^{13}C chemical shift data of the four possible stereoisomers with this structure was undertaken as well as comparison with the recorded MP and $[\alpha]_D$[93] and this led to the identification of (**111**).

2-C-(Hydroxymethyl)-D-ribonic acid-γ-lactone (**111**) has not been isolated from *Scaevola* species before.

COMPOUND O :L-*threo*-Guaiacyl glycerol (112)



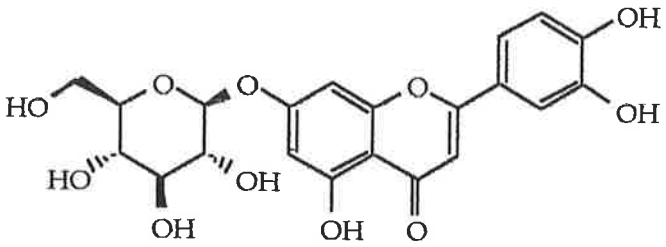
(112)

L-*threo*-Guaiacyl glycerol was isolated by reverse phase HPLC of the more polar extracts.

Its structure was determined using routine NMR experiments. The absolute stereochemistry of the chiral carbons was determined by comparison with the MP and $[\alpha]_D$ of the literature values for all four isomers.[94-96]

L-*threo*-Guaiacyl glycerol has not been previously isolated from *Scaevola spinescens* or from any species of *Scaevola*. The structure determination of L-*threo*-guaiacyl glycerol (**112**) is discussed in Chapter 9, 9.4, pg 133.

COMPOUND P : Luteolin-7-*O*-glucoside (113)

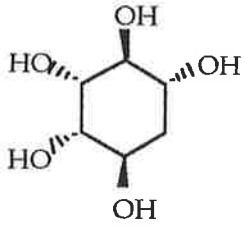


(113)

Luteolin-7-*O*-glucoside (**113**) was isolated as crystals (80% chloroform/20% methanol) from the more polar fractions. Its structure was determined using NMR experiments. A comparison of all NMR data confirmed the structure. This is discussed in Chapter 10, 10.2, pg 136.

Luteolin-7-*O*-glucoside (**113**) has not been isolated from any species of the *Scaevola* genus. However luteolin-7-*O*-glucuronide methyl ester (**110**) was also isolated from the more polar extracts of *Scaevola spinescens* in this study (see Chapter 9, 9.2, pg 129).

COMPOUND Q : 2-Deoxy-D-*chiro*-inositol (114)



(114)

This compound crystallised out of solution (75% chloroform/25% methanol) from the more polar extracts. Its structure was determined by NMR spectroscopy and by a comparison of recorded ^{13}C values of cyclohexanepentols.[97]

The relative stereochemistry of each chiral carbon was determined by a combination of coupling constants and nOe correlations. The MP and $[\alpha]_D$ of (**114**) matched the literature values for (**114**).[78,98] This compound is discussed in Chapter 10, 10.3. pg 138.

Testing for the presence of Alkaloids.

All crude fractions were tested with Meyer's reagent (testing for the presence of alkaloids) with negative results.

Biological testing.

A range of biological tests were carried out on both crude extracts, semi - purified fractions and certain isolated and purified compounds, as outlined in Chapter 11, pg 143.

Crude Fractions 4, 8 and 9 showed moderate anti bacterial activity. One novel coumarin - emmarin (**98**) - and vanillic acid (**99**) were isolated from Fraction 4.

The four iridoids - loganin (**5**), scaevoloside (**6**), alidyjosioside (**108**) and katecateroside (**109**) - were isolated from Fraction 8.

Luteolin-7-*O*-glucuronide methyl ester (**110**), 2-C-(hydroxymethyl)-D-ribonic acid-γ-lactone (**111**) and L-*threo*-guaiacyl glycerol (**112**) were isolated from Fraction 9. These compounds need to be tested to determine whether they have anti bacterial activity.

The initial cytotoxicity test used - Procedure A - indicated a massive stimulatory effect at high concentrations of the more polar extracts on three cancer cell lines, while at lower concentrations there was some inhibition of growth. However these results could not be reproduced. Other cytotoxicity tests were inconclusive.

The anti viral tests showed no activity.

CHAPTER 13 EXPERIMENTAL

13.1 General procedures

Analytical TLC was performed using Merck Kieselgel 60 F254 silica on aluminium backing. Preparative TLC was performed using 20 cm X 20 cm Merck Kieselgel 60 F254 silica on glass (1 mm thickness), while preparative chromatography was performed using positive pressure flash and squat chromatography with Merck Kieselgel 60 (230-400 mesh ASTM).

HPLC was carried out using a Waters 6000A solvent pump, a Waters U6K Injector, a Waters model 441 absorbance detector operating at different wavelengths, in conjunction with an ICI DP-700 database.

Analytical HPLC was performed using a Waters Nova-Pak HR Silica column (100 x 8 mm, 10 μm) or a Waters Nova-Pak HR C18 column (100 x 8 mm, 10 μm) with either isocratic or gradient conditions. All solvents were analytical grade and pre filtered twice.

Preparative HPLC was performed using Waters Nova-Pak HR Silica column (100 x 25 mm, 10 μm) or a Waters Nova-Pak HR C18 column (100 x 25 mm, 10 μm) with either isocratic or gradient conditions using a range of solvents.

NMR spectra were recorded on either a Bruker ACP (300 MHz ^{1}H), a Varian Gemini 2000 (300 MHz ^{1}H) or a Varian INOVA (600 MHz ^{1}H) spectrometer. A number of different pulse sequences were used, including routine ^{1}H, ^{13}C and DEPT sequences, COSY, HMQC, HSQC, HMBC, HSQC-TOCSY, NOESY and ROESY sequences. 2D Spectra acquired at higher field (600 MHz) used gradient coherence selection for COSY, HMQC, HSQC, HSQC-TOCSY and HMBC.

Spectra were recorded in either $CDCl_3$ (TMS-internal standard), d_6-DMSO, d_4-methanol or D_2O (for D_2O exchange experiments).

Chemical shift values were recorded as parts per million (ppm) downfield from the internal standard. Multiplicities are abbreviated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; dd, doublet of doublets, etc.

Mass spectral analysis was conducted at the Central Science Laboratory, Tasmania, where MS and HRMS were recorded using a range of experiments (EI, LSIMS, CI, ESI).

IR spectra were recorded on an ATI Mattson, Genesis series FTIR instrument. UV spectra were recorded on a PYE UNICAM SP8-100 ultraviolet spectrometer.

MP were determined on a Kofler hot-stage apparatus equipped with a Reichart microscope and are uncorrected.

Optical rotations were recorded on a POLA AR 21 polarimeter set at 589 nm, using a 0.15 dm cell.

All solvents were distilled before use, with drying and purification of solvents and reagents performed using standard laboratory procedures.[68, 69]

Visualisation of TLC plates was achieved by use of UV light in addition to the use of ammonium molybdate, vanillin, Meyers reagent or I_2 vapour.

13.2 Plant collection, verification and extraction

12.7 kg of *Scaevola spinescens* was collected from a site 10 km west of Morgan, in South Australia, on the Morgan-Eudunda Road. A sample was sent to the South Australian State Herbarium and verified as *Scaevola spinescens* (Voucher No. AD 99702040).

The 12.7 kg was ground into a powder using a ball mill and then soaked in 40 L of solvent for 2 weeks. The solvents used were hexane, ethyl acetate, methanol and distilled water. The solvents were then removed, using a cyclone evaporator and the hexane, ethyl acetate and aqueous fractions stored under nitrogen.

A total of 828 g of the methanol extract was then subjected to flash chromatography (silica, sequential, 90% hexane/10% ethyl acetate through to 50% ethyl acetate/50% methanol). The flash column used was 14 cm in diameter; 200 g of silica was used in each of the columns. The extract was pre-loaded onto silica and 2 L of each solvent was used for each elution (4 column volumes). **Table 3.1.1**, Chapter 3.1, pg 47, indicates the amount of the methanol extract loaded onto each of the seven separate columns.

The isolation of these ten fractions was illustrated in **Diagram 3.1.1**, Chapter 3.1, pg 48.

Each of the seven columns underwent the same solvent extraction sequence and the equivalent fractions from each column were then combined. Initially, 2 L of 90% hexane/10% ethyl acetate was run through the column and a total of 5 g was collected (after the solvent was removed). This crude fraction was labelled Fraction 1. The next nine fractions, the eluting solvents and the total mass of each fraction (when all seven columns were combined) are outlined in **Table 3.1.2**, Chapter 3.1, pg 48.

All crude fractions (F.1 - F.10) were then tested with Meyers reagent, to determine whether any alkaloids were present.

All crude fractions gave a negative result to Meyers reagent.

13.3 Compounds from *Scaevola spinescens*

13.3.1 Initial treatment FRACTION 1

Fraction 1 [5 g] was separated from the original methanol extract [828 g] using 90% hexane/10% ethyl acetate as the eluting solvent.

5 g of Fraction 1 was subjected to flash chromatography, (silica, sequential, 100% hexane - 50% hexane/50% ethyl acetate) to yield ten fractions.

A flash column (silica, isocratic, 100% hexane) of Fraction 1.2 [5 mg] yielded Fraction 1.2.3 that was found to be hexadecanoic acid, methyl ester (**95**) [less than one mg] by GC/MS analysis and comparison with the known MS spectrum.

Fraction 1.4 [150 mg] was subjected to flash chromatography (silica, isocratic, 98% hexane/2% ethyl acetate) to yield seven fractions. Fraction 1.4.1 [50 mg] was subjected to flash chromatography (silica, isocratic, 98% hexane/2% ethyl acetate) to yield Fraction 1.4.1.4 [22 mg] which was found to be a mixture when analysed using GC/MS. Fraction 1.4.1.4.1 - a major peak- was found to be taraxerol acetate (**92**).

Fraction 1.6 [150 mg] formed crystals in 100% hexane that were labelled 1.6.2 [50 mg].

Fraction 1.6.2 [50 mg] was subjected to flash chromatography (silica, isocratic, 95% hexane/5% ethyl acetate) and separated into three fractions. When fraction 1.6.2.1 was left standing, a white solid crystallized out of solution (100% hexane). This solid [45 mg] was then recrystallized in 99% hexane/1% methanol to give fine crystals of judarrylol (**96**) [40 mg].

The mother liquor was labelled 1.6.1 [100 mg]. It underwent flash chromatography (silica, isocratic, 95% hexane/5% ethyl acetate) and was separated into ten fractions. When Fraction 1.6.1.8 was left standing, a white solid crystallized out of solution (100% hexane). This solid [42 mg] was then recrystallized in 99% hexane/ 1% methanol to give fine white crystals. Fraction 1.6.1.8 [32 mg] was pure and was identified as taraxerol (**73**) [32 mg].

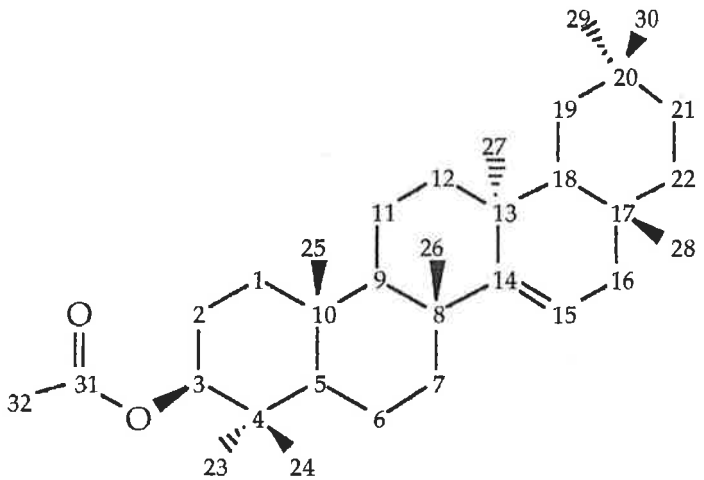
This procedure is outlined in **Diagram 4.1**, Chapter 4.1, pg 53.

13.3.2 Hexadecanoic acid, methyl ester

(95)

Less than one mg of (**95**) was isolated as a white amorphous solid. Identified by MS peak matching to known compound; $C_{17}H_{34}O_2$; *m/z* (EI): 270 (M^+ 50%), 239 (25), 227 (45), 143 (40), 129 (39), 74 (100), 86 (90).

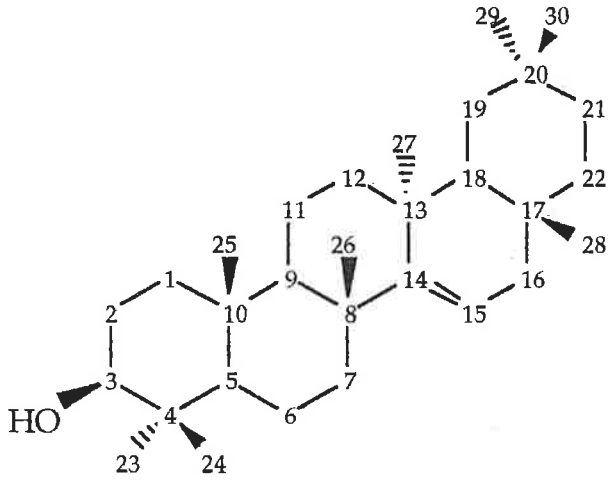
13.3.3 Taraxerol acetate



(92)

22 mg of a mixture with (**92**) present was isolated as a white crystalline solid. Identified by MS peak matching and comparison of ^{1}H and ^{13}C data with recorded data of taraxerol acetate; $C_{32}H_{52}O_2$; exact mass calculated for $C_{32}H_{52}O_2$: 468.3957, found 468.3964, (+/- 0.0007); **MP**[78]: 304 - 305°C; $[\alpha]_D^{78}$: + 9° ($CHCl_3$); **FTIR** (nujol): 1720 (C=O), 1650 (C=C); **^{1}H NMR** (600 MHz/$CDCl_3$); selected data, δH: 0.809 (3H, s), 0.849 (3H, s), 0.867 (3H, s), 0.906 (3H, s), 0.920 (3H, s), 0.934 (3H, s), 1.049 (3H, s), 1.081 (3H, s), 2.36 (1H, m, H_{16a}), 4.47 (3H, s, H_{32}), 5.55 (1H, dd, 3.6, 8.6, H_{15}); **^{13}C NMR** (150 MHz /$CDCl_3$); δC: 16.3 (C_{25}), 16.4 (C_{24}), 18.3 (C_{11}), 19.5 (C_6), 22.1 (C_{30}), 23.6 (C_{32}), 25.2 (C_{27}), 26.9 (C_2), 28.8 (C_{20}), 29.1 (C_{23}), 30.1 (C_{28}), 30.6 (C_{26}), 32.8 (C_{22}), 33.9 (C_{21}), 34.1 (C_{29}), 34.9 (C_{12}), 35.6 (C_7), 37.4 (C_1), 38.2 (C_{13}), 38.3 (C_{16}), 38.5 (C_{10}), 38.5 (C_{17}), 38.7 (C_4), 39.5 (C_8), 41.3 (C_{19}), 49.0 (C_{18}), 49.5 (C_9), 56.0 (C_5), 79.1 (C_3), 117.4 (C_{15}), 158.3 (C_{14}), 166.7 (C_{31}); *m/z* (EI): 468 (M^+ 20%), 344 (44), 329 (42), 269 (61), 204 (100), 189 (49), 135 (38).

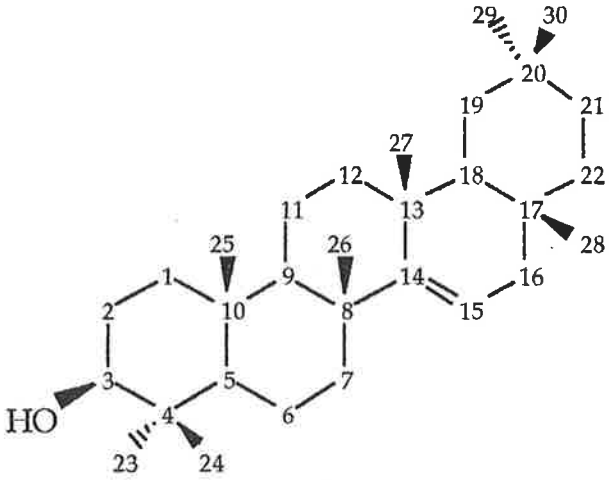
13.3.4 Taraxerol



(**73**)

32 mg of (**73**) was isolated as white crystals. Identified by MS peak matching and comparison of ^{13}C chemical shift values with known values; $C_{30}H_{50}O$; exact mass calculated for $C_{30}H_{50}O$: 426.3864, found 426.3859 (+/- 0.0005); **MP**: 280 - 284°C (lit.[78], 282 - 283°C); $[\alpha]_D$: + 0.69° (c 0.1, $CHCl_3$), (lit.[78], + 0.72° (c 0.974, $CHCl_3$)); **FTIR** (nujol): 3420 (OH), 1660 (C=C); **^{13}C NMR** (150 MHz, $CDCl_3$); δC: 15.2 (C_{24}), 15.2 (C_{25}), 17.8 (C_{11}), 19.1 (C_6), 21.7 (C_{30}), 25.0 (C_{27}), 27.2 (C_{23}), 27.3 (C_2), 29.5 (C_{20}), 29.5 (C_{26}), 29.7 (C_{28}), 32.8 (C_{22}), 33.4 (C_{29}), 33.9 (C_{21}), 35.1 (C_{12}), 35.3 (C_7), 36.4 (C_{16}), 37.0 (C_{13}), 37.9 (C_{10}), 38.2 (C_1), 38.6 (C_{17}), 38.7 (C_8), 39.8 (C_4), 41.7 (C_{19}), 48.2 (C_9), 50.3 (C_{18}), 55.2 (C_5), 78.9 (C_3), 117.3 (C_{15}), 159.1 (C_{14}); *m/z* (EI): 426 (M^+ 15%), 302 (40), 287 (38), 204 (100), 135 (39).

13.3.5 Judarrylol



(96)

40 mg of (**96**) was isolated as white crystals. Identified by assignment of all ^{1}H and ^{13}C NMR signals including stereochemistry and conformation of all proton signals; $C_{30}H_{50}O$; exact mass calculated for $C_{30}H_{50}O$: 426.3874, found 426.3876 (+/- 0.0002); **MP**: 191 - 193°C; $[\alpha]_D$: + 58.6° (c 0.1, $CHCl_3$); FTIR (nujol): 3410 (OH), 1660 (C=C); **^{1}H NMR** (600 MHz, $CDCl_3$); δH: 0.715 (1H, dd, 2.4, 12.0, $H_{5\alpha}$), 0.734 (3H, s, $H_{24\beta}$), 0.754 (3H, s, $H_{28\beta}$), 0.841 (3H, s, $H_{27\beta}$), 0.841 (3H, s, $H_{30\beta}$), 0.860 (3H, s, $H_{25\beta}$), 0.881 (3H, s, $H_{29\alpha}$), 0.890 (1H, m, $H_{1\alpha}$), 0.902 (1H, m, $H_{18\alpha}$), 0.910 (3H, s, $H_{23\alpha}$), 0.920 (1H, m, $H_{19\alpha}$), 0.952 (1H, m, $H_{22\alpha}$), 1.022 (3H, s, $H_{26\beta}$), 1.172 (1H, m, $H_{21\alpha}$), 1.224 (1H, m, $H_{19\beta}$), 1.271 (1H, m, $H_{21\beta}$), 1.271 (1H, m, $H_{7\beta}$), 1.305 (1H, m, $H_{22\beta}$), 1.349 (1H, m, $H_{9\alpha}$), 1.412 (1H, m, $H_{11\beta}$), 1.412 (1H, m, $H_{6\beta}$), 1.475 (1H, m, $H_{12\beta}$), 1.486 (1H, m, $H_{2\beta}$), 1.522 (1H, m, $H_{2\alpha}$), 1.531 (1H, m, $H_{1\beta}$), 1.547 (1H, m, $H_{6\alpha}$), 1.560 (1H, m, $H_{12\alpha}$), 1.563 (1H, m, $H_{11\alpha}$), 1.575 (1H, m, $H_{16\alpha}$), 1.841 (1H, dd, 3.0, 15.0, $H_{16\beta}$), 1.960 (1H, dd, 3.0, 12.6, $H_{7\alpha}$), 3.125 (1H, d, 11.4, $H_{3\alpha}$), 5.464 (1H, dd, 3.0, 8.4, H_{15}) (OH_3 not observed); **^{13}C NMR** (150 MHz, $CDCl_3$); δC: 15.4 (C_{24}), 15.4 (C_{25}), 17.5 (C_{11}), 18.8 (C_6), 21.3 (C_{30}), 25.9 (C_{26}), 27.1 (C_2), 28.0 (C_{23}), 28.8 (C_{20}), 29.7 (C_{28}), 29.9 (C_{27}), 33.1 (C_{29}), 33.3 (C_{21}), 33.7 (C_{12}), 35.1 (C_{22}), 35.7 (C_{17}), 36.6 (C_{19}), 37.5 (C_{13}), 37.7 (C_1), 37.7 (C_{16}), 38.0 (C_{10}), 38.7 (C_4), 38.9 (C_8), 41.3 (C_7), 48.7 (C_{18}), 49.2 (C_9), 55.5 (C_5), 79.0 (C_3), 116.8 (C_{15}), 158.0 (C_{14}); *m/z* (EI): 426 (M^+ 4%), 302 (40), 287 (40), 204 (100), 189 (78) 135 (39) 133 (88).

13.3.6 Initial treatment FRACTION 3

Fraction 3 [11 g] was separated from the original methanol extract [828 g] using 50% hexane/50% ethyl acetate as the eluting solvent.

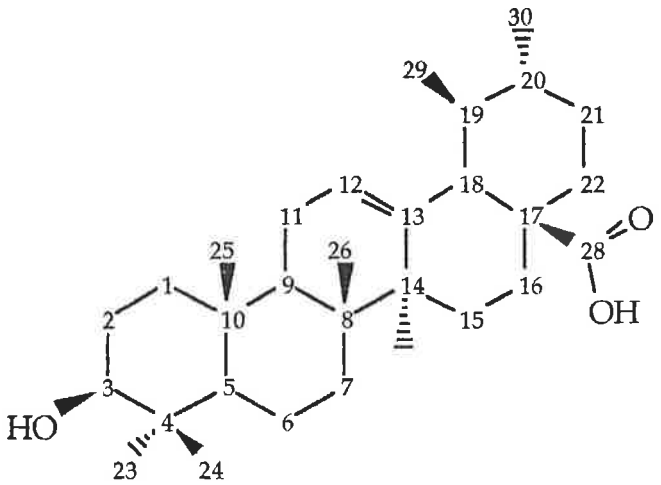
11 g of Fraction 3 was initially subjected to flash chromatography (silica, sequential, 100% hexane - 50% hexane/50% ethyl acetate) to give nine fractions.

Fraction 3.6 [900 mg] was then further fractionated by flash chromatography (silica, sequential, 100% chloroform - 95% chloroform/5 % methanol) to yield five fractions.

Fraction 3.6.3 [72 mg] was then separated using flash chromatography (silica, sequential, 100% chloroform - 95% chloroform/5 % methanol) to give ten fractions.

Fraction 3.6.3.4 [42 mg] underwent a further purification step (recrystallization, 100% chloroform) to give ursolic acid (**97**) [7 mg]. This separation process is outlined in **Diagram 5.1**, Chapter 5.1, pg 70.

13.3.7 Ursolic acid



(**97**)

7 mg of (**97**) was isolated as white crystals. Identified by assignment of ^{1}H and ^{13}C chemical shift data and comparison with recorded literature values for ursolic acid; $C_{30}H_{48}O_3$; exact mass calculated for $C_{30}H_{48}O_3$: 456.3605; found, 456.3599 (+/- 0.0006); **MP**: 282 - 285°C (lit.[80], 285 - 288°C); $[\alpha]_D$: + 69° (c 0.1, $CHCl_3$) (lit.[80], + 67.5° (c 1 ethanol, NaOH)); **FTIR** (nujol): 3410 (OH), 3000 (COOH), 1730 (C=O), 1660 (C=C); **^{1}H NMR** (600 MHz, $CDCl_3$); δH: 0.667 (3H, s, $H_{23\alpha}$), 0.713 (1H, m, $H_{5\alpha}$),0.741 (3H, s, $H_{26\beta}$), 0.802 (3H, d, 6.6, $H_{29\beta}$), 0.857 (3H, s, $H_{25\beta}$), 0.886 (3H, s, $H_{24\beta}$), 0.904 (3H, d, 6.0, $H_{30\alpha}$), 0.924 (1H, m, H_{7b}), 0.976 (1H, m, H_{11b}), 1.030 (3H, s, $H_{27\alpha}$), 1.081 (1H, m, H_{15b}), 1.267 (1H, m, H_{22b}), 1.272 (1H, m, $H_{19\alpha}$), 1.392 (1H, m, H_{6b}), 1.408 (1H, m, H_{22a}), 1.432 (1H, m, $H_{9\alpha}$), 1.435 (1H, m, H_{7a}), 1.461 (1H, m, H_{2a}), 1.463 (1H, m, H_{15a}), 1.467 (1H, m, H_{21b}), 1.466 (1H, m, H_{1b}), 1.503 (1H, m, H_{21a}), 1.507 (1H, m,

$H_{20\beta}$), 1.544 (1H, m, H_{6a}), 1.546 (1H, m, H_{2b}), 1.546 (1H, m, H_{16b}), 1.558 (1H, m, H_{1a}), 1.613 (1H, m, H_{16a}), 1.815 (1H, m, H_{11a}), 2.092 (1H, d, 11.4, $H_{18\beta}$), 2.980 (1H, br t, $H_{3\alpha}$), 5.118 (1H, br d, H_{12}) (OH_3, COOH not observed); ^{13}C NMR (150 MHz, $CDCl_3$); δC: 14.7 (C_{25}), 15.1 (C_{24}), 16.4 (C_{26}), 17.6 (C_{29}), 17.6 (C_6), 20.5 (C_{30}), 22.8 (C_{11}), 23.5 (C_{27}), 26.5 (C_{15}), 27.5 (C_2), 27.5 (C_{16}), 28.8 (C_{23}), 30.0 (C_{21}), 32.3 (C_{17}), 36.0 (C_{22}), 36.2 (C_{10}), 38.0 (C_1), 38.0 (C_4), 38.3 (C_{20}), 38.8 (C_7), 39.0 (C_{19}), 39.8 (C_8), 41.3 (C_{14}), 46.8 (C_9), 52.0 (C_{18}), 54.5 (C_5), 77.5 (C_3), 124.4 (C_{12}), 137.6 (C_{13}), 179.1 (C_{28}); *m/z* (EI): 456 (M^+ 25%), 438 (32), 410 (56), 248 (100), 203 (60), 133 (53).

13.3.8 Initial treatment FRACTION 4

Fraction 4 [14 g] was separated from the original methanol extract [828 g] using 100% ethyl acetate as the eluting solvent.

Initially 1 g of Fraction 4 was partitioned between hexane (100 ml) and a methanol (90 ml)/water (10 ml) mixture. The methanol/water extract was freeze-dried, redissolved in methanol and then repartitioned using chloroform (100 ml), methanol (90 ml) and water (10 ml). The chloroform fraction was kept and labelled 4.E [45 mg]. 4.E was subjected to flash chromatography (silica, isocratic, 99% chloroform/1 % methanol) to yield nine fractions.

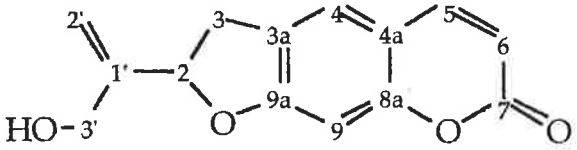
Fraction 4.E.6 [22 mg] was then subjected to normal phase analytical HPLC (isocratic, 50% hexane/50% ethyl acetate, 330 nm, 1 mL/min., 30 min.) and emmarin (**98**) [2 mg] was collected and identified.

The rest of Fraction 4 [13 g] was subjected to the same partition process as outlined above. The chloroform fraction was labelled 4.C [200 mg] and then subjected to flash chromatography (silica, isocratic, 99% chloroform/1 % methanol) to give nine fractions.

4.C.3 [100 mg] was then subjected to flash chromatography (silica, isocratic, 99% chloroform/1% methanol) to yield 4.C.3.3 [63 mg] . This fraction was then subjected to normal phase preparative HPLC (isocratic, 50% hexane/50% ethyl acetate, 330 nm, 5 mL/min, 30 min.) to yield more of (**98**) [25 mg].

Fraction 4.C.6 [27 mg] was subjected to normal phase analytical HPLC (isocratic, 50 % hexane/50 % ethyl acetate, 256 nm, 1 mL/min., 30 min.) to isolate vanillic acid (**99**) [4 mg]. This separation system is outlined in **Diagrams 6.1.1** and **6.1.2**, Chapter 6.1, pgs 75, 76.

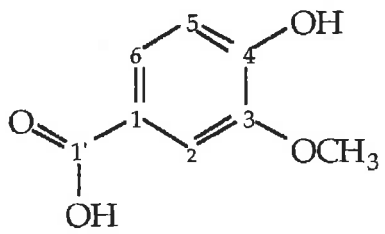
13.3.9 Emmarin



(98)

25 mg of (98) was isolated as white crystals. Identified by complete assignment of ^{1}H and ^{13}C spectral data; $C_{14}H_{12}O_4$; exact mass calculated for $C_{14}H_{12}O_4$: 244.0736, found, 244.0733 (+/-0.0003); **MP**: 123 - 125°C; $[\alpha]_D$: - 10° (c 0.1, $CHCl_3$); **FTIR** (nujol): 3360 (OH), 1720 (C=O), 1660 (C=C); **UV**: λ_{max}, (methanol) 225, 248, 258, 335 ; **^{1}H NMR** (600 MHz, d_6-DMSO); δH: 3.137 (1H, dd, 7.2, 15.6, H_{3b}), 3.392 (1H, dd, 9.6, 15.6, H_{3a}), 4.209 (2H, m, $H_{3'}$), 4.915 (1H, br s, $C_{3'}$-OH), 5.219 (1H, br s, $H_{2'b}$), 5.321 (1H, br s, $H_{2'a}$), 5.394 (1H, dd, 7.2, 9.6, H_2), 6.151 (1H, d, 9.6, H_6), 6.703 (1H, s, H_9), 7.162 (1H, br s, H_4), 7.527 (1H, d, 9.6, H_5); **^{13}C NMR** (150 MHz, d_6-DMSO); δC: 34.1 (C_3), 63.1 ($C_{3'}$), 85.4 (C_2), 98.4 (C_9), 112.6 (C_6), 113.3 ($C_{2'}$), 116.1 (C_{4a}), 123.7 (C_4), 124.6 (C_{3b}), 143.9 (C_5), 146.6 ($C_{1'}$), 155.1 (C_{8a}), 161.5 (C_7), 163.0 (C_{9a});*m/s* (EI): 244 (M^+ 25%), 213 (28), 186 (18), 167 (100).

13.3.10 Vanillic acid



(99)

4 mg of (99) was isolated as a white amorphous solid. Identified by complete assignment of ^{1}H and ^{13}C spectral data; $C_8H_8O_4$; exact mass calculated for $C_8H_8O_4$: 168.0422, found, 168.0425 (+/- 0.0003); **MP**: 211 - 214°C (lit.[80], 210 - 213°C); **FTIR** (nujol): 3460 (OH), 1730 (C=O), 1660 (C=C); **UV**: λ_{max}, ($CHCl_3$) 235, 322; **^{1}H NMR** (600 MHz, $CDCl_3$); δH: 3.893 (3H, s, OCH_3), 5.986 (1H, s, C_4-OH), 6.905 (1H, d, 8.4, H_5), 7.507 (1H, d, 1.2, H_2), 7.638 (1H, dd, 1.2, 8.4, H_6) ; **^{13}C NMR** (150 MHz, $CDCl_3$); δC: 56.0 (OCH_3), 112.0 (C_2), 114.6 (C_5), 121.5 (C_1), 125.2 (C_6), 146.3 (C_3), 151.2 (C_4), 170.5 ($C_{1'}$); *m/s* (EI): 168 (M^+ 100%), 153 (60), 57 (63).

13.3.11 Initial treatment FRACTION 5

Fractions 5 [4 g] was separated from the original methanol extract [828 g] using 99% ethyl acetate/1% methanol as the eluting solvent while Fraction 6 [4 g] was

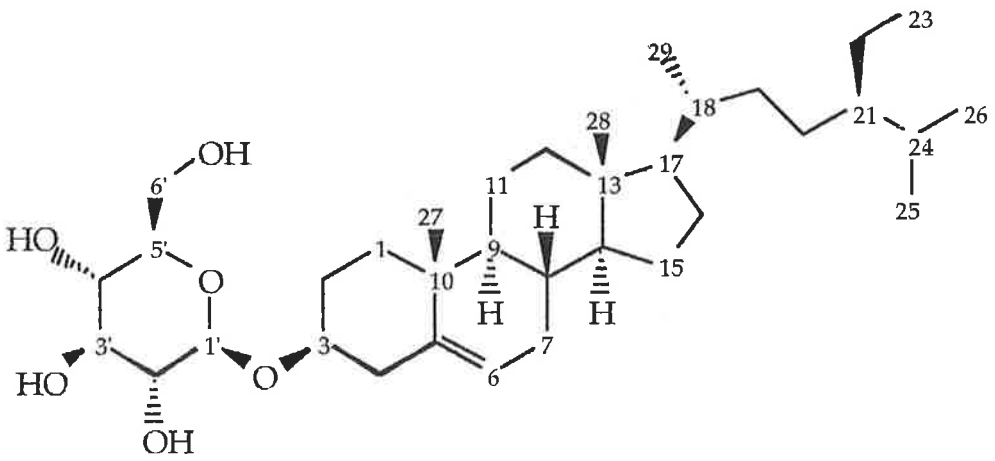
separated from the original methanol extract [828 g] using 98% ethyl acetate/2% methanol as the eluting solvent.

Fractions 5 [4 g] and 6 [3 g] were very similar by TLC and so were combined. The combined total [7 g] was dissolved in 98% ethyl acetate/2% methanol. 250 mg of a white crystalline solid precipitated out and on recrystallization (100% ethyl acetate) 100 mg of a white crystalline solid was collected and labelled 5.1.

Fraction 5.1 was then subjected to flash chromatography (silica, isocratic, 100% ethyl acetate) and 3 fractions were collected.

Fraction 5.1.3 [52 mg] was then recrystallized in 100% ethyl acetate and the crystals collected to give daucesterol (**100**) [26 mg]. This separation system is outlined in **Diagram 7.1**, Chapter 7.1, pg 90.

13.3.12 Daucesterol



(**100**)

26 mg of (**100**) was isolated as a white crystalline solid. Identified by complete assignment of ^{1}H and ^{13}C spectral data; $C_{35}H_{60}O_6$; exact mass calculated for $M+NH_4^+$, $C_{35}H_{64}NO_6$: 594.4686, found, 594.4737 (+/- 0.0051); **MP**: 280 - 286°C (lit.[78], 283 - 286°C); $[\alpha]_D$: - 44° (c 0.1, $CHCl_3$) (lit.[78], - 41.5° (c 0.4, Py))**FTIR** (nujol): 3210 (OH), 1660 (C=C); **^{1}H NMR** (600 MHz, d_6-DMSO); δH: 0.642 (3H, d, 6.6, Me_{26}), 0.780 (3H, s, $Me_{27\beta}$), 0.820 (3H, s, $Me_{28\beta}$), 0.861 (1H, m, $H_{14\alpha}$), 0.890 (3H, m, Me_{23}), 0.918 (1H, m, $H_{17\alpha}$), 0.949 (3H, d, 6.6, Me_{29}), 0.979 (1H, m, H_{19b}), 0.979 (1H, m, H_{16b}), 0.990 (3H, d, 6.6, Me_{25}), 1.035 (1H, m, H_{11b}), 1.105 (1H, m, H_{20b}), 1.105 (1H, m, $H_{9\alpha}$), 1.131 (1H, m, H_{24}), 1.131 (1H, m, H_{15b}), 1.114 (1H, m, H_{1b}), 1.215 (1H, m, H_{12b}), 1.224 (1H, m, H_{22b}), 1.262 (1H, m, H_{20a}), 1.286 (1H, m, H_{16a}), 1.308 (1H, m, H_{18}), 1.381 (1H, m, H_{21}), 1.417 (1H, m, H_{15a}), 1.417 (1H, m, H_{2b}), 1.439 (1H, m, H_{7b}), 1.496

(1H, m, H_{11a}), 1.496 (1H, m, $H_{8\beta}$), 1.635 (1H, m, H_{22a}), 1.770 (1H, m, H_{19a}), 1.770 (1H, m, H_{12a}), 1.794 (1H, m, H_{2a}), 1.893 (1H, m, H_{7a}), 1.922 (1H, m, H_{1a}), 2.116 (1H, m, H_{4b}), 2.350 (1H, dd, 3.0, 13.8, H_{4a}), 2.880 (1H, dd, 7.8, 9.0, $H_{2'}$), 3.008 (1H, t, 9.0, $H_{4'}$), 3.050 (1H, ddd, 3.0, 5.4, 9.0, $H_{5'}$), 3.114 (1H, t, 9.0, $H_{3'}$), 3.399 (1H, dd, 5.4, 11.4, $H_{6b'}$), 3.444 (1H, m, $H_{3\alpha}$), 3.630 (1H, dd, 3.0, 11.4, $H_{6a'}$), 4.210 (1H, d, 7.8, $H_{1'}$), 4.409 (1H, br s, $C_{6'}$ -OH), 4.842 (1H, br s, $C_{2'}$ -OH), 4.846 (1H, br s, $C_{4'}$ -OH), 4.884 (1H, br s, $C_{3'}$ -OH), 5.310 (1H, br t, 3.0, H_6); **^{13}C NMR** (150 MHz, d_6-DMSO); δC: 14.2 (C_{26}), 18.5 (C_{23}), 18.8 (C_{27}), 19.0 (C_{29}), 20.5 (C_{15}), 20.8 (C_{28}), 21.0 (C_{25}), 22.4 (C_{20}), 23.8 (C_{11}), 25.4 (C_{24}), 27.7 (C_{12}), 28.6 (C_{22}), 29.2 (C_2), 31.3 (C_7), 31.3 (C_{21}), 33.3 (C_{16}), 35.4 (C_{18}), 36.4 (C_{10}), 36.7 (C_{19}), 38.3 (C_4), 40.0 (C_1), 41.8 (C_{13}), 45.7 (C_{17}), 49.5 (C_{14}), 50.5 (C_8), 55.4 (C_9), 61.0 ($C_{6'}$), 70.0 ($C_{4'}$), 73.4 ($C_{2'}$), 76.7 ($C_{5'}$), 76.7 ($C_{3'}$), 76.9 (C_3), 100.7 ($C_{1'}$), 121.1 (C_6), 140.4 (C_5); *m/s* (EI): 594 ($M+NH_4^+$, 100%), 396 (34), 223 (10).

13.3.13 Initial treatment FRACTION 7

Fraction 7 [16 g] was separated from the original methanol extract [828 g] using 95% ethyl acetate/5 % methanol as the eluting solvent.

Fraction 7 [16 g] was subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) to yield ten fractions, which were analysed by TLC. Fractions 7.3 and 7.4 appeared to contain the same compounds and were added together to give 10.75 g and labelled 7.(3).

Fraction 7.(3) was analysed using analytical reverse phase HPLC (gradient, 100% water - 100% methanol, 254 nm, 1 mL/min., 30 min.) which indicated the presence of four compounds. Fraction 7.(3) [10.75 g] was then subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) to give six fractions, of which Fraction 7.(3).5 [192 mg] appeared to contain the four compounds identified by reverse phase HPLC.

Fraction 7.(3).5 was then subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) to give eight fractions.

Fraction 7.(3).5.4 was analysed by reverse phase analytical HPLC (gradient, 100% water to 100% methanol, 254 nm, 1 mL/min., 30 min.) and found to be pure, isolating alidyjosioside (**108**) [26 mg].

Fraction 7.(3).5.5 [60 mg] was subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) to give ten fractions.

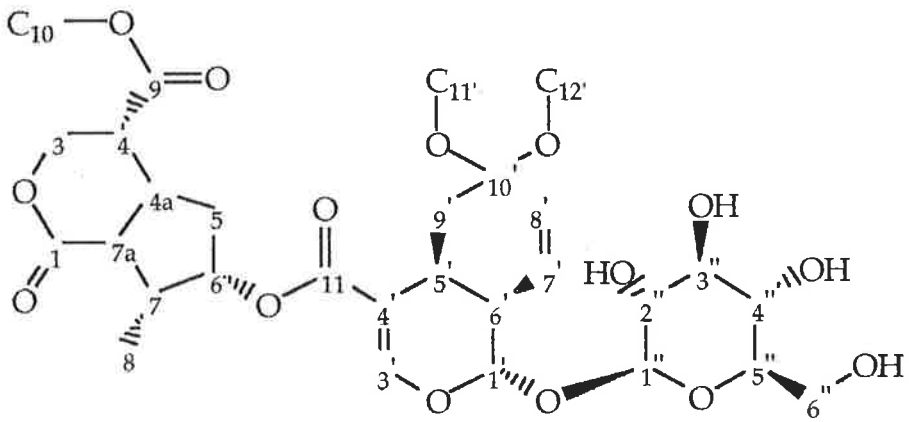
Fraction 7.(3).5.5.5 appeared to be pure and was recrystallized using 90% chloroform / 10% methanol to give scaevoloside (**6**) [10 mg].

Fraction 7.(3).5.5.6 was pure by analytical reverse phase HPLC (gradient, 100% water - 100% methanol, 254 nm, 1 mL/min., 30 min.) to yield katecateroside (**109**) [12 mg].

Fractions 7.(3).5.5.8 and 7.(3).5.5.9 were combined to give 7.(3).5.5.9.2b [15 mg] which was then purified by analytical reverse phase HPLC (gradient, 100% water - 100% methanol, 254 nm , 1 mL/min., 30 min.) to yield loganin (**5**) [10 mg].

The purity of these compounds was analysed by analytical reverse phase HPLC under two gradient systems and an isocratic system. The first gradient system was 100% water to 100% methanol (254 nm, 1 mL/min., 30 min.). The second gradient system was 70% water/30 % methanol to 30% water/70 % methanol (254 nm, 1 mL/min., 30 min.). The isocratic system was 50% water/50% methanol (254 nm, 1 mL/min., 30 min.). (**5**), (**6**), (**108**) and (**109**) all appeared as one peak under all three systems. This isolation procedure is outlined in **Diagram 8.1**, Chapter 8.1, pg 94.

13.3.14 Alidyjosioside

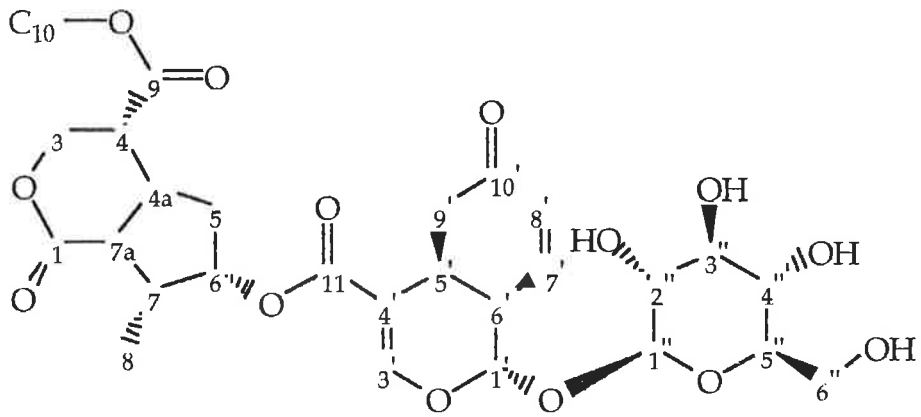


(**108**)

26 mg of (**108**) was isolated as a white amorphous solid. Identified by complete assignment of ^{1}H and ^{13}C spectral data; $C_{29}H_{42}O_{15}$; exact mass calculated for $M+Na^{+}$, $C_{29}H_{42}ONa$: 653.2424, found, 653.2364 (+/- 0.0060); **MP**: 94 - 96°C; $[\alpha]_D$: - 64° (c 0.1, MeOH); **FTIR** (nujol): 3400 (OH), 1735 (C=O), 1680 (C=C); **^{1}H NMR** (600 MHz, $CDCl_3$); δH: 1.21 (3H, d, 7.2, Me_8), 1.62 (1H, ddd, 3.6, 11.4, 13.8, $H_{5\beta}$), 1.67 (1H, ddd, 4.8, 7.2, 13.8, $H_{9'b}$), 2.04 (1H, ddd, 6.6, 7.2, 13.8, $H_{9'a}$), 2.20 (1H, dd, 7.8, 13.8, $H_{5\alpha}$), 2.48 (1H, ddq, 7.2, 7.2, 11.4, H_7), 2.55 (1H, td, 3.6, 9.6, H_4), 2.71 (1H, dt, 5.4, 5.4, 14.4,

$H_{6'}$), 2.77 (1H, dd, 9.6, 11.4, H_{7a}), 2.90 (1H, ddd, 5.4, 6.6, 7.2, $H_{5'}$), 3.04 (1H, dddd, 7.8, 9.6, 9.6, 11.4, H_{4a}), 3.292 (3H, s, $Me_{11'}$), 3.298 (3H, s, $Me_{12'}$), 3.39 (1H, dd, 7.2, 9.0, $H_{2''}$), 3.43 (1H, ddd, 3.0, 8.4, 9.0, $H_{5''}$), 3.58 (1H, t, 9.0, $H_{3''}$), 3.63 (1H, t, 9.0, $H_{4''}$), 3.75 (3H, s, Me_{10}), 3.86 (1H, dd, 8.4, 11.4, $H_{6''b}$), 3.93 (1H, dd, 3.0, 11.4, $H_{6''a}$), 4.30 (1H, dd, 9.6, 11.4, $H_{3\alpha}$), 4.46 (1H, dd, 3.6, 11.4, $H_{3\beta}$), 4.49 (1H, dd, 4.8, 7.2, $H_{10'}$), 4.72 (1H, d, 7.2, $H_{1''}$), 5.25 (1H, dd, 3.6, 7.2, H_6), 5.32-5.29 (2H, m, $H_{8'}$), 5.36 (1H, d, 5.4, $H_{1'}$), 5.68 (1H, m, $H_{7'}$), 7.34 (1H, d, 1.2, $H_{3'}$) ($OH_{2''}$, $OH_{3''}$, $OH_{4''}$, $OH_{6''}$ not observed); **^{13}C NMR** (150 MHz, $CDCl_3$); δC: 15.2 (C_8), 29.6 ($C_{5'}$), 32.2 ($C_{9'}$), 37.0 (C_{4a}), 38.5 (C_5), 43.7 (C_7), 44.5 ($C_{6'}$), 46.5 (C_4), 47.5 (C_{7a}), 52.29 ($C_{12'}$), 52.28 ($C_{11'}$), 53.8 (C_{10}), 62.5 ($C_{6''}$), 68.0 (C_3), 70.5 ($C_{4''}$), 72.9 ($C_{2''}$), 75.9 ($C_{5''}$), 76.7 ($C_{3''}$), 78.2 (C_6), 97.6 ($C_{1'}$), 98.6 ($C_{1''}$), 103.7 ($C_{10'}$), 110.8 ($C_{4'}$), 120.6 ($C_{8'}$), 133.9 ($C_{7'}$), 154.9 ($C_{3'}$), 166.3 (C_{11}), 171.4 (C_9), 173.3 (C_1);*m/z* (LRP + LSIMS): 653 (M+ Na^+, 23%), 599 (100), 507 (6), 388 (12)

13.3.15 Scaevoloside

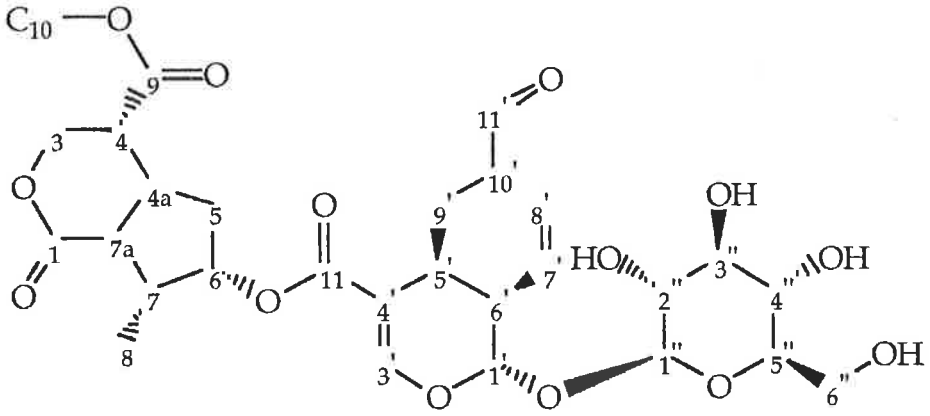


(6)

10 mg of (6) was isolated as a white amorphous solid. Identified by complete assignment of ^{1}H and ^{13}C spectral data. Structure determination via ^{1}H and ^{13}C analysis and comparison with known data; $C_{27}H_{36}O_{14}$; exact mass calculated for M+Na^+, $C_{27}H_{36}O_{14}Na$: 607.2006, found, 607.2013 (+/- 0.0007); **MP**: 120 - 124°C; $[\alpha]_D$: - 64° (c 0.1, MeOH) (lit.[44], - 61° (c 0.2, MeOH)); **FTIR** (nujol): 3400 (OH), 1735 (C=O), 1680 (C=); **^{1}H NMR** (600 MHz, $CDCl_3$); δH: 1.16 (3H, d, 7.2, Me_8), 1.54 (1H, m, $H_{5\beta}$), 2.24 (1H, m, $H_{5\alpha}$), 2.41 (1H, m, $H_{9'b}$), 2.44 (1H, m, H_7), 2.53 (1H, m, H_4), 2.71 (1H, m, H_{7a}), 2.78 (1H, m, $H_{6'}$), 2.96 (1H, m, $H_{9'a}$), 3.00 (1H, m, H_{4a}), 3.39 (1H, m, $H_{5'}$), 3.39 (1H, m, $H_{5''}$), 3.42 (1H, m, $H_{2''}$), 3.56 (1H, t, 9.0, $H_{3''}$), 3.61 (1H, t, 9.0, $H_{4''}$), 3.72 (3H, s, Me_{10}), 3.83 (1H, m, $H_{6''b}$), 3.86 (1H, m, $H_{6''a}$), 4.27 (1H, dd, 9.6, 11.4, $H_{3\alpha}$), 4.34 (1H, dd, 3.6, 11.4, $H_{3\beta}$), 4.69 (1H, d, 7.8, $H_{1''}$), 5.21 (1H, m, H_6), 5.22- 5.25 (2H, m, $H_{8'}$), 5.31 (1H, d, 3.6, $H_{1'}$), 5.53 (1H, m, $H_{7'}$), 7.42 (1H, d, 1.2, $H_{3'}$), 9.71 (1H, s, $H_{10'}$) ($OH_{2''}$, $OH_{3''}$, $OH_{4''}$, $OH_{6''}$ not observed); **^{13}C NMR** (150 MHz, $CDCl_3$); δC: 16.0 (C_8), 29.3 ($C_{5'}$), 39.7 (C_{4a}), 40.1 (C_5), 44.0 (C_4), 44.8 ($C_{9'}$), 45.6 (C_7), 46.0 (C_{7a}), 47.8 ($C_{6'}$), 52.5 (C_{10}), 62.0

($C_{6''}$), 67.0 (C_3), 70.0 ($C_{4''}$), 73.8 ($C_{5''}$), 75.6 ($C_{2''}$), 76.3 ($C_{3''}$), 76.3 (C_6), 96.2 ($C_{1'}$), 98.1 ($C_{1''}$), 110.0 ($C_{4'}$), 122.2 ($C_{8'}$), 135.7 ($C_{7'}$), 154.6 ($C_{3'}$), 166.7 (C_{11}), 172.4 (C_9), 173.6 (C_1), 201.2 ($C_{10'}$); *m/s* (EI): 607 ($M+Na^+$, 100%), 585 (26), 423 (68), 391 (24), 369 (20).

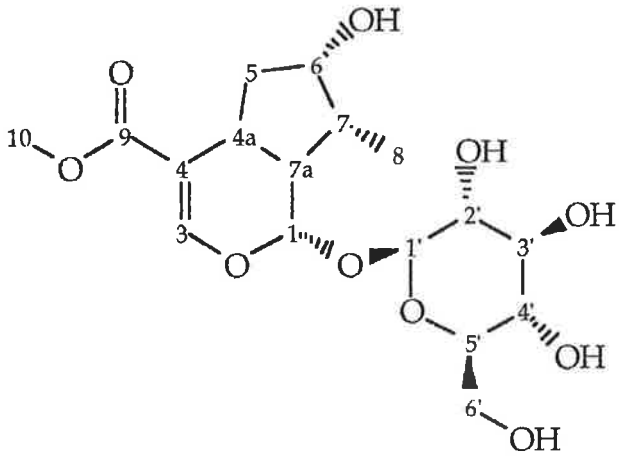
11.3.16 Katecateroside



(**109**)

12 mg of (**109**) was isolated as a white amorphous solid. Identified by complete assignment of ^1H and ^{13}C spectral data; $C_{28}H_{38}O_{14}$; exact mass calculated for $M+H^+$, $C_{26}H_{39}O_{14}$: 599.2341, found, 599.2361 (+/- 0.0020); **MP**: 148 - 150°C; $[\alpha]_D$: - 64° (c 0.1, MeOH); **FTIR** (nujol): 3400 (OH), 1730 (C=O), 1660 (C=C); **^1H NMR** (600 MHz, CD_3OD); δH: 1.06 (3H, d, 6.6, Me_8), 1.56 (1H, m, $H_{9'b}$), 1.60 (1H, m, $H_{5\beta}$), 1.86 (1H, m, $H_{9'a}$), 2.10 (1H, m, $H_{5\alpha}$), 2.34 (1H, m, H_4), 2.40 (1H, m, $H_{10'b}$), 2.56 (1H, m, H_7), 2.60 (1H, m, H_{7a}), 2.60 (1H, m, $H_{10'a}$), 2.79 (1H, dd, 10.2, 11.4, $H_{6'}$), 2.87 (1H, m, $H_{5'}$), 2.97 (1H, m, H_{4a}), 3.09 (1H, m, $H_{5''}$), 3.15 (1H, m, $H_{4''}$), 3.15 (1H, m, $H_{2''}$), 3.26 (1H, dd, 8.4, 9.0, $H_{3''}$), 3.55 (1H, m, $H_{6''b}$), 3.62 (3H, s, Me_{10}), 3.79 (1H, dd, 3.6, 11.4, $H_{6''a}$), 4.27 (1H, dd, 9.6, 11.4, $H_{3\alpha}$), 4.40 (1H, dd, 3.6,11.4, $H_{3\beta}$), 4.53 (1H, d, 7.8, $H_{1''}$), 5.12 - 5.22 (2H, m, $H_{8'}$), 5.14 (1H, m, H_6), 5.42 (1H, d, 3.0, $H_{1'}$), 5.64 (1H, m, $H_{7'}$), 7.37 (1H, d, 1.2, $H_{3'}$), 9.58 (1H, br t, $H_{11'}$) ($OH_{2''}$, $OH_{3''}$, $OH_{4''}$, $OH_{6''}$ not observed); **^{13}C NMR** (150 MHz, CD_3OD); δC: 14.5 (C_8), 29.8 ($C_{5'}$), 37.4 ($C_{9'}$), 37.8 (C_{4a}), 38.7 (C_5), 43.8 (C_4), 45.3 (C_7), 46.1 ($C_{10'}$), 46.3 (C_{7a}), 47.8 ($C_{6'}$), 52.7 (C_{10}), 63.1 ($C_{6''}$), 68.3 (C_3), 71.5 ($C_{4''}$), 74.5 ($C_{5''}$), 77.9 ($C_{2''}$), 78.3 ($C_{3''}$), 78.8 (C_6), 97.7 ($C_{1'}$), 98.0 ($C_{1''}$), 111.9 ($C_{4'}$), 120.5 ($C_{8'}$), 134.8 ($C_{7'}$), 153.3 ($C_{3'}$), 167.7 (C_{11}), 173.2 (C_9), 176.2 (C_1), 203.0 ($C_{11'}$); *m/s* (LSIMS) : 599 ($M+H^+$, 40%), 211 (100), 195 (73), 166 (32), 151 (42), 107 (30).

13.3.17 Loganin



(5)

10 mg of (5) was isolated as a white amorphous solid. Identified by complete assignment of ^{1}H and ^{13}C spectral data; $C_{17}H_{26}O_{10}$; exact mass calculated for M+H$^+$, $C_{17}H_{27}O_{10}$: 391.1604, found, 391.1614 (+/- 0.0010); **MP**: 222 - 225°C (lit.[78, 80], 223°C); $[\alpha]_D$: - 80° (c 0.1, MeOH) (lit.[80], - 82.1° (H_2O)); **FTIR**: 3430 (OH), 1722 (C=O), 1660 (C=C); **^{1}H NMR** (600 MHz, d_6-DMSO); δH: 0.97 (3H, d, 6.6, Me_8), 1.22 (2H, br s, OH), 1.43 (1H, ddd, 3.6, 11.4, 13.8, $H_{5\beta}$), 1.70 (1H, ddd, 6.6, 9.6, 11.4, H_7), 1.83 (1H, ddd, 4.8, 9.6, 9.6, H_{7a}), 2.05 (1H, dd, 7.8, 13.8, $H_{5\alpha}$), 2.93 (1H, dd, 7.8, 9.0, $H_{2'}$), 2.96 (1H, dddd, 1.2, 7.8, 9.6, 11.4, H_{4a}), 3.10 (1H, ddd, 3.6, 5.4, 9.0, $H_{5'}$), 3.13 (1H, dd, 9.0, 9.6, $H_{3'}$), 3.20 (1H, dd, 9.0, 9.6, $H_{4'}$), 3.41 (1H, dd, 5.4, 11.4, $H_{6'b}$), 3.61 (3H, s, Me_{10}), 3.64 (1H, dd, 3.6, 11.4, $H_{6'a}$), 3.86 (1H, dd, 3.6, 11.4, H_6), 4.40 (1H, br d, OH), 4.46 (1H, d, 7.8, $H_{1'}$), 4.54 (1H, br t, OH_6), 5.04 (1H, br d, $OH_{2'}$), 5.11 (1H, d, 4.8, H_1), 7.35 (1H, d, 1.2, H_3); **^{13}C NMR** (150 MHz, d_6-DMSO); δC: 13.5 (C_8), 30.8 (C_{4a}), 39.8 (C_7), 42.0 (C_5), 44.7 (C_{7a}), 50.7 (C_{10}), 61.1 ($C_{6'}$), 70.1 ($C_{4'}$), 72.1 (C_6), 73.1 ($C_{2'}$), 76.7 ($C_{3'}$), 77.3 ($C_{5'}$), 96.1 (C_1), 98.5 ($C_{1'}$), 111.9 (C_4), 150.5 (C_3), 167.1 (C_9);*m/s* (LRP +LSIMS): 413 (M+Na$^+$, 73%), 391 (M+H$^+$, 5), 304 (10), 282 (20), 242 (100), 242 (100), 205 (8), 179 (37), 142 (18).

13.3.18 Initial treatment FRACTION 8

23 g of Fraction 8 was separated from the original methanol extract [828 g] using 90% ethyl acetate/10% methanol as the eluting solvent.

5 g of Fraction 8 was subjected to flash chromatography (silica, isocratic, 90% ethyl acetate/10% methanol) with twelve fractions being separated. Fractions 8.3 and 8.4 were combined to form 8.(3) [1.44 g].

Fraction 8.(3) [1.44 g] was dissolved in 100 mL chloroform - 100 mL water and the two layers separated. The water layer was labelled 8.(3).H_1 [250 mg]. The water was removed (freeze dried) and the residue was then placed in two vials and labelled 8.(3).H_1.MV.[X] [100 mg] and 8.(3).H_1.MV [150 mg].

100 mg of 8.(3).H_1.MV.[X] was subjected to flash chromatography (silica, isocratic, 90% chloroform/10% methanol) with two fractions being collected.

Fraction 8.(3).H_1.MV.[X].2 was then analysed by analytical normal phase HPLC (gradient, 100% ethyl acetate to 90% ethyl acetate/10% methanol, 254 nm, 2 mL/min., 30 min.) where two clearly defined, UV active components could be observed. The sample was then subjected to analytical normal phase HPLC (gradient, 100% ethyl acetate to 90% ethyl acetate/10% methanol, 254 nm, 2 mL/min., 30 min.) where two fractions were collected and shown to be pure.

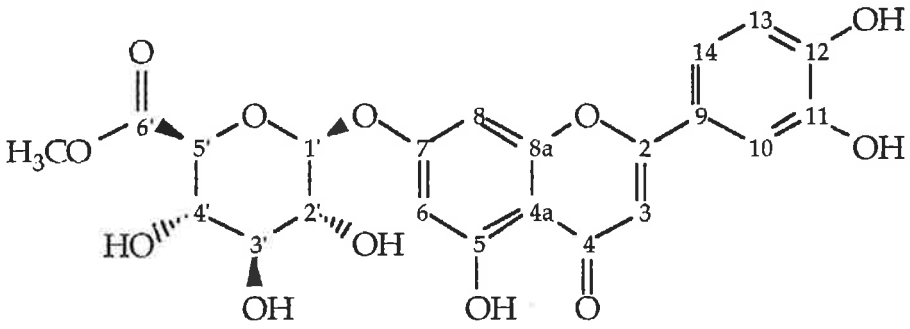
Luteolin-7-*O*-glucuronide methyl ester (**110**) [26 mg] was isolated and its purity confirmed by normal phase analytical HPLC (R_f = 6.75 min, gradient, 100% ethyl acetate to 90% ethyl acetate/10% methanol, 254 nm, 2 mL/min., 30 min.).

2-C-(Hydroxymethyl)-D-ribonic acid-γ-lactone (**111**) [5 mg] was isolated and its purity confirmed by analytical normal phase HPLC (R_f = 8.56 min., gradient, 100% ethyl acetate to 90% ethyl acetate/10% methanol, 254 nm, 2 mL/min., 30 min.).

Fraction 8.(3).H_1.MV [150 mg] was analysed by reverse phase analytical HPLC (gradient, 100% water to 30% water/70% methanol, 254 nm, 2 mL/min., 30 min.), where two peaks were observed.

Preparative reverse phase HPLC (gradient, 100% water - 30% water/70% methanol, 254 nm, 5 mL/min. 30 min.) was then used to purify these compounds with the result that 6 mg of each compound was purified. One of these compounds was L-*threo*-guaiacyl glycerol (**112**) [6 mg]. The compound collected from the second peak was unstable and its structure could not be determined. This separation procedure is outlined in **Diagram 9.1**, Chapter 9.1, pg 128.

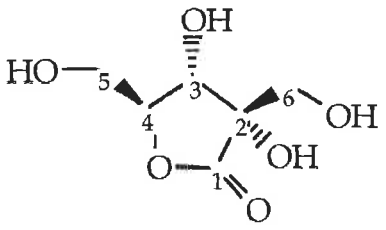
13.3.19 Luteolin-7-*O*-glucuronide methyl ester



(**110**)

26 mg of (**110**) was isolated as yellow crystals. Identified by complete assignment of ^{1}H and ^{13}C spectral data; $C_{22}H_{20}O_{12}$; exact mass calculated M+H$^+$, $C_{22}H_{21}O_{12}$: 477.1032, found, 477.1016 (+/- 0.0016); **MP**: 278-280°C; $[\alpha]_D$: + 1.3° (c 0.1, MeOH); **UV**: λ_{max}, (methanol) 254, 267, 346 (lit.[91], 254, 268, 348); **FTIR**: 3400 (OH), 1730 (C=O), 1660 (C=C); **^{1}H NMR** (600 MHz, d_6-DMSO/D_2O); δH: 3.26 (1H, dd, 7.8, 9.0, $H_{2'}$), 3.37 (1H, t, 9.0, $H_{3'}$), 3.40 (1H, dd, 9.0, 9.6, $H_{4'}$), 3.59 (3H, s, OCH_3), 4.23 (1H, d, 9.6, $H_{5'}$), 5.31 (1H, d, 7.8, $H_{1'}$), 6.45 (1H, d, 2.4, H_6), 6.72 (1H, s, H_3), 6.80 (1H, d, 2.4, H_8), 6.87 (1H, d, 8.4, H_{13}), 7.39 (1H, d, 1.2, H_{10}), 7.42 (1H, dd, 1.2, 8.4, H_{14}) (OH_5, OH_{11}, OH_{12}, $OH_{2'}$, $OH_{3'}$, $OH_{4'}$ not observed); **^{13}C NMR** (150 MHz, d_6-DMSO/D_2O); δC: 52.4 ($C_{7'}$), 71.9 ($C_{4'}$), 73.4 ($C_{2'}$), 74.7 ($C_{3'}$), 75.2 ($C_{5'}$), 94.2 (C_8), 98.6 ($C_{1'}$), 99.7 (C_6), 103.2 (C_3), 105.9 (C_{4a}), 113.2 (C_{10}), 115.5 (C_{13}), 119.2 (C_{14}), 121.6 (C_9), 145.5 (C_{11}), 149.3 (C_{12}), 156.0 (C_{8a}), 161.8 (C_5), 162.2 (C_7), 164.7 (C_2), 169.6 ($C_{6'}$), 181.4 (C_4); *m/s* (LSIMS in glycerol): 477.1 (M+H$^+$, 50%), 287 (100), 239 (15).

13.3.20 2-C-(Hydroxymethyl)-D-ribonic acid-γ-lactone

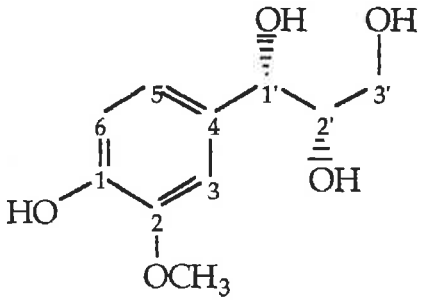


(**111**)

5 mg of (**111**) was isolated as a yellow amorphous solid. Identified by complete assignment of ^{1}H and ^{13}C spectral data; $C_6H_{10}O_5$, exact mass calculated for M+H$^+$, $C_6H_{11}O_5$: 179.0555, found, 179.0538 (+/- 0.0017); **MP**: 82 - 89°C (lit.[93], 88 - 89°C); $[\alpha]_D$: - 76° (c 0.1, MeOH) (lit.[93], - 74° (c 2.1, H_2O); **FTIR** (nujol): 3440 (OH); **^{1}H NMR** (600 MHz, d_6-DMSO/D_2O); δH: 3.30 (1H, d, 10.2, H_{6b}), 3.40 (1H, d, 10.2, H_{6a}), 3.44 (1H, dd, 5.4, 12.6, H_{5b}), 3.72 (1H, dd, 2.4, 12.6, H_{5a}), 4.07 (1H, d, 8.4, H_3), 4.11 (1H, ddd, 2.4, 5.4, 8.4, H_4) (OH_2, OH_3, OH_5, OH_6 not observed); **^{13}C NMR** (150 MHz, d_6-

DMSO/D_2O); δC: 60.2 (C_5), 60.5 (C_6), 67.9 (C_3), 73.8 (C_2), 83.4 (C_4), 175.9 (C_1); *m/z* (LRP +LSMIS): 179 ($M+H^+$, 100%), 149 (50), 123 (70).

13.3.21 L-*threo*-Guaiacyl glycerol



(112)

6 mg of (**112**) was isolated as a white amorphous solid. Identified by complete assignment of ^1H and ^{13}C spectral data; $C_{10}H_{14}O_5$; exact mass calculated for $C_{10}H_{14}O_5$: 214.0084, found, 214.0080 (+/- 0.0004); **MP**: 131 - 134°C (lit.[94,95],132 - 133 °C); $[\alpha]_D$: + 20° (c 0.1, MeOH) (lit.[94,5], + 23° (c 0.6, ethanol); **FTIR** (nujol): 3450 (OH), 1660 (C=C); **UV**: λ_{max}, ($CHCl_3$) 235, 322 (lit.[95], 235, 322); **^1H NMR** (600 MHz, d_6-DMSO, CD_3OD); δH: 3.850 (3H, s, OCH_3), 3.314 (1H, dd, 4.8, 10.2, $H_{3'b}$), 3.440 (1H, dd, 3.0, 10.2, $H_{3'a}$), 3.613 (1H, ddd, 3.0, 4.8, 6.6, $H_{2'}$), 4.468 (1H, d, 6.6, $H_{1'}$), 6.760 (1H, dd, 1.8, 7.8, H_5), 6.724 (1H, d, 7.8, H_6), 6.919 (1H, d, 1.8, H_3) (OH_1, $OH_{1'}$, $OH_{2'}$, $OH_{3'}$ not observed); **^{13}C NMR** (150 MHz, d_6-DMSO, CD_3OD); δC: 56.0 (OCH_3), 64.9 ($C_{3'}$), 75.7 ($C_{1'}$), 78.5 ($C_{2'}$), 110.4 (C_3), 116.7 (C_6), 121.0 (C_5), 134.5 (C_4), 147.3 (C_1), 149.7 (C_2); *m/s* (LSIMS): 237 ($M+Na^+$, 100%).

13.3.22 Initial treatment FRACTION 9

Fraction 9 [75 g] was separated from the original methanol extract [828 g] using 75% ethyl acetate/25% methanol as the eluting solvent.

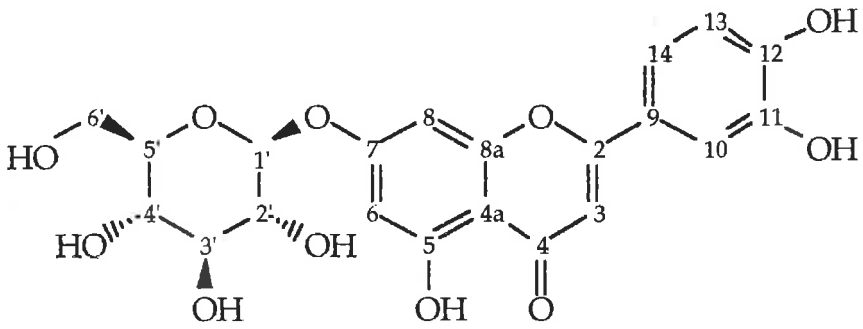
5 g of Fraction 9 was subjected to flash chromatography (silica, sequential, 80% chloroform/20 % methanol then 60% chloroform/40% methanol). Ten fractions in total were collected.

TLC analysis indicated that both Fractions 9.4 [60 mg] and 9.7 [45 mg] were relatively pure. Both these fractions were independently dissolved in 80% chloroform/20% methanol and left overnight.

Fraction 9.4a crystallized out to form yellow needles and was identified as luteolin-7-*O*-glucoside (**113**) [27 mg]. Its purity was confirmed on analytical reverse phase HPLC (gradient, 100% water to 100% methanol, 254 nm, 1 mL/min., 30 min., R_f = 13.45 min.).

9.7c formed a white solid [38 mg] and was recrystallized using 75% chloroform /25% methanol and the crystals were removed. These crystals were found to be 2-deoxy-D-*chiro*-inositol (**114**) [28 mg]. This separation system is outlined in **Diagram 10.1**, Chapter 10.1, pg 135.

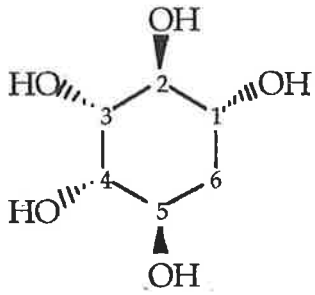
13.3.23 Luteolin - 7-*O*-glucoside



(**113**)

27 mg of (**113**) was isolated as yellow crystals. Identified by complete assignment of ^1H and ^{13}C spectral data; $C_{21}H_{20}O_{11}$; **MP**: 251 - 253°C (lit.[78, 80], 256-58°C); $[\alpha]_D$: + 1.3° (c 0.1, MeOH) (lit.[78], + 2° (H_2O/Me_2CO); **UV**: λ_{max}, (methanol) 254, 268, 348; **^1H NMR** (600 MHz, d_6-DMSO); δH: 3.17 (1H, t, 9.0, $H_{4'}$), 3.25 (1H, dd, 7.2, 9.6, $H_{2'}$), 3.29 (1H, dd, 9.0, 9.6, $H_{3'}$), 3.44 (1H, ddd, 5.4, 9.0, 9.6, $H_{5'}$), 3.48 (1H, dd, 5.4, 11.4, $H_{6'b}$), 3.71 (1H, dd, 9.6, 11.4, $H_{6'a}$), 5.06 (1H, d, 7.2, $H_{1'}$), 6.43 (1H, d, 2.4, H_6), 6.73 (1H, s, H_3), 6.77 (1H, d, 2.4, H_8), 6.88 (1H, d, 8.4, H_{13}), 7.40 (1H, d, 2.4, H_{10}), 7.43 (1H, dd, 2.4, 8.4, H_{14}), 12.97 (1H, s, C_5-OH_5); (OH_{11}, OH_{12}, $OH_{2'}$, $OH_{3'}$, $OH_{4'}$, $OH_{6'}$ not observed); **^{13}C NMR** (150 MHz, d_6-DMSO); δC: 60.5 ($C_{6'}$), 69.5 ($C_{4'}$), 73.0 ($C_{2'}$), 76.3 ($C_{3'}$), 77.1 ($C_{5'}$), 94.6 (C_8), 99.4 (C_6), 100.0 ($C_{1'}$), 103.1 (C_3), 105.2 (C_{4a}), 113.5 (C_{10}), 115.9 (C_{13}), 119.1 (C_{14}), 121.2 (C_9), 145.7 (C_{11}), 149.9 (C_{12}), 156.8 (C_{8a}), 161.0 (C_5), 162.9 (C_7), 164.4 (C_2), 181.8 (C_4); *m/s* (ESI): 448 (100%).

13.3.24 2-Deoxy-D-*chiro*-inositol



(114)

28 mg of (**114**) was isolated as white crystals. Identified by complete assignment of ^{1}H and ^{13}C spectral data; $C_6H_{12}O_5$; molecular weight (-ve ion electrospray), 164; **MP**: 234 - 239°C (lit.[78], 235 - 237); $[\alpha]_D$: + 28° (c 0.1, MeOH) (lit.[78,98], + 25.6° (H_20); **FTIR**: 3340 (OH); **^{1}H NMR** (600 MHz, d_6-DMSO); δH: 1.55 (1H, ddd, 2.6, 10.7, 13.3, $H_{6\beta}$), 1.69 (1H, ddd, 4.3, 4.3, 13.3, $H_{6\alpha}$), 3.26 (1H, dd, 3.0, 3.6, H_4), 3.37 (1H, dd, 3.0, 9.0, H_3), 3.41 (1H, ddd, 2.6, 3.6, 4.3, H_5), 3.56 (1H, dd, 8.6, 9.0, H_2), 3.65 (1H, ddd, 4.3, 8.6, 10.7, H_1), 4.20 (1H, d, 6.0, C_3-OH_3), 4.31 (1H, d, 4.8, C_5-OH_5), 4.37 (1H, d, 4.2, C_4-OH_4), 4.45 (1H, d, 3.6, C_2-OH_2), 4.68 (1H, d, 3.0, C_1-OH_1); **^{13}C NMR** (150 MHz, d_6-DMSO); δC: 34.8 (C_6), 68.3 (C_1), 68.8 (C_5), 71.3 (C_3), 72.7 (C_2), 74.9 (C_4); *m/s* (ESI): 164 (100%).

13.4 Biological testing

13.4.1 Anti bacterial screening

Samples of *Staphylococcus aureus, Staphylococcus epididemus, Escherichia coli* and *Candida albicans* were obtained from the Pharmacy Department of the University of South Australia, as were suitably prepared agar plates.

1. Preparation of culture plates

Culture plates were prepared using the 'Streak Plate Method'.[99a]

- Nutrient agar was liquefied, cooled to 50°C and then poured into the bottom of the agar plate
- The agar plate was gently rotated, so that the media was evenly distributed
- The agar was allowed to cool, so as to slowly solidify
- The plate was then streaked using a continuous streak method.
- The bacterial samples were added to the plate using the loop method.
  a. The bacterial culture was supplied in a plastic tube, which was gently shaken.
  b. A loop of wire was heated in a Bunsen Burner until it was red-hot.

c. The cap of the tube was removed and the neck of the tube was flared with the flame.
d. When the loop was cooled (five seconds), the loop was dipped in the tube full of organisms, avoiding touching the sides of the tube.
e. Starting at the edge of the plate, with a loopful of organisms, the organisms were spread in a single continuous movement.
f. The plate was then rotated 180°. Without flaring the loop, the streaking of the other half of the plate was continued.

- The agar plates were left in an incubator until needed.

2. Preparation of extracts

All samples of crude extracts and semi - pure or pure samples were prepared in the same way.[99b]

- The samples were weighed accurately and then dissolved in a suitable solvent, ranging from hexane to methanol/water. The concentration of the sample was then determined
- Filter discs were then dipped half way into the sample, removed and then allowed to dry.
- The filter discs were then placed on the prepared agar plate, and incubated for twenty four to forty eight hours, when the plates were checked to determine if any inhibition of bacterial growth had occurred.

13.4.2 Cytotoxicity test

Procedure A

1. Preparation of plant extracts

- The plant extracts were weighed and solubilised in either water, ethanol, methanol or DMSO to form an initial concentration of 100 mg/mL.
- Stock solutions of each extract were prepared at 100 mg/mL and stored at - 20°C.
- Three-fold dilutions were initially used to determine the presence of any potential cytotoxicity.
- Controls for methanol, ethanol and DMSO were also included to ensure that any cytotoxicity was not due to the solvents, but to the extract.

2. Cell lines

Three cell lines were used.

- MM418E is an adherent human melanoma cell line.

- 5637 is an adherent human bladder carcinoma cell line.
- MM418E and 5637 were kept growing in RMPI 1640 medium, supplemented with 10% heat inactivated foetal calf serum (FCS), 2 mM L-glutamine, 50 μg/mL penicillin /streptomyocin and 10^{-5} M 2-mercaptoethanol.
- MDA MB 231 is an adherent human breast adenocarcinoma cell line.
- MDA MB 231 was kept growing in DMEM medium, supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 50 μg/mL penicillin/streptomycin, 10^{-5} M 2-mercaptoethanol and 50 μg/mL insulin.
- All cell lines were incubated at 37°C and 5 % CO_2.

3. Bringing up frozen cells

- The cryotube containing frozen cells was taken out of - 70°C storage.
- The cryotube was then held in the hand until warm
- It was then placed in a 37°C water bath until completely thawed.
- The contents of the cryotube was added to 8-9 mL of culture medium.
- This suspension was then mixed and then centrifuged at 1000 rpm for 10 minutes.
- The supernatant was removed and new culture media was added
- The pellet was then resuspended and mixed by inverting several times to wash cells.
- This suspension was then centrifuged again for a further 10 minutes at 1000 rpm.
- The supernatant was removed and the pellet resuspended in 3-4 mL of culture media
- This solution was then transferred to a 25 mL flask, with the cryotube repeatedly washed to ensure all cells were transferred.
- Culture medium was added to a final volume of 10 mL.
- The cells were allowed to culture at 37°C.

4. Subculture of cells.
MM418E and 5637 cell lines.

- Cells were observed for confluence.
- If confluent, adhering cells were scraped with a sterile, disposable cell scraper.
- The volume of the cell suspension was distributed equally between two or three flasks.
- The flasks were then returned to the incubator.

MDA MB 231 cell line

- This cell line showed little adherence and hence splitting was accomplished by simply distributing the volume of cell suspension between two flasks.

5. Freezing down cells

- The cell suspension was centrifuged at 1000 rpm for ten minutes
- The supernatant was removed and the cells resuspended in 10 mL culture media.
- The haemocytometer was loaded and then the cells counted using 40x magnification.
- The suspension was then centrifuged at 1000 rpm again.
- The suspension was then diluted with freezing medium (90% foetal calf serum, 10% DMSO) to produce a final concentration of 1×10^7 cells/mL.
- The pellet was then resuspended and one mL aliquot's added to each cryotube (properly labelled with cell type, date, concentration and initials).
- These cryotubes were then placed in the fridge for 30 minutes, then at - 20°C for a further 30 minutes and finally stored at - 70°C. (They were stored under nitrogen for longer storage).

6. Cytotoxicity assay.

- For two day incubations the cells were plated at 1×10^4 cells/well, while for five day incubations, the cells were plated at 2×10^4 cells/well.
- Cell viability was determined using an "aqueous, non-radioactive cell proliferation assay kit", supplied by Promega, category no. G5430. This colorimetric assay uses the addition of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) at a concentration of 333 μg/mL and 25 μM phenazine methosulfate (PMS). MTS is converted to formazin by the dehydrogenase enzyme found in metabolically active cells. The quantity of formazin (as measured by the UV absorbance at 490 nm) produced is directly proportional to the number of living cells in the culture.

7. Plate preparation.

- The plates used for this experiment were 96-well, flat-bottomed plates.
- The outer 36 wells were plated with 250 μL of sterile water to avoid evaporation effects, while the inner 60 wells were used.
- Column two of each plate was used as a negative control and contained 100 μL of serum - free culture medium and 100 μL of cells suspended in 2 % serum to produce a final concentration of 1 % serum.

- Column three of each plate was used as a positive control. 22:6 (a polyunsaturated fatty acid) proved to be the best positive control as it showed the most cytotoxicity against the cell lines.
- Columns four to eleven contained serial dilutions of the extract stock (initial concentration of 100 mg/mL).
- 150 µL of the first dilution was plated to column four and 50 µL was then removed and added to column five that contained 100 µL of serum free medium.

Procedure B

1. Cell lines
- Two cell lines were used.
- 5637 is a primary adherent bladder carcinoma cell line.
- 5637 was kept growing in RMPI 1640 medium, supplemented with 10% heat inactivated foetal calf serum (FCS), 2 mM L-glutamine, 12 µg/mL penicillin, 20 mM HEPES buffer, 16 µg/mL gentamicin, 12 µg/mL penicillin and 10^{-5} M 2-mercaptoethanol.
- HEL is a diploid human fibroblast, human embryonic lung cell line.
- HEL was kept growing in Dulbecco's modified eagle medium (DMEM) with 3.7 g/L sodium bicarbonate, 4.5 g/L glucose, 15 µM HEPES buffer, 10 % v/v FCS, 2 mM L-glutamine, 16 µg/mL gentamicin and 12 µg/mL penicillin.
- All cell lines were incubated at 37°C and 5 % CO_2.

2. Preparation of plant extracts
- The plant extracts were a crude water extract and crude fractions 4 and 9. These fractions had been found to have cell proliferation activity when tested previously.
- These extracts were weighed and dissolved in cell culture media containing 1% v/v FCS to form an initial concentration of 4 mg/mL.
- In a 96 - microtiter plate, 1/3 dilutions of this stock were produced to cover the concentration range of 0.914 to 2000 µg/mL in a volume of 100 µL/well.
- Six replicates were performed for each dilution.
- Cell culture medium was added to six wells of each plate to act as controls.
- To each well, 100 µL of a cell suspension containing 1 x 10^5 cells/mL (either 5637 or HEL cells) was added, to give a final volume of 200 µL/well.
- The final concentration of the extract covered the range 0.457 to 1000 µg/mL.
- Plates were incubated at 37°C in a humidified atmosphere of 5 % CO_2 in air for two to five days.

- After the incubation period cytotoxicity was assessed using the neutral red or MTT cytotoxicity assay.

3. MTT assay[100, 101]

i. Preparation of the stock solution of MTT

- MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide was dissolved at a concentration of 5 mg/mL in sterile PBS, then sterilised by filtration through a 0.22 μm sterile filter.
- The solution was stored for up to one month at 4°C, protected from light.

ii. Preparation of solubilisation solution[101]

- SDS was dissolved at a concentration of 20 % w/v in a solution of 50% DMF (N,N-dimethyl formamide), in purified water.
- The SDS was dissolved in the DMF solution at 37°C with gentle agitation.
- Once the SDS had dissolved, 2.5% of an 80% acetic acid solution was added.
- The pH of the solution was then adjusted to 4.7 by the drop wise addition of 1M HCl. The solution was stored at room temperature.

iii. Preparation of MTT/media

- Immediately prior to use, MTT was mixed with the appropriate media for the cell line to be tested.
- A concentration of 1.0 mg/mL was used for HEL and 5637 cells (ie. 2 mL of MTT stock mixed with 8 mL of HEL media).

iv. Cytotoxicity assay

- Cells were incubated in 96-well plates for the appropriate time period with the cytotoxic substance or media.
- After the given incubation period, the media was flicked out of the 96-well plate under sterile conditions.
- To each well (except the first row, which serve as a blank and contain no cells) was added 100 μL of the appropriate MTT/media for the cell line used.
- To each of the blank wells, 100 μL of media only was added.
- The plate was then incubated in a humidified atmosphere of 5 % CO_2 at 37°C for four hours.
- After the four hour incubation period, the plate was removed from the incubator and 100 μL/well of the solubiliser/stop solution was added to all wells, including the blanks.
- The plate was shaken at medium speed (Ratek Instruments Plate Shaker) for twenty minutes at room temperature.
- The absorbance (blanked against the first row), was measured at 570 nm (Multiscan ELISA reader).

4. Neutral Red Assay method.[102]

i. Preparation of Neutral Red stock solution

- A 0.4% neutral red solution was prepared by dissolving 40 mg of neutral red in purified water (Milli-Q), to 10 mL.
- The solution was then filtered through a 0.22 μm filter to sterilise it.

ii. Preparation of cell fixative

- A 10% calcium chloride solution was prepared by adding 13.23 g of $CaCl_2$ to 100 mL of Milli-Q water and stored at 4°C.
- 1.25 mL of a 40% formaldehyde solution was added to 10 mL of the 10 % calcium chloride solution and then made up to 100 mL using milli-Q water.
- The solution was stored at room temperature.

iii. Preparation of solubilisation solution

- 1 mL of glacial acetic acid was added to 50 mL of ethanol and 49 mL of Milli-Q water and stored at room temperature.

iv. Preparation of neutral red/media (day before testing).

- Neutral red was added to complete media at a final concentration of 40 μg/mL (ie 0.20 mL of 4% neutral red to 19.80 mL media).
- The media was then incubated overnight at 37°C to precipitate any dye crystals.
- The neutral red/media was then centrifuged at 2000 rpm (Clements 2000 centrifuge) for 10 minutes to remove the precipitated dye.

v. Cytotoxicity assay

- After the incubation period of cells with extract, the media was flicked out of the 96-well plate under sterile conditions.
- To each well (except the first row which will serve as a blank and contained no cells) was added 200 μL of neutral red/media.
- The plate was then incubated in a humidified box for three hours at 37°C, gassed with 5 % CO_2.
- After the three hour incubation, the neutral red/media solution was flicked out of the plate.
- To each well 100 μL of cell fixative was added, except for the control wells and left for 90 seconds.
- The fixative solution was removed by flicking the plate and tapping it on a dry absorbent cloth.
- Solubilisation solution (200 μL/well) was then added to each well including the blank wells.
- The plate was shaken at medium speed (Ratek Instruments Plate Shaker) for ten minutes at room temperature.

- The absorbance (blanked against the first row of cells) was measured at 540 nm (Multiscan ELISA reader).

Procedure C

1. Cell lines
- Two cell lines were used, HEL cells and Caco-2.
- HEL and Caco-2 were kept growing in Dulbecco's modified eagle medium (DMEM) with 3.7 g/L sodium bicarbonate, 20 mM HEPES buffer, 10 % FCS, 2 mM L-glutamine, 16 μg/mL gentamicin and 12 μg/mL penicillin.
- All cell lines were incubated at 37°C and 5 % CO_2.

2. Preparation of plant extracts
- The crude hexane, crude ethyl acetate extracts and fractions 8, 9, 10 and 11 were retested against two cell lines.
- These extracts were dissolved in cell culture to give an extract concentration of 1 mg/mL and sterilised by filtration through a 0.22 μm filter (Sartorius).
- Serial 1:2 dilutions of the extracts in media were prepared in a volume of 100 μm/well in a 96-well microtiter plate (three replicates per concentration).
- Controls of media only (100 μL/well) were performed in triplicate.
- 100 μL of a cell suspension containing 8 x 10^4 cells/mL was added to each well, including the controls, to give a final volume of 200 μL/well.
- The final concentration of each extract ranged from 15.6 to 500 μg/mL.
- The plates were incubated at 37°C, in a humidified atmosphere of 5 % CO_2 in air for seven days when cells in the control cells had grown to form a confluent monolayer.
- Cytotoxicity was assessed using the neutral red cytotoxicity assay.

3. Neutral Red Assay method - see previous outline of method, p 190.[102]

Procedure D

1. Cell lines
- Two cell lines were used, HEL cells and Caco-2.
- HEL and Caco-2 was kept growing in Dulbecco's modified eagle medium (DMEM) with 3.7 g/L sodium bicarbonate, 20 mM HEPES buffer, 10 % FCS, 2 mM L-glutamine, 16 μg/mL gentamicin and 12 μg/mL penicillin.
- All cell lines were incubated at 37°C and 5 % CO_2.

2. Preparation of plant extracts

- Eleven boiled aqueous extracts of various combinations of *Scaevola spinescens with Codonocarpus cotinifolius* were prepared and lyophilised.
- These extracts were stored at - 60°C and then thawed to room temperature when needed.
- Extracts were dissolved in cell culture to give an extract concentration of 1 mg/mL and sterilised by filtration through a 0.22 μm filter (Sartorius).
- Serial 1:2 dilutions of the extracts in media were prepared in a volume of 100 μm/well in a 96-well microtiter plate (three replicates per concentration).
- Controls of media only (100 μL/well) were performed in triplicate.
- 100 μL of a cell suspension containing 8 x 10^4 cells/mL was added to each well, including the controls, to give a final volume of 200 μL/well.
- The final concentration of each extract ranged from 15.6 to 500 μg/mL.
- The plates were incubated at 37°C, in a humidified atmosphere of 5 % CO2 in air for seven days when cells in the control cells had grown to form a confluent monolayer.
- Cytotoxicity was assessed using the neutral red cytotoxicity assay.

3. Neutral Red Assay method - see previous outline of method, p 190.[102]

13.4.3 Anti viral testing

1. Preparation of plant extracts

- Six compounds were tested for anti viral activity.
- They were tested at a concentration of 50 μg/mL in cell culture media in anti viral assays.
- To aid solubilisation in cell culture media, the compounds were pre dissolved in either ethanol, methanol or DMSO.
- The final concentrations of these solvents did not exceed 1 % v/v. At this concentration, none of the solvents had antiviral activity against the three viruses tested.

2. Viruses

- Three viruses, representing three different viral families were chosen, herpes simplex type 1 (HSV1), Ross River virus strain T48 (RRV), and poliovirus type 2.
- HSV1 and RRV were grown in Vero cells (African green monkey kidney) and poliovirus was grown in BGM (Buffalo green monkey kidney) cells.

- Cells were grown with Dulbecco's modified eagle medium (DMEM) with 3.7 g/L sodium bicarbonate, 4.5 g/L glucose, 15 mM HEPES buffer, 10 % FCS, 2 mM L-glutamine, 16 μg/mL gentamicin, 12 μg/mL penicillin and heat inactivated FCS (5 % v/v).
- All cell lines were grown in a humidified atmosphere of 5 % CO_2, in air.

3. Anti viral and cytotoxicity assays.

- To test the extracts for anti-HSV, anti-RRV and anti-polio activity, inhibition of virus-induced cytopathic effect was measured.
- Vero (HSV and RRV assay) or BGM cells (polio assay) were seeded into a 96-well microtiter plate at an initial concentration of 8×10^3 cells/well.
- Cells were incubated for four to six hours at 37°C in a humidified CO_2 atmosphere.
- One series of triplicate wells was then infected with virus at a multiplicity of infection (MOI) of approximately 0.02 $TCID_{50}$ units/cell.
- The second series of wells was mock infected with media only.
- Two-fold serial dilutions of the compounds were then added in the two series of triplicate wells to allow simultaneous determination of anti viral and cytotoxic effects.
- Controls of mock infected cells without extract treatment and untreated cells with virus were included in triplicate on each plate.
- Cultures were incubated until the wells containing untreated cells infected with virus showed complete (100 %) cytopathic effect (40 - 48 hours for polio; 50 hours for RRV and 96 hours for HSV1) and cells in mock-infected, untreated cells had grown to form a near confluent monolayer.
- Mock-infected cells treated with the serial dilutions of the extract were examined microscopically for cytotoxic effects.
- Microtiter plates were then washed with PBS to remove any detached cells and cell debris, fixed and stained with crystal violet, 0.5 % in water : ethanol : formaldehyde: sodium chloride (400 : 190 : 7 : 1).
- Cell viability was quantified using crystal violet cytotoxicity assay method.[103]

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APPENDICES

Appendix A Botanical description of *Scaevola spinescens*.

From: 'Flora of Australia', Volume 35, (Australian Government Publishing Service: Canberra, 1992), p. 97-99.[12]

Scaevola spinescens R.Br., Prodr. 586 (1810)

Crossotoma spinescens (R.Br.) Vriese, *Natuurk, Verh. Holl. Maatsch. Wetensch. Haarlem* ser. 2, 10: 36 (1854); *Lobelia spinescens* (R.Br.) Kuntze, Revis. *Gen. Pl.* 2: 378 (1891). T: near the shores of Fowler Bay, [S.A.], R.Brown; lecto: BM, *fide* R.C.Carolin, *Telopea* 3: 491 (1990); *isolecto*: K.

S. oleoides DC., *Prodr.* 7(2): 512 (1839); *Crossotoma oleoides* (DC.) Vriese, *Natuurk. Verh. Holl. Maatsch. Wetensch. Haarlem* ser. 2, 10: 37 (1854). T: interior of N.S.W., *A.Cunningham;* holo: G-DC; iso: BM?,K.

S. lycoides DC., *Prodr.* 7(2): 512 (1839); *Crossotoma lycoides* (DC.) Vriese, *Natuurk. Verh. Holl. Maatsch. Wetensch. Haarlem* ser. 2, 10: 38 (1854). T: Dampier Archipelago, [W.A.], *A.Cunningham;* holo: G-DC; iso: K.

S. spinescens var. *rufa* E.Pritzel in F.L.E. Diels and E.Pritzel, *Bot. Jahrb. Syst.* 35: 568 (1905). T: near Kalgoorlie, W.A., Nov. 1900, *L.Diels 1685;* holo: ?B (destroyed).

Illustrations: K. Krause, *Pflanzenr.* 54: 137, fig. 26H-L (1912); E.R.Rotherham *et al., Fl. Pl. New South Wales & S. Queensland* 150, fig. 488 (1975); G.M.Cunningham *et al., Pl. W. New South Wales* 638 (1981).

Rigid, divaricate shrub to 2 m tall, glabrous or with substellate scurfy hairs, greyish when young; dwarf branchlets often spinescent. Leaves often clustered on branchlets, sessile, obovate to linear, entire, thick; lamina 9-36 mm long, 1-6 mm wide. Flowers solitary in axils; peduncle slender, 5-20 mm long; bracteoles linear, 2-5 mm long, +/- equal to ovary. Sepals rim-like, to 1 mm long. Corolla 9-16 mm long, scurfy or glabrous outside, densely bearded inside, white or yellowish, occasionally with purple veins; barbulae broad, prominent, simple; wings to 1 mm wide, laciniate. Indusium to 2 mm wide, with scattered hairs above. Fruit

ovoid, 5-8 mm long, glabrous, black or purplish. $n = 8$, W.J.Peacock, *Proc. Linn. Soc. New South Wales* 88: 8 (1963). *Currant Bush, Maroon Bush.* Figs 40E, 53.

Occurs throughout the drier parts of all mainland States; grows on hillsides and on stony plains. Flowers most of the year.

WA: c. 14.5 km N of Learmouth, *A.S.George 1278* (PERTH). N.T.: 3 km S of Yuendumu, *T.S.Henshall 2896* (DNA). S.A.: Dalhousie Springs, *D.E.Symon 9324* (AD). Qld: Whynot Stn, c. 38 km WSW of Quilpie, 15 Nov. 1954, *L.S.Smith* (BRI). N.S.W.: Mt Oxley near Bourke, 13 July 1958, *C.K.Ingram* (NSW). Vic.: Barneys Track, Hattah Lakes Natl Park, *G.W.Anderson 16* (MEL).

A very variable species. The dwarf branchlets distinguish it from all other shrubby species except *S. tomentosa* but that species has markedly stellate hairs. Used by Aborigines for various medicinal purposes including, in W.A., an infusion from the form on the Eastern Goldfields for alleviating the pain of cancer (E.Reid, *Rec. W. Austral. Pl. Used by Aboriginals as Medicinal Agents*, Western Australian Institute of Technology, 1977; E.V. Lassak and T.McCarthy, *Austral. Medicinal Pl.* 138, 1983).

Appendix B Phytochemical screening of *Scaevola* genera

From: Collins, D.J., Culvenor, C.C.J., Lamberton, J.A., Loder, J.W. and Price, J.R., 'Plants for Medicines', (CSIRO Publications : Victoria, 1990), p. 42-43.[11]

Table B.1 Results of the CSIRO screening of twelve species of *Scaevola*[11]

Family, Species	Locality[A], Voucher No[B]	Plant part[C]	Alkaloid Test[D]			Pharmacol.(P)[E]
			Mayers	Silicotungstic	Other	Anti-tumour(AT)
Goodeniaceae						
Scaevola aemula R.Br.	near Augathella, Q (WTJ 1920)	wp	++	++		AT-
		Lf,st	-		0.006%	
Scaevola albida (Smith) Druce	near Rendlesham, SA (Court 2673)	Lf,st	-	-		AT-
Scaevola angustata Carolin [S.nitida R.Br.]	Beachport, SA (Court 2703)	Lf,st	-	-		AT-
Scaevola crassifolia Labill.	Boddy's Nursery, Geelong, V	Lf	-			
Scaevola densevestita Domin	near Cloncurry, Q (CCJC 391)	Lf	++	-		
Scaevola bispida Cav.	Stanthorpe, Q (WTJ 2155)	Lf,st			0.001%	AT-
Scaevola microcarpa Cav.	Boddy's Nursery, Geelong, V	Lf	-	-		
Scaevola oppositifolia R.Br. [S. enantiophylla F.Muell.]	Mission Beach, Q (W.T. Jones)	wp	-	-		AT-
Scaevola ovalifolia R.Br.	near Devil's Marbles, NT (CCJC 341)	Lf	-	-		
Scaevola parviflora F.Muell.ex Benth.	near Barrow Creek, NT (CCJC 327)	Lf	-			
Scaevola sericea Vahl [S.frutescens (Mill.) Krause]	Mission Beach, Q (WTJ 805)	rt,Lf,st			Webb-	AT-

Key : wp = whole plant, Lf = leaf, st = stem, rt = root