BIOLOGICALLY ACTIVE NATURAL PRODUCTS FROM SOUTH AFRICAN MARINE INVERTEBRATES

THESIS

Submitted in Fulfilment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

of Rhodes University

by

GREGORY JOHN HOOPER

July 1996

DEDICATED TO MY PARENTS

WHO TAUGHT ME THAT:

"to love life through labour is to be intimate

with one of life's innermost secrets"

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

CHAPTER FOUR :   68

NEW METABOLITES FROM THE SOUTH AFRICAN SOFT CORAL *CAPNELLA THYRSOIDEA*

CHAPTER FIVE :   98

NEW METABOLITES FROM TWO SOUTH AFRICAN SOFT CORALS *ALCYONIUM* SPECIES A AND SPECIES B

CHAPTER SIX :

SESQUITERPENE HYDROQUINONES FROM THE SOUTH AFRICAN SOFT CORAL
ALCYONIUM FAURI

CHAPTER SEVEN :

EXPERIMENTAL

LIST OF FIGURES, SCHEMES AND TABLES

TABLE   Page

LIST OF ABBREVIATIONS

CIEIMS	Chemical Ionisation Electron Impact Mass Spectrometry
COSY	^{1}H - ^{1}H homonuclear COrrelation SpectroscopY
DEPT	Distortionless Enhancement by Polarisation Transfer
EIMS	Electron Impact Mass Spectrometry
FABMS	Fast Atom Bombardment Mass Spectometry
GC-MS	Gas Chromatography - Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectometry
HRFABMS	High Resolution Fast Atom Bombardment Mass Spectometry
IR	InfraRed
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Enhancement
NOEDS	Nuclear Overhauser Enhancement Difference Spectroscopy
ROESY	Rotating frame nuclear Overhauser Enhancement SpectroscopY
RT	Room Temperature (25 ± 2 °C)
SCUBA	Self Contained Underwater Breathing Apparatus
TLC	Thin Layer Chromatography
UV	UltraViolet

br	broad (used in conjunction with s, d or t)
d	doublet
eV	electron Volt
m	multiplet
q	quartet
s	singlet
t	triplet

ABSTRACT

This thesis describes the chemical and biological investigation of the extracts of six different marine invertebrate organisms collected along the South African coastline. The work on these extracts has resulted in the isolation and structural elucidation of twenty-one previously undescribed secondary metabolites.

The history of marine natural product chemistry in South Africa has not previously been reviewed and so a comprehensive review covering the literature from the 1940's up until the end of 1995 is presented here.

The marine ascidian *Pseudodistoma* species collected in the Tsitsikamma Marine Reserve was shown to contain four new unsaturated amino alcohols [47], [48], [49] and [50] which were isolated as their acetyl derivatives. These compounds exhibited strong antimicrobial activity.

Four new pyrroloiminoquinone alkaloids, the tsitsikammamines A [90] to D [93], were isolated from a new genus of Latrunculid sponge collected in the Tsitsikamma Marine Reserve. These highly pigmented compounds also possessed strong antimicrobial activity.

An investigation of two phenotypic colour variants of the soft coral *Capnella thyrsoidea* resulted in the isolation of the known steroid 5α-pregna-1,20-dien-3-one [97] and an additional six new metabolites, 16β-hydroxy-5α-pregna-1,20-dien-3-one 16-acetate [98], 3α,16β-dihydroxy-5α-pregna-1,20-diene 3,16-diacetate [99] and four xenicane diterpenes, the tsitsixenicins A [100] to D [103]. This is the first reported isolation of xenicane diterpenes from the soft coral family Nephtheiidae. Tsitsixenicin A and B showed good anti-inflammatory activity by inhibiting superoxide production in both rabbit and human cell neutrophils.

A further four new metabolites were isolated from two soft corals which could only be identified to the genus level and were designated *Alcyonium* species A and species B. *Alcyonium* species A was collected in the Tsitsikamma Marine Reserve and yielded two new polyhydroxysterols, cholest-5-ene-3β,7β,19-triol 19-acetate [121] and cholest-5,24-diene-3β,7β,19-triol 19-acetate [122]. The soft coral *Alcyonium* species

B was collected off Aliwal Shoal and was found to contain two known xenicane diterpenes, 9-deacetoxy-14,15-deepoxyxeniculin **[110]** and zahavin A **[16]**, and two new xenicane diterpenes, 7-epoxyzahavin A **[123]** and xeniolide C **[124]**. Compounds **[110]**, **[16]** and **[123]** exhibited strong anti-inflammatory activity and compounds **[110]** and **[16]** showed good antithrombotic activity.

The endemic soft coral *Alcyonium fauri* collected at Riet Point near Port Alfred yielded the new sesquiterpene hydroquinone rietone **[141]** in high yield, together with the minor compounds 8'-acetoxyrietone **[142]** and 8'-desoxyrietone **[143]**. Rietone exhibited moderate activity in the NCI's *in-vitro* anti-HIV bioassays.

CHAPTER ONE

INTRODUCTION

1

1.1 INTRODUCTION

Natural products from terrestrial sources have long provided a valuable source of useful pharmaceuticals, for example, penicillin from bread mould, aspirin from the bark of willow trees, cardiac glycosides from the *digitalis* plant, morphine from opium poppies and insulin from cattle and sheep. It has been estimated that marine species comprise approximately half of the total global biodiversity[1] and hence it seems reasonable to assume that the marine environment offers an enormous potential resource for novel compounds with possible pharmaceutical value. Added to this is the fact that marine invertebrate organisms have evolved over millions of years in a fairly stable environment and so have had the luxury of time to divert their energy resources into the development of toxic secondary metabolites as an integral part of a chemical survival strategy[2].

It is only in the last three decades that marine natural products as a research field has evolved and grown considerably. There are many reasons which contribute to this late development. Firstly, man is primarily land based and so has concentrated on utilizing his immediate terrestrial resources. Secondly, the use of SCUBA and submersibles which have given access to the marine environment is relatively new technology, and thirdly, there is a distinct lack of marine taxonomic literature and taxonomic studies in many parts of the world. The first comprehensive review of marine natural products did not appear until 1973[3] and since then a host of papers have been published detailing the identification of novel marine natural products often with interesting biological activity. The field has been reviewed with regularity, in particular through the efforts of Faulkner[4-14] and Scheuer[3,15-23]. Faulkner has recently estimated that approximately 6500 marine natural products have been isolated[24] and these have the following phylogenetic distribution (**FIGURE 1.1**).

From these data it is clear that the international research effort has concentrated on soft bodied, sessile invertebrate phyla. The rationale behind this trend is the fact that these phyla produce an abundance of secondary metabolites for use in predator

defence, antifouling, inhibition of overgrowth, protection from ultraviolet radiation and as mediators in the competition for settling space[25,26]. It is these metabolites which may be of pharmaceutical value.

25%

33%

24%

18%

ALGAE
SPONGES
COELENTERATES
OTHER (ascidians;opisthobranch molluscs;echinoderms;bryozoans)
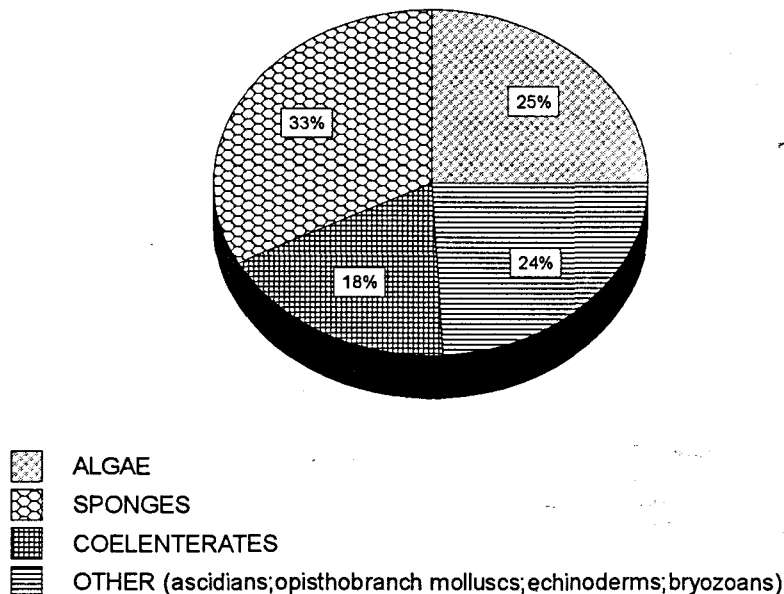
FIGURE 1.1 Phylogenetic distribution of marine natural products isolated by researchers worldwide until 1994.

With the exponential growth in marine natural product research several questions have arisen and problems have been encountered which require urgent attention. It has been suggested that many marine natural products are in fact not produced independently by the invertebrate but are a consequence of a symbiotic relationship between the host and micro-organisms[27]. This has opened up a whole new research area fraught with new problems. Symbionts often have unknown nutritional needs so that although the culture of marine microbes has the potential to provide large quantities of natural products[1] only around 10 per cent of marine bacteria observed by microscopic examination can be cultured in media employing methods designed for terrestrial bacteria[28]. Faulkner has suggested that the problem of defining the role of symbionts in producing marine natural products will indeed challenge researchers for years to come[24].

3

A major hindrance in the search for new drugs is that the yields of pure compounds from marine invertebrates are so small that the natural resource cannot provide sufficient material for commercial development. Although extensive *in vitro* testing of a drug can be accomplished with milligram quantities, usually several grams are required for full *in vivo* evaluation and hence an adequate supply of the drug can become a major limiting factor in the completion of clinical trials[29]. Previously the approach has been to collect larger quantities of the material. For example, it is claimed that 1600 kg of the sea hare *Dolabella auricularia* was collected to obtain 10 mg of dolastatin 13 which shows activity against melanoma[30], in excess of 2400 kg of the Indo-Pacific sponge *Spirastrella spinispirulifera* was required to yield a few milligrams of the spongistatin anticancer compounds[31] and 10 000 gallons of a bryozoan was needed to provide sufficient bryostatin 1 for clinical trials[32].

Recently this approach has incited criticism with protests that the ocean is being plundered in the name of science[30] and rightly so taking into account the above examples. There is a definite need to consider environmental implications as little is known of just how fragile reef ecosystems are and how rare the collected invertebrates may be. It is also often the developing countries which are sacrificing their precious natural resources[33]. The need to address these problems has fortunately been recognised with the United Nations Biodiversity Convention providing a basis to try and resolve these issues[34]. Strict control measures need to be introduced into legislation and a greater effort made to investigate the possible aquaculture of marine organisms to ensure a better relationship between environmentalists, scientists and multinational pharmaceutical companies.

1.2 HISTORY OF MARINE NATURAL PRODUCT RESEARCH IN SOUTH AFRICA

Arguably, the start of marine natural product chemistry, *per se*, in South Africa began during the 1940's and gained momentum during the 1950's. At this time the National Chemical Research Laboratory in Pretoria was internationally recognised as

a leading laboratory for the isolation and synthesis of long chain fatty acids. Initial work focused on the characterisation of highly unsaturated fatty acids and alcohols derived from South African fish oils[35-39]. The quality of this research was acknowledged in the Annual Report of the Chemical Society for 1954[40], which called the paper by Silk and Hahn[39], on the the isolation of hexadeca-6,9,12,15-tetraenoic acid from pilchard oil, an "outstanding achievement".

In Grahamstown, in the late 1950's, Nunn and co-workers pioneered research into the polysaccharides of seaweeds. Particular attention was paid to red seaweeds, resulting in the isolation of several polysaccharides from *Gracilaria confervoides*[41], *Porphyra capensis*[42] and *Hypnea specifera*[43]. This work continued in the 1960's and into the early 1970's. During this time Parolis completed an investigation of the sulphated polysaccharides produced by the red seaweeds *Aeodes orbitosa* and *Phyllymena cornea*[44], and then joined Nunn in supervising further research into sulphated polysaccharides from the red seaweeds *Pachymenia carnosa*[45], *Anatheca dentata*, *Chaetangium erinaceum*[46], and *Aeodes ulvoida*[47].

In 1972 a report was compiled by the South African Prime Minister's Planning Advisory Council's Committee on Ocean Affairs entitled "Drugs from the Sea"[48] which provided a fairly detailed review of the medicinal potential of marine plants and animals and concluded with the following statement:

> "Very little attention has as yet been paid in South Africa to the recovery of drugs from the sea. This field offers exciting and rewarding challenges to South African scientists. Once research brings down the unit cost, the sea may offer a vast potential for the production of drugs for South Africa."

Unfortunately, little heed was taken of this report and, with the exception of the research of Nunn and co-workers, the field of South African marine natural product chemistry remained largely unexplored. It was only in 1977 that consideration was

once again given to the abundant potential of the South African coast line for natural product research and it was around this time that a Natural Product Research Group was established at the University of Cape Town (UCT) under the direction of Elsworth and Cragg[49]. The aim of this group was to investigate primarily the terrestrial, and to a lesser extent, the marine natural product chemistry of indigenous South African species.

Initial marine research involved analysing the sterol composition of two oceanic and three intertidal species of annelid by combined GC-MS techniques in collaboration with Ballantine in Swansea[50]. It was reported that the oceanic annelids had more complex sterol profiles containing small quantities of 5α-stanols while the intertidal species had simpler profiles with the absence of 5α-stanols. It was also found that lugworm species from South Africa and south Wales exhibited almost identical sterol compositions. This research was further extended to the steroidal analysis of two sea anemones, the ribbed mussel *Aulacomya ater*, starfish *Marthasterias glacialis* and *Henricia ornata*, and the sea cucumber *Cucumeria frauenfeldii*[51,52]. More challenging projects involved attempts to characterise toxins from the sea hare *Notarchus leachi* and the puffer fish *Amblyrhynchotes honckenii*[52]. Difficulty was experienced in isolating and determining the pure compounds responsible for activity.

In 1979 Pettit visited UCT, providing fresh input and enthusiasm, and by the end of the year a nearly-pure neurotoxin had been isolated from *Notarchus leachi*[53]. It was obtained as a yellow oil with the molecular formula $C_{14}H_{29}NO_6$. Unfortunately the complete structure remained undetermined. Elsworth, while on sabbatical leave in Pettit's laboratory, investigated marmorastatin 1, a toxic glycoside from the sea cucumber *Bohadschia marmorata*[54]. FABMS was used to confirm the molecular formula of this compound as $C_{67}H_{110}O_{32}$. Acid hydrolysis of the isolate suggested a mixture of genins with marmorastatin 1 as the predominant glycoside. After various hydrogenation and acetolysis studies the partial structures [1], [2] and [3] were proposed for marmorastatin 1.
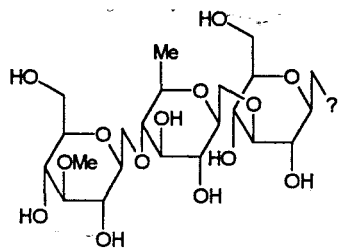
[1]
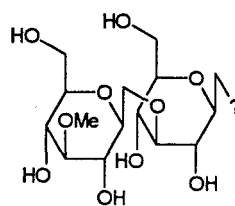
[2]
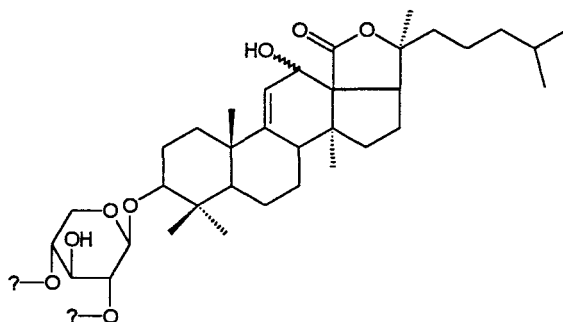
[3]

At the end of 1983 Elsworth put forward a proposal for establishing a separate Marine Natural Product Chemistry Unit at UCT. He argued that, with considerable funding available to other marine sciences such as oceanography, oceanology and the Benguela Project, it was high time that marine natural products received much needed attention[54]. Unfortunately his plea went unheeded.
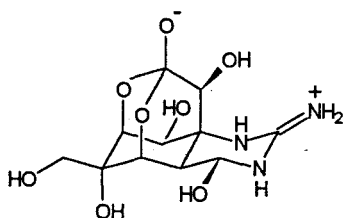
[4]
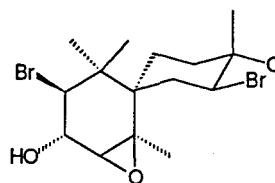
[5]

In 1986 results from the toxicity studies on the South African puffer fish *Amblyrhynchotes honckenii* were published[55]. It was assummed that tedrodotoxin **[4]**, previously isolated from a Japanese puffer fish, was the toxic principle. A new chamigrane **[5]** was isolated in 1989 from the red seaweed *Laurencia glomerata* by Elsworth in collaboration with Thomson in Aberdeen[56]. This brought an end to the contributions of UCT to South African marine chemistry.

7

Perhaps the most pharmacologically successful South African marine natural products to be isolated so far, as well as among the most environmentally controversial, are several spongistatins reported by Pettit[31,57]. An investigation of the antineoplastic constituents of a brightly coloured sponge *Spirastrella spinispirulifera* collected off the south coast of Africa was begun in 1973. However, increasingly large (up to 360 kg) recollections of the sponge up until 1980 proved inadequate for the chemical/biological research. A further 2409 kg of the sponge was recollected and this afforded 10.7 mg of colourless spongistatin 4 [6] and 12.9 mg of spongistatin 5 [7][31]. Evaluation of these two compounds against the US National Cancer Institute's (NCI) panel of 60 human cancer cell lines gave dramatic results and [6] and [7] were reported as being amongst the most potent of all substances tested in the NCI screen. Further work-up yielded 1.8 mg of spongistatin 8 [8] and 5.4 mg of spongistatin 9 [9][57] which revealed an overall potency comparable to or exceeding that of the previously isolated spongistatins. Importantly, spongistatin 9 along with spongistatin 1 (isolated from a Republic of Maldives' *Spongia* species[58]) appear to be the most potent cancer cell growth inhibitory antimitotic substances discovered to date.

The decline of marine chemistry at UCT again left the South African coast wide open for marine natural product research. In the early 1990's two different groups of researchers emerged to fill the void. At Rhodes University in Grahamstown, Davies-Coleman founded a Marine Pharmaceutical Discovery Programme in collaboration with Faulkner in California and later with SmithKline Beecham Pharmaceuticals (SKB) in the US[34]. Initial marine invertebrate collections were undertaken around the south east coast of South Africa but later extended to include the entire coast (**FIGURE 1.2**). At about the same time Kashman in Israel began to analyse marine invertebrate specimens supplied by Schleyer of the Oceanographic Research Institute in Durban and collected in the Sodwana Bay Marine Reserve situated on the north east coast of South Africa.

[6]

[7]

[8] R = H
[9] R = Cl

9

	RESEARCH GROUP	YEAR	LOCATION
1	Rhodes / SKB*	1994/5	Ponto do Ouro, Mozambique
2	Kashman	1993 -	Sodwana Bay Marine Reserve
3	Rhodes / SKB / Faulkner	1994	Aliwal Shoal, Umkomaas
4	Faulkner	1992	Wild Coast, Transkei
5	Rhodes	1992 -	Riet Point, Port Alfred
6	Rhodes	1992 -	Algoa Bay, Port Elizabeth
7	Rhodes	1992 -	Tsitsikamma Marine Reserve
8	Rhodes	1995	Mossel Bay
9	Rhodes / SKB	1996	Hout Bay, Cape Town

SKB*- SmithKline Beecham Pharmaceutical's marine drug discovery under the direction of Dr Brad Carté.

FIGURE 1.2 Marine invertebrate collection sites around the coast of southern Africa for the purpose of marine natural product research.

The following reviews the chemical compounds isolated and characterised by the two research groups until the end of 1995 but excludes compounds isolated by the author which are covered in later chapters. The research efforts of both groups have largely focused on the chemistry of soft corals, sponges, ascidians and molluscs and hence the work is presented here in this phylogenetic order.

In 1992, a survey of the marine invertebrates off the Wild Coast of the Transkei, which lies on the east coast of South Africa, resulted in the collection of ninety different sponges, ascidians and soft corals. Screening of the extracts of these invertebrates by Faulkner and co-workers revealed a rather disappointing incidence of bioactivity. A secondary screening of the crude extracts by ^{1}H NMR spectroscopy led to the investigation of a yellow soft coral, *Alcyonium valdiviae*. This apparently endemic soft coral[59] yielded the valdivone diterpene esters [10] - [14][60]. Although the valdivones proved inactive against a standard panel of bacteria and fungi, it was shown that valdivone A and B exhibited fairly strong inhibition of chemically-induced inflammation in the mouse ear assay. A curious observation in the report was the close relationship between the valdivones and the sarcodictyins[61,62], despite the fact that the producing organisms, *Alcyonium valdiviae* (Order Alcyonacea) and *Sarcodictyon roseum* (Order Stolonifera), belong to different orders of the class Alcyonaria. Williams reports that the only previous record of the occurrence of the genus *Sarcodictyon* in southern Africa is also from a location on the Transkei coast[59]. It would be interesting to ascertain whether this stoloniferous octocoral also contains diterpene esters analogous to the valdivones.
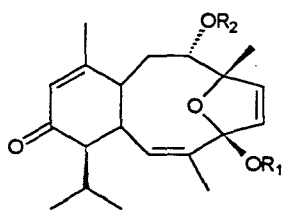
[10] R₁ = H   R₂ = a
[11] R₁ = H   R₂ = b
[12] R₁ = Me R₂ = a
[13] R₁ = Me R₂ = b
[14] R₁ = H   R₂ = c
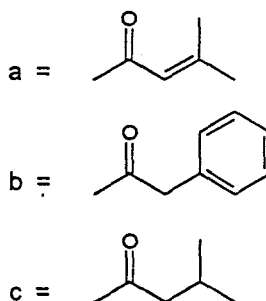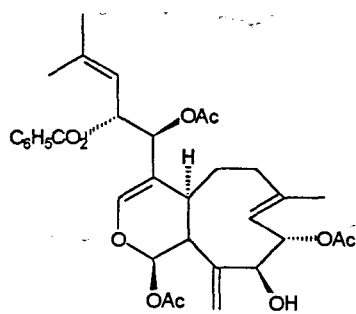
[15]
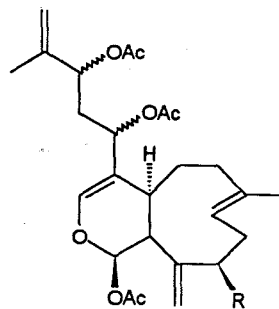
[16] R = H
[17] R = OH

Kashman and co-workers have had an ongoing interest in the comparison of Red Sea soft corals with their Indo-Pacific counterparts and have recently investigated two Indo-Pacific soft corals from two different reef-inhabiting genera, *Anthelia glauca* and *Alcyonium aureum*, collected from Sodwana Bay[63]. This species of *A. glauca*, in contrast to the Red Sea species[64], contained a single diterpenoid, antheliatin **[15]**. The second soft coral, reported as the newly described *A. aureum*, yielded two new xenicane diterpenes, zahavins A **[16]** and B **[17]**. Immediately prior to the publication of these results, the author had completed the isolation of several metabolites from an undescribed *Alcyonium* species (designated *Alcyonium* species B - see chapter five) collected from Aliwal Shoal and found that this *Alcyonium* species also contained zahavin A. All three compounds **[15]** - **[17]** were cytotoxic towards mouse leukaemia, human lung carcinoma, human melanoma and human colon carcinoma cell lines.
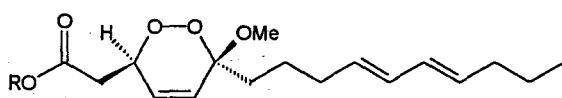
[18] R = H
[20] R = Me
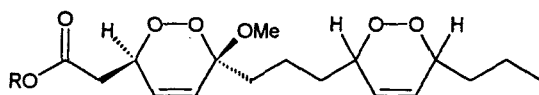
[19] R = H
[21] R = Me

Although widespread in all tropical and subtropical seas, a collection of the sponge *Plakortis simplex* from Sodwana Bay was the first report of this sponge's occurrence along the South African coast. The sponge afforded four new C_{16} 1,2-

12

dioxene polyketides [18] - [21][65]. A number of similar oxygenated polyketides have previously been reported from sponges of the genus *Plakortis*[66,67]. The facile acid catalysed decomposition of [18] and [19] was suggested as a reason for the instability of these compounds even when stored at low temperature under nitrogen. Although this instability precluded an examination of the bioactivity of [18] and [19], their more stable methyl esters [20] and [21] exhibited cytotoxicity towards cultured murine leukaemia cells.

[22]

[23]

[24]
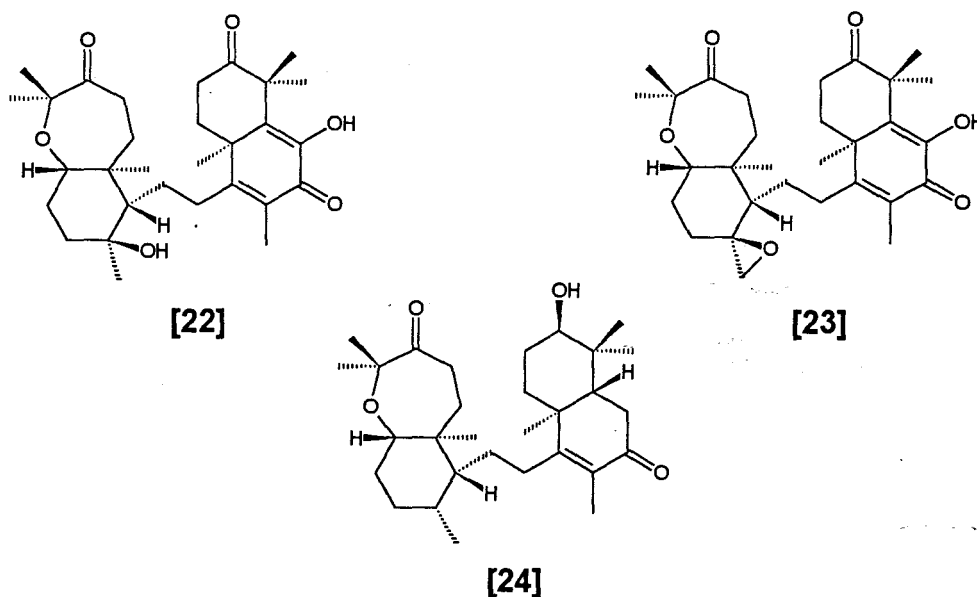
Six triterpenoids, sodwanones A-F, were isolated from the Indo-Pacific purple-brown fan sponge, *Axinella weltneri*, collected in Sodwana Bay[68,69]. The structure of sodwanone A [22] was unambiguously determined by X-ray diffraction studies and it was found that [22] appears in higher concentration (0.1%) relative to the other sodwanones which exist in the sponge in minute amounts (0.01-0.005%). The sodwanones are closely related to the previously reported sipholenols[70], siphonellinol[71] and neviotine A[72], isolated from the Red Sea sponge *Siphonochalina siphonella*, and the raspacionins[73], isolated from the Mediterranean red sponge *Raspaciona aculeata*, providing further evidence of a close affinity between metabolites isolated from Sodwana Bay marine invertebrates and those from other subtropical marine environments. A recent recollection of *A. weltneri* has yielded a further three triterpenoids, sodwanones G [23], H [24] and I[74], which exhibited cytotoxic activity against several cancer cell lines.
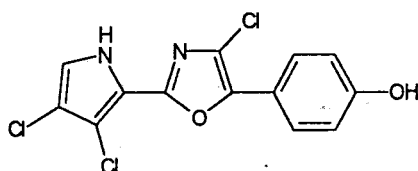
[25]

Fractionation of the EtOAc extract of the Indo-Pacific sponge *Phorbas aff. clathrata* led to the isolation of four novel oxazole derivatives, the phorbazoles A-D[75]. The structure of phorbazole A [25] was determined by X-ray diffraction studies on its dimethyl derivative. Although there are numerous reports of oxazoles, pyrroles and bromopyrroles being isolated from marine sponges, these compounds appear to be the first examples where chlorination of the oxazole and pyrrole ring systems has occurred.
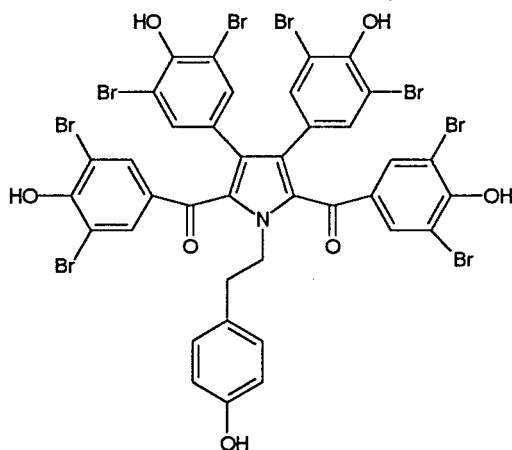
[26]
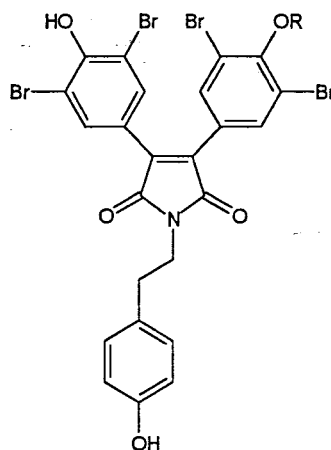
[27] R = H
[28] R = Me

A newly discovered translucent white colonial ascidian, *Polycitor* species, yielded three novel compounds, polycitone A [26] and polycitrins A [27] and B [28][76]. The structures were established by NMR spectroscopy and, in the case of [26], also by single-crystal X-ray diffraction analysis. These compounds represent novel marine alkaloids with unprecedented skeletons and Kashman and co-workers proposed a plausible biogenetic relationship between these compounds and the lamellarins, which were first isolated from the prosobranch mollusc *Lamellaria* species[77] and then from the ascidian *Didemnum chartaceum*[78].

14

Gastropod molluscs of the subclass Nudibranchia lack the usual protective molluscan shell and so have developed an alternative defense mechanism which involves the sequestering of dietary chemicals[79,80]. These chemicals are generally obtained from sponges, soft corals and bryozoans.

[29]   **[30]**   **[31]**   **[32]**
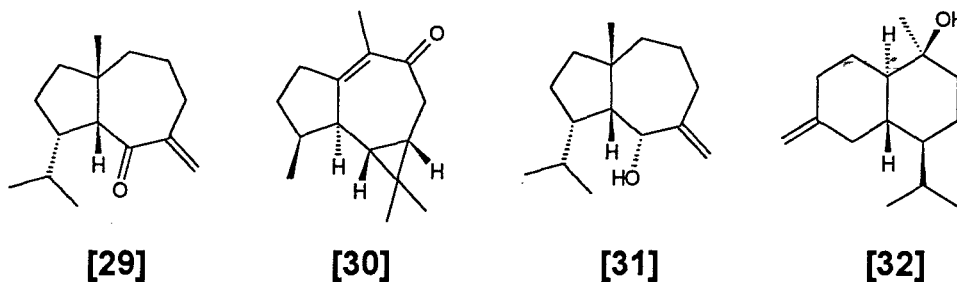
A chemical investigation of the endemic nudibranch, *Leminda millecra*[81], collected off the Transkei coast, resulted in the isolation of four new sesquiterpenes, millecrone A **[29]** and B **[30]** and millecrol A **[31]** and B **[32]**. These metabolites are typical of those found in soft corals and an investigation of the digestive gland of *L. millecra* revealed spicules belonging to the soft corals *Alcyonium foliatum*, *A. valdiviae* and *Capnella thyrsoidea*. *Alcyonium* and *Capnella* genera are known to produce sesquiterpenes[82,83]. It is interesting to note, however, that an investigation of the chemistry of *A. valdiviae* by Faulkner[60] and the chemistry of *C. thyrsoidea* by the author[84] (see chapter four), both collected off the South African coast, yielded diterpenes but no sesquiterpenes.

[33]   **[34]**
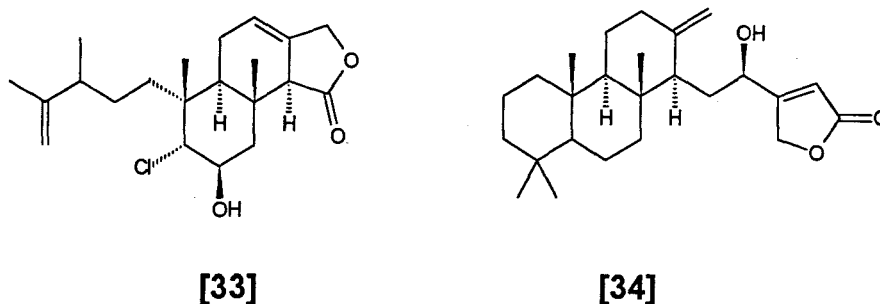
Specimens of the brilliantly coloured nudibranch, *Chromodoris hamiltoni*, were collected off Aliwal Shoal using SCUBA. Extracts were found to contain four unusual chlorinated homo-diterpenes, hamiltonins A **[33]**, B, C and D, and a new sesterterpene

hamiltonin E [34][85]. The ability of nudibranchs to use dietary chemicals for defense was demonstrated with the additional isolation of the known toxins latrunculin A and B. An inspection of the nudibranch gut contents revealed spicules typical of the genus *Latrunculia*. Latrunculins A and B were previously reported from the sponge *Latrunculia magnifica*[86] and, as part of their biological activity, cause disruption of cytoskeletal proteins.

1.3   THESIS OBJECTIVE

This aim of the work presented in this thesis was to isolate and identify biologically active natural products from South African marine invertebrates. This research forms an integral part of a programme to systematically collect, classify and screen South African marine invertebrates and their extracts for interesting chemistry and biological activity. Based on these results individual invertebrates are selected for further chemical analysis with the aim of discovering agents of potential pharmaceutical benefit.

1.4   COLLECTION AND SCREENING

Coastal southern Africa, from the Skeleton Coast of Namibia to southern Mozambique, has a remarkably rich and diverse marine fauna and flora with over 10 000 species or almost 15 per cent of all the coastal marine species known worldwide[87]. The benthic environment of southern Africa is extremely varied with a high degree of endemism. Day reported that approximately 66 per cent of the benthic invertebrate species from False Bay are South African endemics[88]. A specific example is the octocoral cnidarians, which are probably the most extensively studied of the South African marine invertebrates, with at least 200 species and as high as 60-70 per cent endemism[59]. This provides an incredibly wealthy and unexplored natural resource for the possible discovery of new pharmaceuticals.

This research thesis describes the chemistry of selected marine invertebrates which have been collected from four different localities on the South African coast ranging from the subtropical conditions of the Aliwal Shoal on the Natal coast to the warm temperate environment of the Tsitsikamma Marine Reserve in the southern Cape. A total of 350 different marine invertebrates were collected by the research group based at Rhodes University between 1992 and 1995. This relatively large collection shows the following phylogenetic distribution (**FIGURE 1.3**).

SPONGES		ASCIDIANS	
SOFT CORALS		GORGONIANS	
NUDIBRANCHS		OTHER	
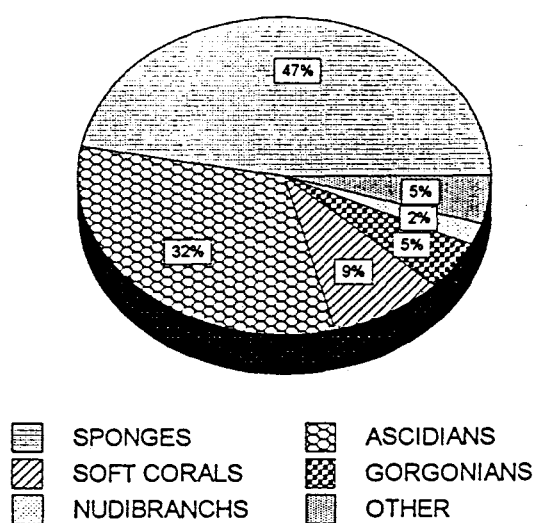
FIGURE 1.3 Phylogenetic distribution of the Rhodes University marine invertebrate collection between 1992 and 1995.

Crude organic and aqueous extracts of each of these 350 marine invertebrates have been screened using both ^{1}H NMR spectroscopy and antimicrobial activity assays. The simple, relatively inexpensive, antimicrobial bioassay is used by many marine natural product research groups as a primary screen for biological activity. Therefore, restricting an investigation by pursuing projects on the basis of good antimicrobial activity contains an increased risk of isolating previously described compounds.

For example, a grey-brown sponge, *Ircinia* species, collected in the Tsitsikamma Marine Reserve afforded the antimicrobial sesterterpene tetronic acid, variabilin [35][†]. First isolated by Faulkner in 1973 from the sponge *Ircinia variabilis*[89], it has subsequently been reported from other *Ircinia* species[90] as well as other sponge genera[91-93].
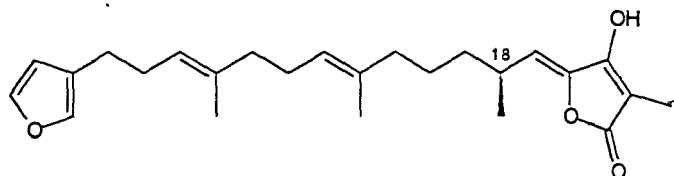
[35]

Remarkably, the stereochemistry at the C-18 position remained undefined for a period of twenty years until there was a sudden recent renewed interest in this compound with two papers simultaneously reporting an 18S-configuration for samples of variabilin isolated from an Okinawan *Amphidmedon* species[93] and from southern Australian sponges[94]. More recently the existence of 18R-variabilin in the Caribbean sponge, *Ircinia felix*[90] has been reported. A matching optical rotation of variabilin from southern Australia[94] and that from the South African *Ircinia* species suggests an 18S-configuration for the latter.
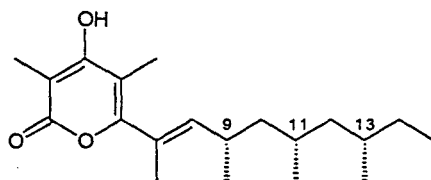
[36]

A further example of the discovery of a known antimicrobial compound, was the isolation of the polypropionate antibiotic pectinatone [36][‡], from the marine mollusc *Siphonaria concinna*. This crystalline compound, containing a γ-hydroxy-α-pyrone ring system, was previously isolated from skin extracts of *Siphonaria pectinata*[95] and reported to have a 9S, 11R, 13S stereochemistry. However, this was later revised to a 9S, 11S, 13S stereochemistry based on X-ray diffraction analysis as well as degradative studies[96].

---

† and ‡ - Unpublished results. Experimental details reported in chapter seven (section 7.2).

The following chapters provide a detailed description of twenty-one new compounds that have been isolated from South African marine invertebrates. Where possible every attempt has been made to explore the full pharmaceutical potential of each of these compounds.

CHAPTER TWO

NEW ANTIMICROBIAL C_{13} AND C_{14} AMINES FROM A SOUTH AFRICAN MARINE ASCIDIAN *PSEUDODISTOMA* SPECIES

20

2.1 INTRODUCTION

Ascidians belong to the phylum Chordata, which encompasses all vertebrate animals, and represent the most highly evolved group of animals commonly investigated by the marine natural product researcher. In the early 1990's there was a surge of interest in ascidian chemistry largely due to the high incidence of biologically active metabolites produced by these organisms. The bioactivity of many ascidian extracts is often attributed to the specialised ability of ascidians to biosynthesise amino acid derivatives, containing as much as 89 per cent by mass of nitrogen[97].

Amongst the nitrogenous compounds produced by ascidians are a number of unsaturated acyclic amines and amino alcohols. Examples include the C_{12} and C_{14} diene and triene amines [37] - [40] from an orange compound ascidian, *Pseudodistoma novaezelandiae*, collected from the North Island of New Zealand[98]. This ascidian was reported to be extremely abundant, reaching up to 25 per cent cover in shallow subtidal reefs. It is suggested that this profusion is probably due to a lack of predation or fouling caused by the presence of high levels of toxic amines in these invertebrates.

[37] [38]

[39]
[40] Δ^{13}
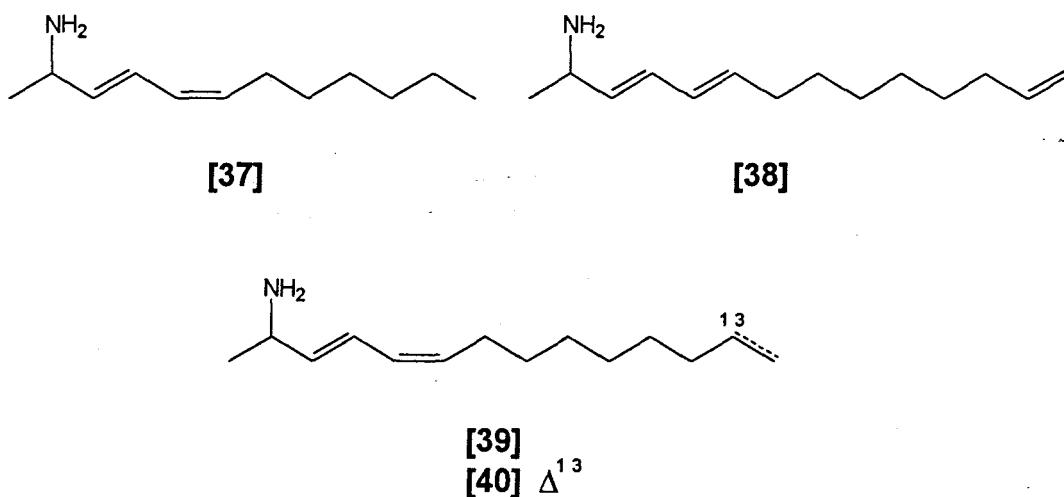
The cytotoxicity and antimicrobial activity displayed by the alcoholic extract of the Mediterranean ascidian, *Pseudodistoma crucigaster*, during a systematic screening exercise, led to its selection for further analysis. This analysis resulted in the isolation of three polyunsaturated amino alcohols, the crucigasterins [41] - [43][99]. Difficulty was

experienced in separating the parent compounds. Although acetylation of a portion of a mixture of the amino alcohols improved the chromatographic separation, it was found that these compounds (both natural products and their acetyl derivatives), especially [41] and [42], were unstable when concentrated in a pure form, even below -20 °C.

[41]
[42] Δ^{17}

[43]

[44] Δ^5
[45] Δ^4
[46] Δ^3
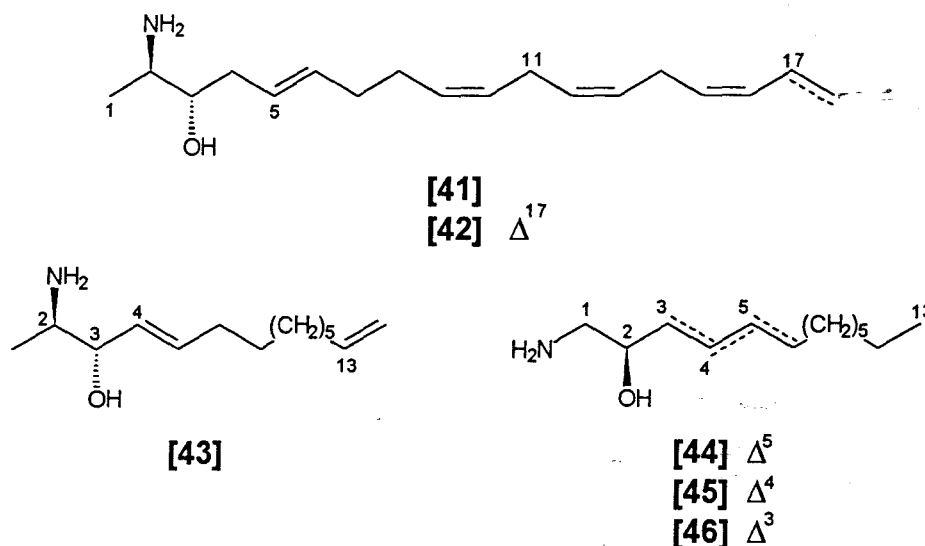
The significant antifungal activity of the extract of an orange encrusting ascidian, *Didemnum* species, collected from the Great Barrier Reef, prompted a chemical investigation of the extract. The study resulted in the isolation and structural elucidation of the major antifungal compound [44][100]. Two minor congeners, [45] and [46], proved refractory to HPLC separation and so a portion of a mixture of these was treated with di-*tert*-butyl dicarbonate followed by separation using HPLC and structural elucidation of the corresponding *N*-Boc derivatives.

Amino alcohols are not only confined to ascidians and also occur in sponges of the genus *Xestospongia*[101,102]. As with the ascidians, the isolation of the parent amino alcohols from these sponges proved problematic. Gulavita and Scheuer[101] relied on mild acetylation followed by HPLC in order to characterise the amino alcohols from a *Xestospongia* sponge collected from Papua-New Guinea. Jiménez and Crews[102], however, working with a sponge of the same genus collected from Fiji, managed to separate the amino alcohols in their native state using reverse phase HPLC, but this involved a slow flow rate (2 mL min^{-1}) and long retention times (40-60 min).

22

Section 2.2 and 2.3 of this chapter describes the isolation and identification of four new antimicrobial, unsaturated amino alcohols, characterised as their acetyl derivatives **[47]** - **[50]**, from the EtOAc extract of a South African marine ascidian, *Pseudodistoma* species[103].

[47] Δ^5, Δ^{13}
[48] Δ^5
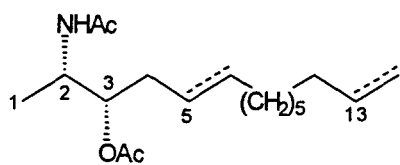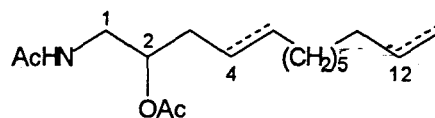
[49] Δ^4, Δ^{12}
[50] Δ^4

2.2 COLLECTION, EXTRACTION AND ISOLATION
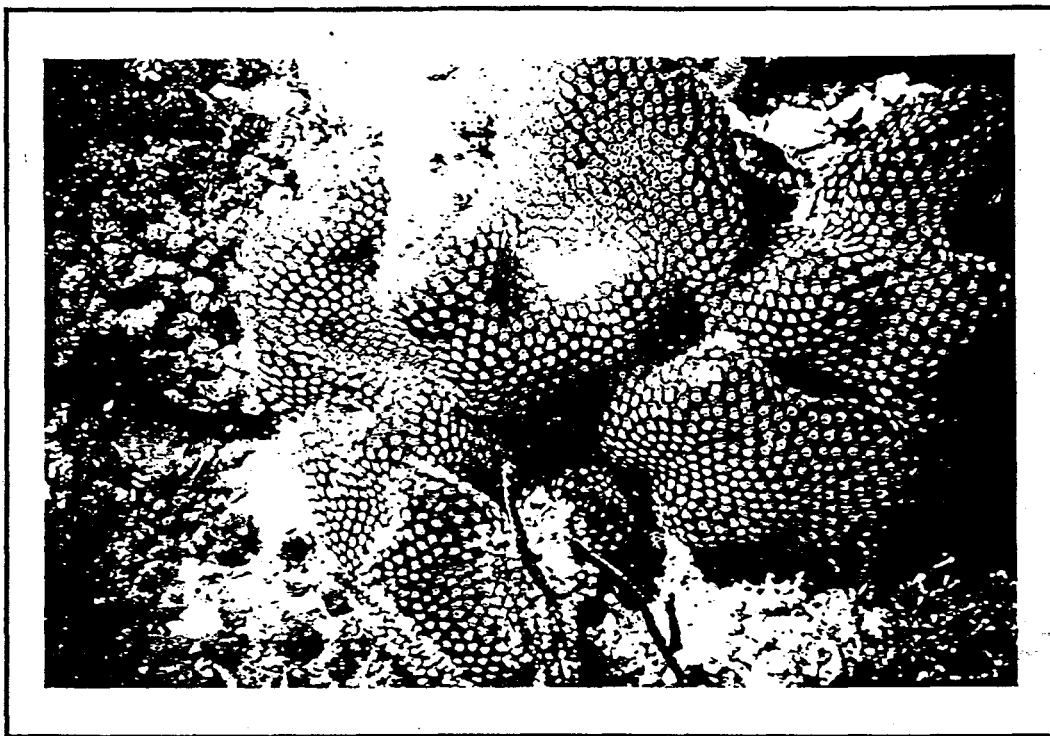
FIGURE 2.1 Colonial ascidian, *Pseudodistoma* species, collected from The Knoll in the Tsitsikamma Marine Reserve.

A pink-orange colonial ascidian, *Pseudodistoma* species (Family: Polyclinidae, Order: Aplausobranchia) (**FIGURE 2.1**), was collected in the Tsitsikamma Marine Reserve in May 1993 using SCUBA from depths of -20 to -30 m and stored frozen. A portion of this ascidian was subsequently freeze-dried (dry weight 256.9 g) and steeped in hexane, EtOAc and MeOH respectively. All three crude extracts showed strong antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subitilis*, and Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, with the greatest activity residing in the EtOAc extract. The antimicrobial activity guided chromatography of a portion of the EtOAc extract was performed according to SCHEME 2.1.

```
                        EtOAc extract
                          (900 mg)
                              │(a)
        ┌─────────────┬───────┘
        │             │        │
50% hexane/EtOAc    EtOAc    MeOH
   (180 mg)        (40 mg)  (319 mg)
                              │(b)
              ┌───────────────┤
              │               │
      15% MeOH/CHCl₃         MeOH
         (90 mg)           (106 mg)
                              │
                       REPEAT OF ABOVE
                          (1.07 g)
                              ⌇
                          (374 mg)
                              │(c)
                          (476 mg)
                              ⌇
                          (284 mg)
                              │(d)
      (e)┌───────────────────────────────┐(g)
        ┌┴──────┬(f)              ┌───────┴──┐(h)
        │       │                 │          │
    ┌───────┐ ┌───────┐      ┌───────┐  ┌───────┐
    │ [47]  │ │ [48]  │      │ [50]  │  │ [49]  │
    │33.0 mg│ │10.7 mg│      │21.1 mg│  │17.3 mg│
    └───────┘ └───────┘      └───────┘  └───────┘

PLEASE NOTE :
  ⌇  Use of this character in the above scheme and subsequent
  ⌇  schemes indicates that a portion of the extract fraction was
     removed before proceeding.
```
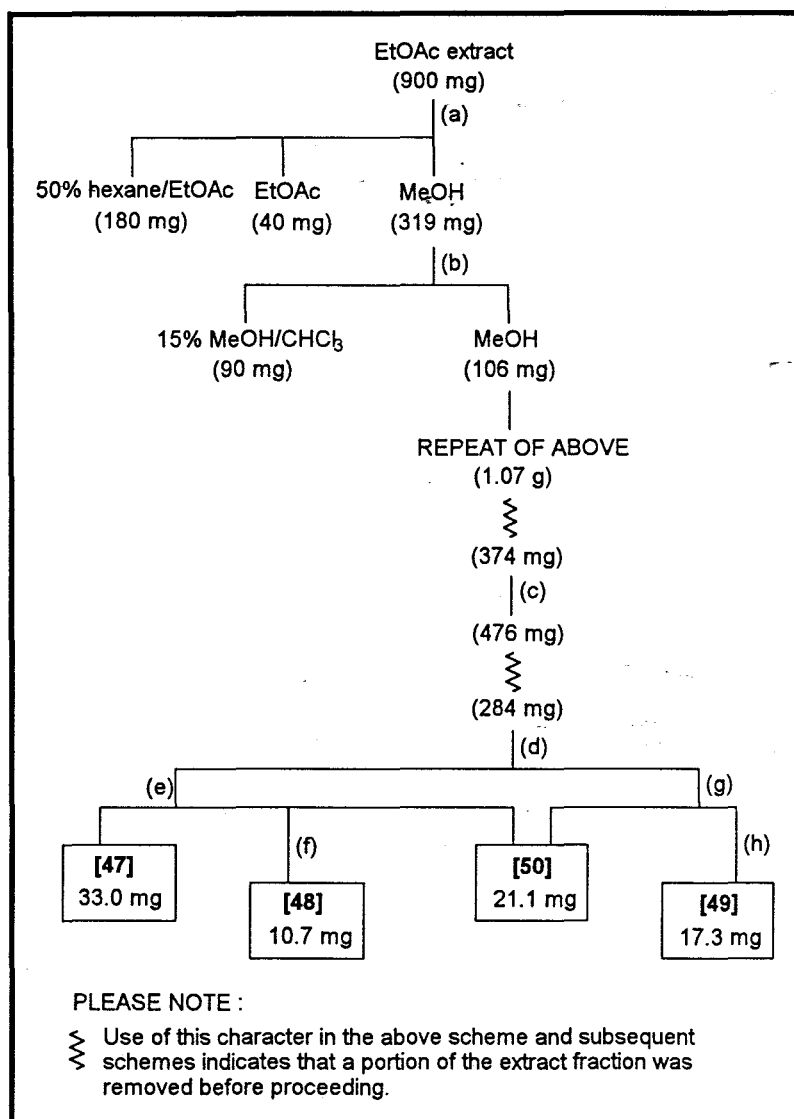
SCHEME 2.1 Chromatographic procedure for the isolation of compounds **[47]** - **[50]**. (a) flash chromatography (silica, 230-400 mesh, 150 g, elution sequence: 50% hexane/EtOAc; EtOAc; MeOH). (b) flash chromatography (silica, 230-400 mesh, 150 g, elution sequence: 15% MeOH/CHCl₃ ; MeOH). (c) acetylation (Ac₂O, RT 12 h, pyridine). (d) HPLC (silica, 10 μ, Whatman, EtOAc, 4 mL min⁻¹). (e) HPLC (C18, 10 μ, Phenomenex, 20% H₂O/MeOH, 4 mL min⁻¹). (f) HPLC (C18, 10 μ, Phenomenex, 30% H₂O/MeOH, 4 mL min⁻¹). (g) HPLC (C18, 10 μ, Phenomenex, 20% H₂O/MeOH, 4 mL min⁻¹). (h) repeat of (g).

25

Although TLC suggested that the MeOH fraction, from flash chromatography, contained a single compound (106 mg), multiple resonances of varying intensity in the ^{13}C NMR spectrum of this fraction suggested a mixture. No normal or reverse phase TLC conditions were found that could separate this mixture. The collapse of a broad singlet at δ 5.9 in the ^1H NMR spectrum of this fraction upon the addition of D_2O, indicated the presence of hydroxyl groups. Esterification is a standard method whereby the negative contribution of lipophobic alcohol and/or amino functionalities to the overall chromatographic properties of a mixture can be reduced. Accordingly, acetylation of a portion of the extract yielded an oil which could be further chromatographed to yield compounds [47] - [50].

2.3 STRUCTURE DETERMINATION AND STEREOCHEMISTRY

2.3.1 Structure determination of the C_{13} and C_{14} amines [47] - [50].
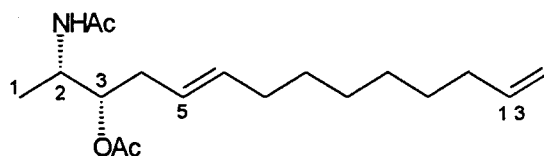
[47]

The major compound, 2-acetamido-3-acetoxy-5,13-tetradecadiene [47], was isolated as an optically active, stable, colourless oil. HREIMS established the molecular formula as $C_{18}H_{31}NO_3$ (m/z 309.2292, Δmmu -8). ^{13}C NMR data (**TABLE 2.1**) indicated two acetate carbonyl (δ 169 and 171) and four olefinic (δ 114, 124, 135 and 139) resonances fulfilling the required four degrees of unsaturation implied by the molecular formula. A further three methyl, seven methylene and two methine signals in the DEPT spectrum of [47] supported a simple, acyclic diacetoxy-tetradecadiene structure for this compound. The ^{13}C chemical shifts of the two methine signals (δ 76 and 47) were assigned to carbon atoms attached to acetoxy and acetamido functionalities respectively, while the olefinic methylene signal (δ 114) in the DEPT spectrum placed one of the double bonds in a terminal position.

The contiguous coupling sequence observed in the COSY spectrum of [47] (**FIGURE 2.2**) from the olefinic proton at δ 5.3 (1H, dt, H-5) through the overlapping allylic methylene protons at δ 2.2 (2H, m, H-4), the acetoxy methine proton at δ 4.8 (1H, m, H-3), the acetamido methine proton at δ 4.2 (1H, m, H-2) to the terminal methyl protons at δ 1.1 (3H, d, $J_{1,2}$ = 6.8 Hz, H-1) placed the second double bond at Δ^5 and established the C-2, C-3 acetamido-acetoxy substructure of [47]. An HMQC experiment enabled full assignment of all the ^{13}C and ^1H signals in concurrence with published data for substituted linear 2-amino-3-alcohols[99,101].

27
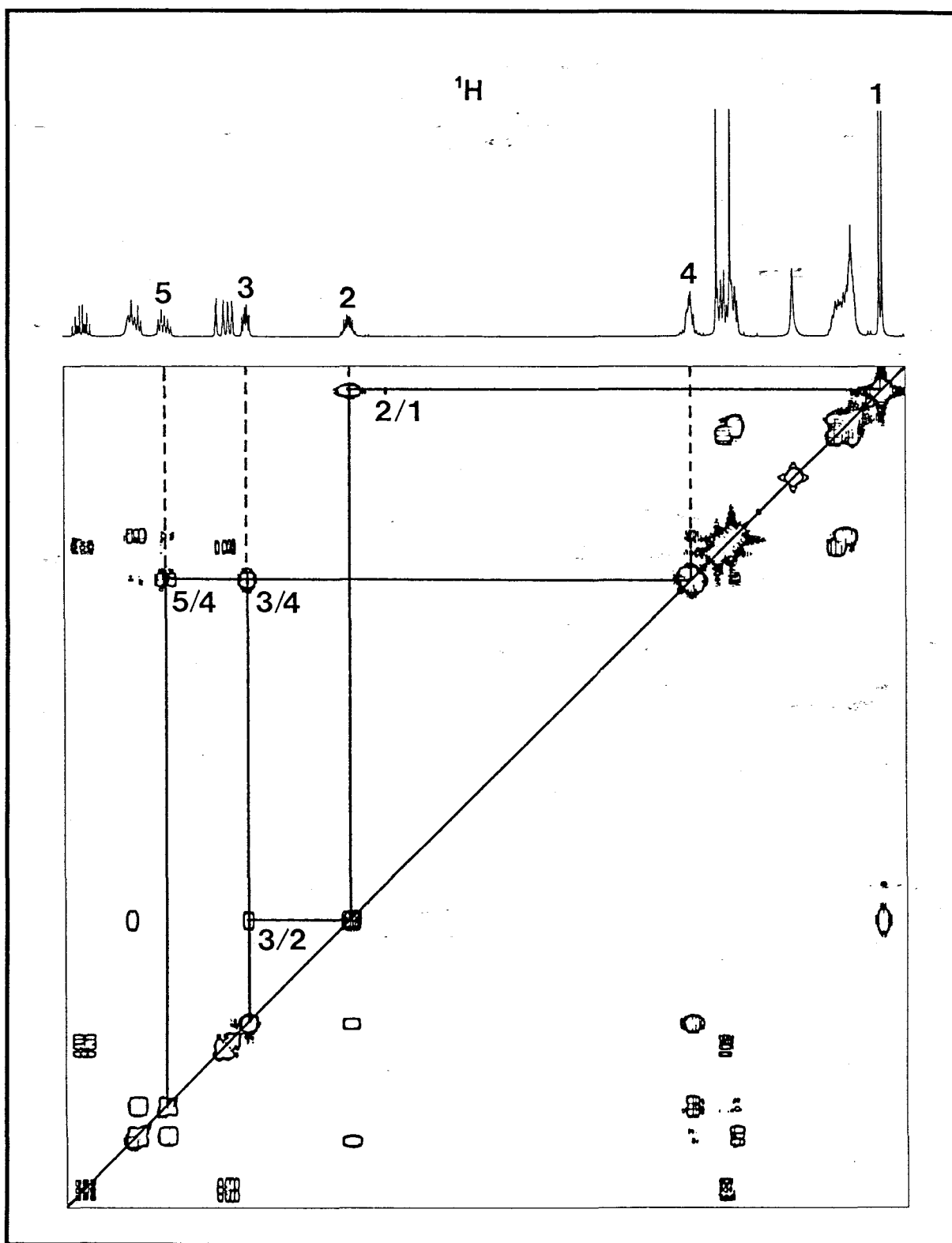
FIGURE 2.2 COSY NMR (400 MHz, CDCl$_3$) spectrum of compound **[47]**.

TABLE 2.1 ^{1}H and ^{13}C NMR spectral data for compounds **[47]** and **[48]**.

	[47] ^{13}C	[47] ^{1}H	[48] ^{13}C	[48] ^{1}H
1	18.4 q	1.09 d (6.8)	18.5 q	1.09 d (6.8)
2	46.7 d	4.20 m	46.8 d	4.21 m
3	76.1 d	4.82 m	76.1 d	4.82 m
4	35.0 t	2.22 m	35.0 t	2.22 m
5	123.9 d	5.30 dt (6.8,15.3)	123.9 d	5.30 dt (7.5,15.0)
6	134.8 d	5.48 dt (6.9,15.3)	134.9 d	5.47 dt (6.7,15.1)
7	32.5 t	1.95 m	31.9 t	2.01 m
8	[a]29.0 t		[b]29.5 t	
9	[a]28.9 t	1.36-1.25 m	[b]29.3 t	
10	[a]28.9 t		[b]29.2 t	1.30-1.23 m
11	[a]29.3 t		[b]28.9 t	
12	33.8 t	2.01 m	32.6 t	
13	139.2 d	5.79 dq	22.7 t	
14	114.1 t	4.94 dd	14.1 q	0.87 t (6.7)
OCOCH₃	170.6 s		170.6 s	
OCO*CH₃*	23.4 q	2.06 s	23.4 q	2.06 s
NHCOCH₃	169.3 s		169.4 s	
NHCO*CH₃*	21.0 q	1.98 s	21.0 q	1.98 s
N*H*		5.50 br s		5.50 br s

Spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$ with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses. All assignments are supported by DEPT, COSY, HMQC and HMBC NMR experiments. Assignments with identical superscripts within a column may be interchanged.

29

NHAc

[48]
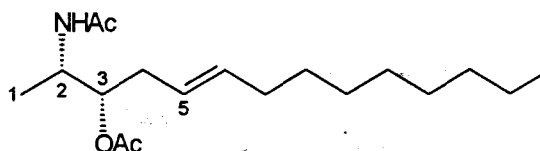
A molecular formula of $C_{18}H_{33}NO_3$ (m/z 311.2478, Δmmu +18) from HREIMS data, supported by a methyl triplet at δ 0.8 (3H, t, $J_{13,14}$ = 6.7 Hz, H-14) in the ^{1}H NMR spectrum of [48] (2-acetamido-3-acetoxy-5-tetradecene) indicated that it was a homologue of [47] with a saturated terminal double bond. The coupling observed from the olefinic proton at δ 5.3 (1H, dt, H-5), through the overlapping methylene protons at δ 2.2 (2H, m, H-4) to the acetoxy methine proton at δ 4.8 (1H, m, H-3) in the COSY spectrum of [48] indicated that the double bond was homoallylic to C-3 and, therefore, situated at C-5.
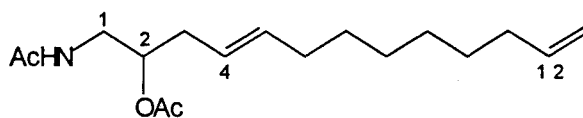
[49]

Compound [49], 1-acetamido-2-acetoxy-4,12-tridecadiene, was isolated as an optically active, stable, colourless oil. The molecular formula $C_{17}H_{29}NO_3$ (295.2156, Δmmu -9) established by HREIMS suggested that this compound was also homologous with [47] but with one carbon atom less in the unsaturated alkyl chain. These HREIMS data were complemented by the ^{13}C NMR data of [49] (TABLE 2.2). The absence of the terminal methyl doublet at δ 1.1 in the ^{1}H NMR spectrum and the presence of an amino-methylene signal at δ 43 in the ^{13}C NMR spectrum of [49] placed the acetamido group at C-1.
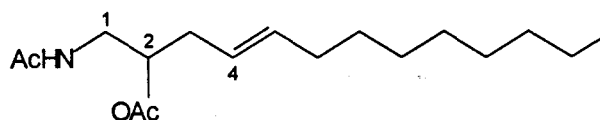
[50]

As discussed earlier for the homologous compounds **[47]** and **[48]**, a molecular formula of $C_{17}H_{31}NO_3$ (297.2305, Δmmu -1) from HREIMS data indicating two additional protons, together with a methyl triplet at δ 0.9 (3H, t, $J_{12,13}$ = 6.6 Hz, H-13) in the ^{1}H NMR spectrum of **[50]**, implied that this compound was a homologue of **[49]** with a saturated terminal double bond.
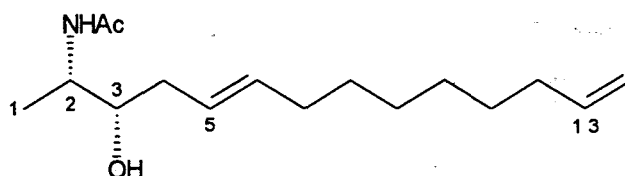
[51]

An attempt was made to isolate the parent amino alcohol of **[47]** by refluxing in strong base. This procedure resulted in a mixture of products with column chromatography yielding only the monoacetate compound **[51]** in a pure form. The upfield shift of the H-3 proton signal together with the absence of the $OCOCH_3$ singlet at δ 2.06 and the presence of the $NHCOCH_3$ singlet at δ 1.98 in the proton NMR spectrum of **[51]** indicated that only the O-acetoxy group had been removed.

2.3.2 Stereochemistry of the C_{13} and C_{14} amines **[47]** - **[50]**.

The modified Karplus equation requires J = 12-18 Hz for an *E* stereochemistry and J = 7-11 Hz for a *Z* stereochemistry in the vicinal coupling of alkenes[104], hence the assignment of an *E* stereochemistry at Δ^5 in **[47]** and **[48]** and Δ^4 in **[49]** and **[50]** followed from the $J_{5,6}$ = 15 Hz and $J_{4,5}$ = 15 Hz coupling constants respectively.

TABLE 2.2 ^{1}H and ^{13}C NMR spectral data for compounds **[49]** and **[50]**.

	[49]		[50]	
	^{13}C	^{1}H	^{13}C	^{1}H
1	42.6 t	3.40 m	42.7 t	3.41 m
2	73.0 d	4.90 m	73.0 d	4.91 m
3	35.3 t	2.25 t (6.8)	35.3 t	2.26 t (6.7)
4	123.6 d	5.31 dt (7.1, 15.3)	123.6 d	5.31 dt (7.1, 15.2)
5	134.9 d	5.48 dt (6.9, 15.3)	135.0 d	5.50 dt (8.2, 15.2)
6	32.5 t	1.98 m	31.9 t	1.98 m
7	[a]29.0 t		[b]29.4 t	
8	[a]28.9 t	1.38-1.26 m	[b]29.4 t	
9	[a]28.9 t		[b]29.3 t	1.31-1.25 m
10	29.3 t		[b]29.2 t	
11	33.8 t	2.02 m	32.6 t	
12	139.1 d	5.79 dq	22.7 t	
13	114.1 t	4.95 m	14.1 q	0.87 t (6.6)
OCOCH$_3$	171.1 s		171.1 s	
OCOCH_3	23.2 q	2.05 s	23.2 q	2.05 s
NHCOCH$_3$	170.1 s		170.1 s	
NHCOCH_3	21.1 q	1.96 s	21.1 q	1.96 s
NH		5.70 br s		5.69 br s

Spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C in CDCl$_3$ with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses. All assignments are supported by DEPT, COSY, HMQC and HMBC NMR experiments. Assignments with identical superscripts within a column may be interchanged.

Various approaches have been used in assigning the relative and absolute stereochemistry at chiral centres in amino alcohol compounds. The relative configuration in aliphatic 2-amino-3-alcohol compounds can be established by preparing oxazolidinone derivatives of these compounds and examining the *cis* or *trans* orientation of the C_2 - C_3 bond using NOE or coupling constant measurements from the NMR spectra of the derivatives.

[52]   **[53]**
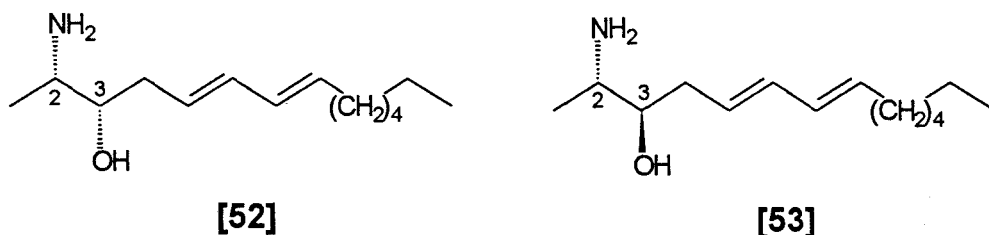
Comparison of the NMR spectra of the two parent amino alcohol compounds **[52]** and **[53]**, isolated by Gulavita and Scheuer[101] from a sponge of the genus *Xestospongia*, implied that the compounds were C-3 epimers. The $2R^*$, $3R^*$ or $2S^*$, $3S^*$ relative stereochemistry of the major epimer **[52]** was established from the significant NOE values between H-2 and CH_2-4 and between Me-1 and H-3 in its oxazolidinone derivative (**FIGURE 2.3**).
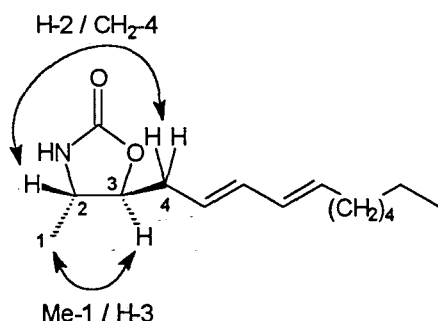
FIGURE 2.3 NOEs observed in the oxazolidinone derivative of **[52]**.

The minor epimer **[53]**, therefore, had a relative stereochemistry of $2R^*$, $3S^*$ or $2S^*$, $3R^*$. The absolute stereochemistry was determined by degradation of **[52]** to *L*-alanine and comparison of the HPLC retention times of this with authentic standards. Based on this HPLC method it was proposed that *n*-12 fatty acids and *L*-alanine **[54]** are the biosynthetic precursors of the *Xestospongia* amino alcohols and established a $2S$, $3S$ and a $2S$, $3R$ configuration for **[52]** and **[53]** respectively.
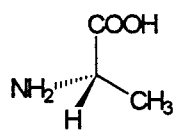
[54]
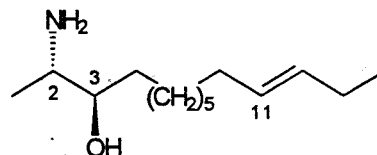
[55]

Jiménez and Crews[102] used the derivatisation of xestaminol A **[55]** to its oxazolidinone in order to establish the relative stereochemistry of this compound. Interpretation here, however, was based on coupling constants rather than observed NOEs, and a *cis* H-2/3 configuration was assigned from the $J_{2,3}$ = 7.5 Hz coupling constant (*trans* configuration requires $J_{2,3}$ = 4.1 - 5.1 Hz) obtained after spin decoupling of the terminal methyl doublet (**FIGURE 2.4**). The coupling constant was accordingly used to propose a 2S^*, 3R^* relative stereochemistry for **[55]**.

$J_{2,3}$ = 7.5 Hz
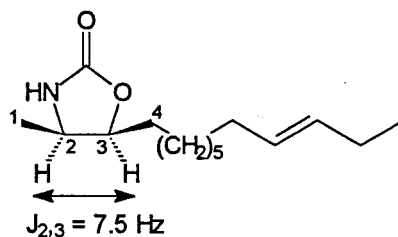
FIGURE 2.4 $J_{2,3}$ coupling in the oxazolidinone derivative of **[55]** (Me-1 decoupled).
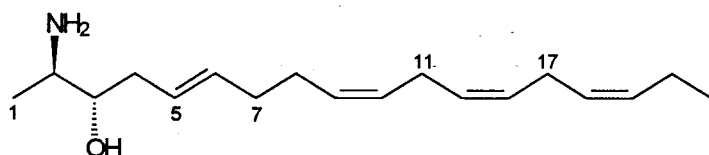
[41]
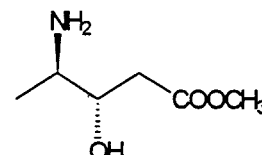
[56]

The amino alcohol portion in **[41]** ($[\alpha]_D^{21}$ +45°), isolated from *Pseudodistoma crucigaster*[99], was assigned a 2R, 3S absolute stereochemistry based on the chiral GC comparison of methyl-3-hydroxy-4-aminopentanoate **[56]**, a chemical degradation product of **[41]**, with a synthetic sample prepared from L-alanine **[54]**. This led to the suggestion that these compounds were, in fact, biosynthesised from D-alanine.
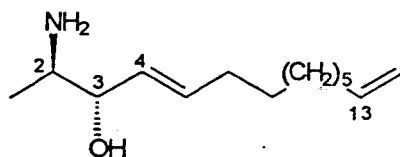
[43]

The stereochemistry of the amino alcohol **[43]** ($[\alpha]_D^{21}$ +36°), also from *P. crucigaster*[99], could not be determined using the same degradative technique due to the difference in substitution at C-4. However, the stereochemistry was tentatively assigned as 2*R*, 3*S* because, as with **[41]**, **[43]** exhibited a positive optical rotation. This reasoning must be questioned considering the fact that the diacetyl derivatives of the C-3 epimers, **[52]** ($[\alpha]_D^{21}$ +17°) and **[53]** ($[\alpha]_D^{21}$ +51°), isolated by Gulavita and Scheuer[101], also have positive optical rotations even though they possess different stereochemistries.

[47] Δ^5, Δ^{13}
[48] Δ^5
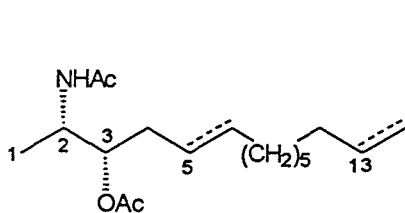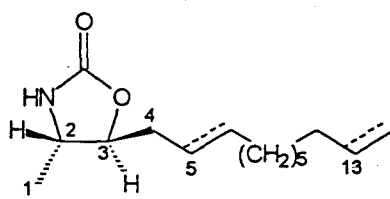
[57] Δ^5, Δ^{13}
[58] Δ^5

The relative stereochemistry of the parent compounds of **[47]** and **[48]** from the South African ascidian, *Pseudodistoma* species, was determined *via* the formation of oxazolidinone derivatives. Treatment of a portion of a mixture of the amino alcohols obtained from flash chromatography with 1,1-carbonyl-di-imidazole followed by extensive normal and reverse phase HPLC of the reaction products gave the oxazolidinones **[57]** and **[58]**. Proton spin decoupling of the C-1 methyl group signal at δ 1.25 in the ^{1}H NMR spectra of **[57]** and **[58]** established a $J_{2,3}$ = 6.1 Hz for both compounds, thus confirming that the two compounds have the same C-2, C-3 stereochemistry. Although the coupling of $J_{2,3}$ = 6.1 Hz suggests a *trans* configuration when compared to the $J_{2,3}$ = 7.5 Hz coupling in the *cis* configuration of xestaminol A oxazolidinone (**FIGURE 2.4**), it was still considered large for a *trans* configuration (*trans* usually requires $J_{2,3}$ = 4.1-5.1 Hz) and hence not conclusive. However, in the ROESY

NMR spectrum of [57] (FIGURE 2.5) a significant NOE between H-2 and CH_2-4, between Me-1 and H-3 and the total absence of any NOE between H-2 and H-3 (see hatched area in FIGURE 2.5) placed H-3 and the C-1 methyl group on the same side of the oxazolidinone ring. This observation implied that the compound was a *trans* isomer with a 2S*, 3S* or 2R*, 3R* relative stereochemistry. Extension of the biosynthetic argument of Gulavita and Scheuer[101] to include similar ascidian compounds intimates a 2S, 3S stereochemistry for [47] and [48].
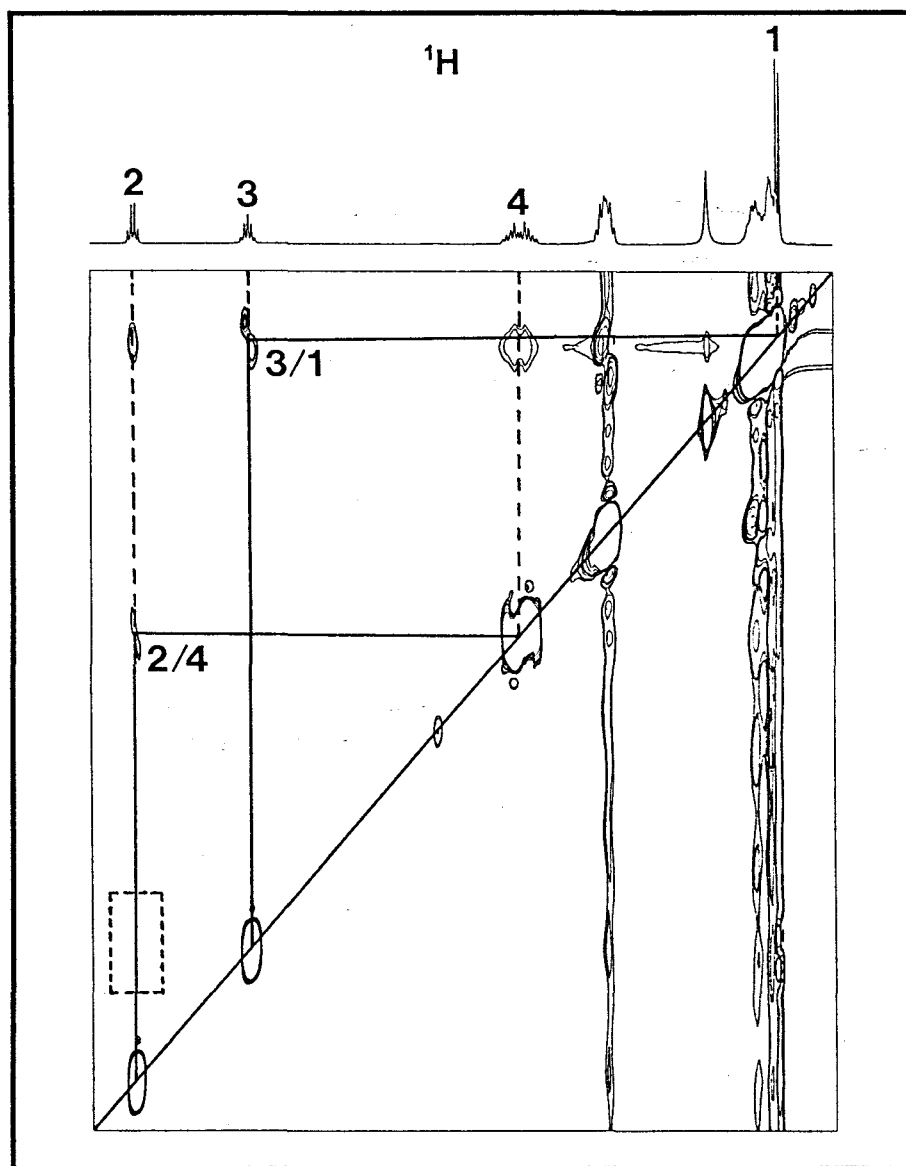
FIGURE 2.5 ROESY NMR (400 MHz, $CDCl_3$) spectrum of compound [57].

Searle and Molinski[100] showed that the stereochemistry of aliphatic 1-amino-2-alcohol compounds can be effectively determined using the circular dichroic exciton coupling method of Harada and Nakanishi[105]. CD spectroscopy of optically active unsaturated organic compounds is a powerful method of studying the 3-D structure of these molecules. Circular dichroism results from the interaction of the individual chromophores of each molecule with an electromagnetic field of light[105]. This interaction causes a "split Cotton effect" due to the chiral exciton coupling. The absolute configuration of a compound can thus be determined by the positive or negative sign of this Cotton effect. The successful application of the dibenzoate chirality method to 2-amino-1-butanol by Nagai and co-workers[106] prompted Molinski to use this method for compound [44] from the ascidian, *Didemnum* species.
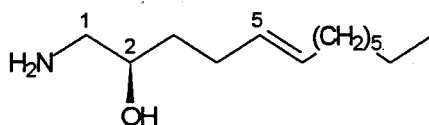
[44]

A negative bisignate Cotton effect observed in the CD spectrum of the *N,O*-dibenzoyl derivative of [44] correlated with a negative helicity from exciton coupling theory and, hence, an *R* configuration for [44]. Isolation difficulties encountered with the parent amino alcohol of [49], from the South African ascidian, *Pseudodistoma* species, prevented the application of the same CD method to this compound. An attempt to isolate the *N,O*-dibenzoyl derivative of [49] by HPLC after treating a portion of a mixture of the amino alcohols with benzoyl chloride and DMAP failed, with only the monobenzoyl derivative (established by CIEIMS) being isolated in pure form.
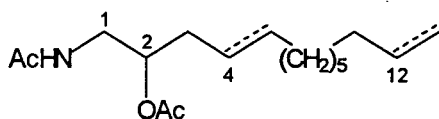
[49] Δ^4, Δ^{12}
[50] Δ^4
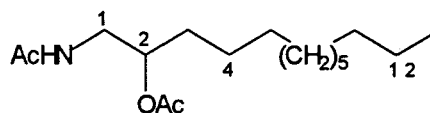
[59]

Although the stereochemistry at C-2 in [49] and [50] remains unassigned, catalytic hydrogenation of each compound gave the same optically active product [59],

$[\alpha]_D^{25}$ -9°, thus establishing the same stereochemistry at the chiral centre in these compounds.

2.4   BIOLOGICAL ACTIVITY

A variety of biological activities have been reported for unsaturated amino alcohols including activity against parasites, the enzyme reverse transcriptase[102], microbes[99] and fungi[100,101]. Compounds [47] - [50] together with various derivatives were tested for antimicrobial activity against the Gram-positive *Bacillus subtilis* and Gram-negative *Escherichia coli* bacteria in the agar plate disc diffusion assay (TABLE 2.3).

TABLE 2.3   Zone of inhibition diameters measured in the screening of the amino alcohol compounds and their derivatives for antimicrobial activity.

SAMPLE	B. subtilis	E. coli
mixture of parent amino alcohols	14.5*	12.0*
semi-pure parent of [47]	10.5	10.0
hydrogenated semi-pure parent of [47]	10.5	9.0
[47]	-	sl
[48]	-	9.0
[49]	11.0	sl
[50]	12.0	sl
hydrogenated [47]	sl	sl
hydrogenated [49]	sl	sl
[57]	-	-
[58]	-	-
gentamycin standard	19.0	19.0

*Inhibition zone diameters in mm, disc size = 8.0 mm and "sl" indicates slight activity (8.0-9.0 mm). Samples tested at 100 µg disc^{-1}. Gentamycin standard = 10 µg disc^{-1}.

Surface active agents (detergents) containing a "lipophobic head" and a "lipophilic tail" may have an affinity for adsorption into a cell membrane, causing disruption of cell membrane integrity and subsequent cell death[107]. It is this amphiphilic nature of the amino alcohol metabolites which is therefore probably responsible for their biological activity and accounts for the lack of predation and fouling experienced by the producer organism. Expanding on the theory proposed by Munro and co-workers for the antimicrobial activity of aliphatic amines[98], it is the combination of a hydrophobic alkyl chain, a basic amino group and a polar hydroxyl group which imparts semi-quantitative antimicrobial properties to the unsaturated acyclic amino alcohol compounds. The testing of a series of synthetic 2-aminoalkanes found that the greatest antimicrobial activity and strongest detergent-like behaviour was caused by C_{13} - C_{16} compounds[108]. From the mode of action of surfactants, it stands to reason, that a reduction in the basicity of the amino, and in the polarity of the alcohol groups, should diminish bioactivity. This was corroborated by the reduced antimicrobial activity observed with the acetylated compounds [47] - [50] and the total loss of activity with the oxazolidinone derivatives [57] and [58] (TABLE 2.3).

CHAPTER THREE

NEW ALKALOIDS FROM A SOUTH AFRICAN LATRUNCULID SPONGE

3.1 INTRODUCTION

Sponges of the family Latrunculidae have recently been a focus of attention by taxonomists[†]. It is believed that in several instances Latrunculid sponges have been misidentified in papers detailing the isolation of their secondary metabolites and, as a result, the taxonomy of this family is presently being revised. This revision will encompass morphologic, chemotaxonomic, biosynthetic and DNA sequencing data[‡].

Three distinct chemical groups are evident in Latrunculid sponges :

(a) Terpene and related compounds found in the Indo-Pacific genera *Diacarnus* and *Sigmosceptrella*. These compounds include the sigmosceptrellins[109], trunculins[110] and other cyclic peroxides[111]. An example is sigmosceptrellin A [60][109].

[60]   [61]   [62]
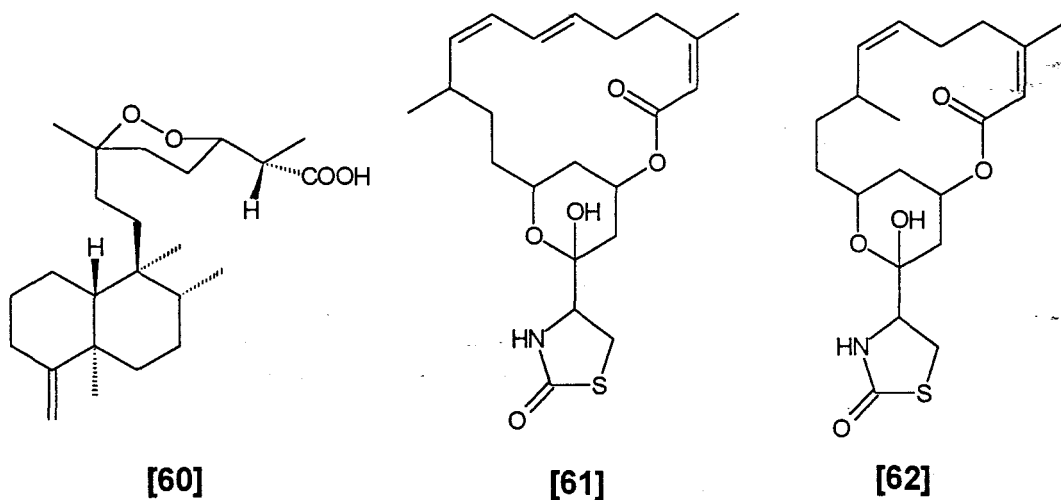
(b) Latrunculins and related compounds from the Indo-Pacific genera *Negombata* and *Hyatella*. The latrunculins A [61] and B [62][86,112] were reported as being the first known 2-thiazolidinone-bearing marine macrolides and were found, as part of their biological activity, to cause major alterations in the cytoskeletal protein actin.

† Presentation at the 8th International Conference on Marine Natural Products, Tenerife (Sept. 1995).
‡ Personal communication with Michelle Kelly-Borges, The Natural History Museum, London.

(c) Discorhabdin and related compounds from the warm-temperate to Antarctic genera *Latrunculia* type 1 and 2, *Zyzzya* and *Batzella*. It is these compounds which are the focus of this chapter.

The discorhabdins are dark pigments characterised by an iminoquinone chromophore, an unsaturated spiro-cyclohexanone system and a pyrrolo[1,7]phenanthroline ring system. The first example of this group of compounds was the highly cytotoxic pigment discorhabdin C [63] isolated by Munro and co-workers[113] from a sponge of the genus *Latrunculia*. This compound was soon followed by the identification of two other major pigments, discorhabdin A [64] and B [65][114], from related sponges of the same genus.
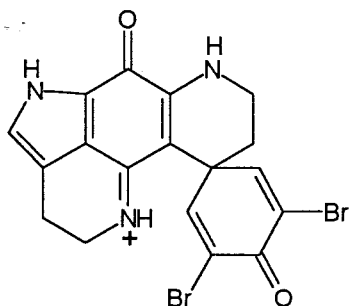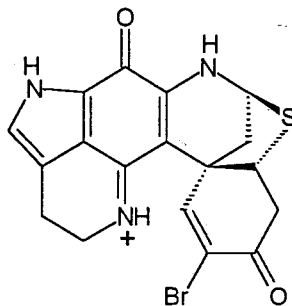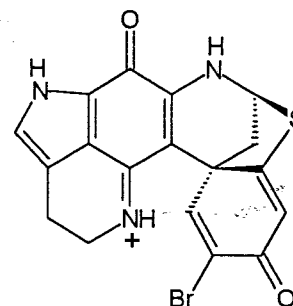
[63]   [64]   [65]

Ironically, the structure of prianosin A, identical to [64], from an Okinawan marine sponge *Prianos melanos* (possibly a misidentified *Zyzza* sponge)† was simultaneously published by Kobayashi and co-workers[115]. As expected, the spectroscopic and bioactivity data of prianosin A were consistent with those of discorhabdin A [64].

Further antitumour alkaloids isolated by these two research groups include discorhabdin D [66] from the sponges *Latrunculia brevis* and *Prianos* species[116], and prianosins B [67], C [68] and D [69] from *Prianos melanos*[117].

---

† Michelle Kelly-Borges - personal communication.

42

O H
H
N 11 N
9 13 S

N
+
R O

[66] R = H
[70] R = OH

O H
H N
N

N

Br O

[67]

OH
H
N 11 N
9 13 S

N

R O
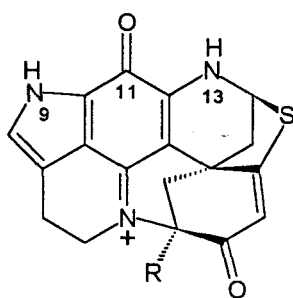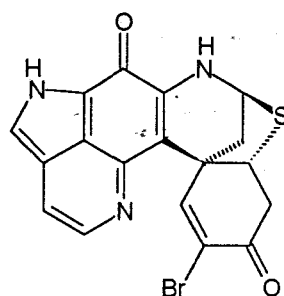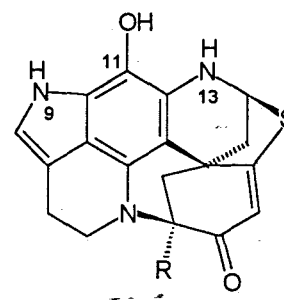
[68] R = OH
[69] R = H

The aminophenol moiety in prianosin D [69] is the reduced form of the iminoquinone functionality of discorhabdin D [66]. Considering that both were isolated independently from what was believed to be the same species of sponge[118] collected from Okinawa led to a re-examination and revision of the structures of prianosins C and D[119]. The original structural elucidation of prianosins C [68] and D [69] was performed on their acetylated derivatives. It seems that during the esterification reaction, the acetyl groups were first introduced at the N-9 and N-13 positions with a subsequent reduction of the iminoquinone occurring to give a phenolic group at C-11. Possible steric hinderance from the acetyl groups at N-9 and N-13 prevented acetylation of the phenolic moiety. Without prior knowledge of the esterification induced reduction it was incorrectly assumed that the aminophenol was a natural product. The revised structures of prianosins C and D are shown as [70] and [66] respectively.

Several highly functionalised pyrroloquinoline alkaloids, the batzellines A [71], B [72] and C [73] and isobatzellines A [74], B [75], C [76] and D [77], were isolated from a deep water sponge Batzella species[120,121]. The two alkaloid groups, batzellines and isobatzellines, possess the same pyrrolo[4,3,2-de]quinoline ring system with the isobatzellines containing an aminoiminoquinone moiety instead of the aminoquinone functionality of the batzellines. Suprisingly, only the isobatzellines exhibit cytotoxic and moderate antifungal activity. Structurally related pigments, the damirones A [78] and B [79], have been isolated from the Palauan sponge Damiria species[122]. The structures

of these compounds were determined by comparison of their spectral data with those of the batzellines.
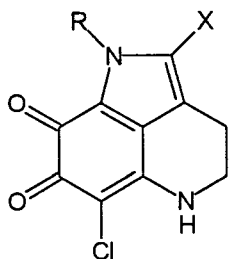
[71] R = Me X = SMe
[72] R = H   X = SMe
[73] R = Me X = H
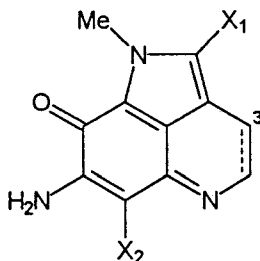
[74] X₁ = SMe X₂ = Cl
[75] X₁ = SMe X₂ = H
[76] X₁ = H   X₂ = Cl
[77] X₁ = SMe X₂ = Cl Δ³
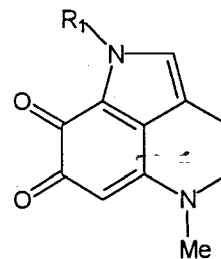
[78] R₁ = Me
[79] R₁ = H

Ireland and co-workers[123] discovered that pyrroloiminoquinone alkaloids are not confined to the phylum Porifera with the isolation of wakayin [80] from the ascidian *Clavelina* species. This compound displayed strong *in vitro* cytotoxicity against the human colon tumour cell line HCT116 and inhibited the topoisomerase II enzyme.
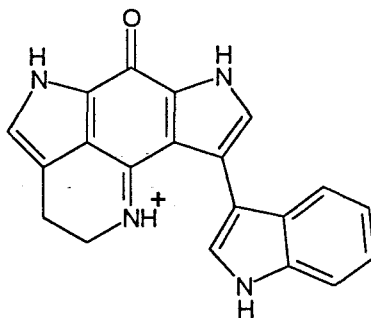
[80]

Topoisomerase II enzymes control the topological state of DNA by mediating DNA interconversions and are therefore implicated in a number of vital cellular processes including DNA replication, DNA transcription and chromosomal segregation[124]. The nature of this function makes the inhibition of the topoisomerase II enzyme an important target for the discovery of new anticancer drugs.
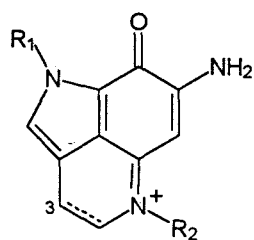
[81] R₁ = Me R₂ = H

[82] R₁ = Me R₂ = H Δ³

[83] R₁ = H   R₂ = Me
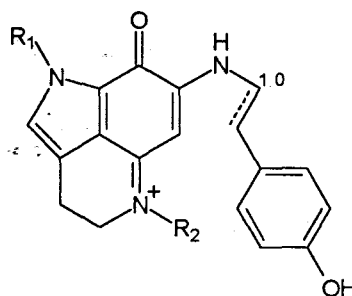
[84] R₁ = H   R₂ = H

[85] R₁ = Me R₂ = H Δ¹⁰

[88] R₁ = Me R₂ = Me Δ¹⁰
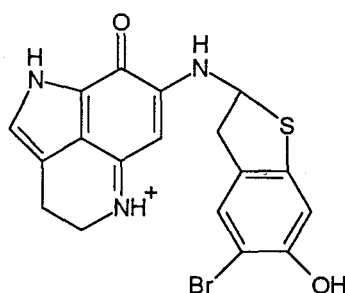
[86]
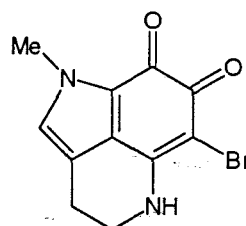
[87]

The isolation of the makaluvamines A [81], B [82], C [83], D [84], E [85] and F [86], makaluvone [87] and the known compounds discorhabdin A [64] and damirone B [79] from the Fijian sponge *Zyzzya* cf. *marsailis*[125], represented the first example of the co-occurrence of unfunctionalised pyrroloiminoquinones with the highly elaborated discorhabdin A. The isolation of, in particular, makaluvamines D [84] and F [86] suggests a plausible biosynthetic interrelationship within the pyrroloiminoquinone family of compounds. Makaluvamine G [88], from an Indonesian sponge *Histodermella* species[126], was reported as being biogenetically derivable from tryptamine and tyramine and structurally related to the discorhabdins.

From recent studies on the biosynthesis of discorhabdin B [65][127], which occurs in the New Zealand sponge *Latrunculia* species B, Munro and co-workers have proposed a loose biogenetic pathway which interrelates the discorhabdin and makaluvamine alkaloids (SCHEME 3.1).
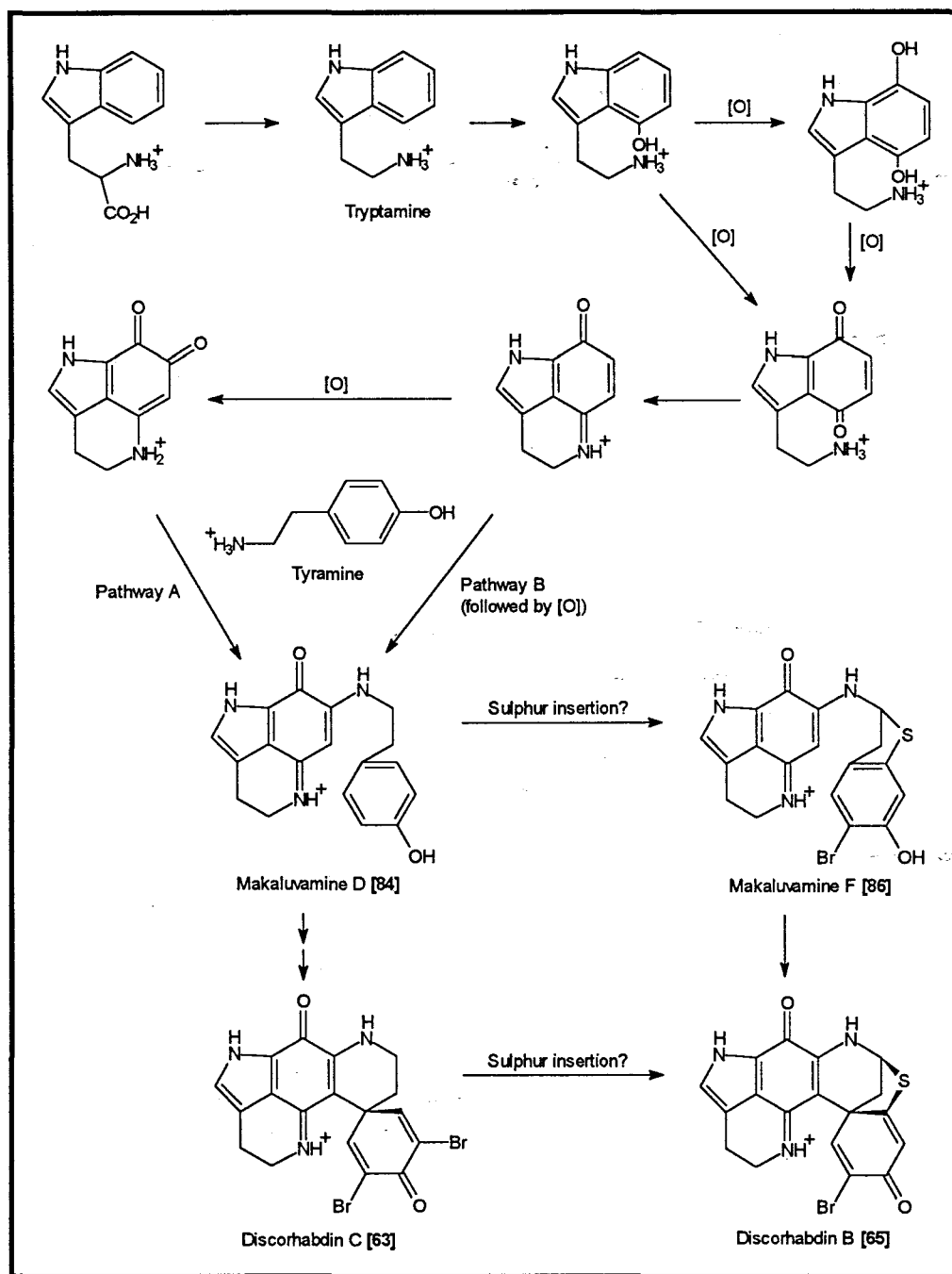
SCHEME 3.1 Postulated biogenesis of the discorhabdins by Munro and co-workers[127].

This pathway utilises the amino acids tryptophan and phenylalanine (*via* tryptamine and tyramine) as the precursors of the pyrroloiminoquinone backbone. Tryptamine, after undergoing appropriate functional changes and oxidation, incorporates tyramine, thereby generating makaluvamine D. Sulphur insertion into,

and/or ring closure of, makaluvamine D, together with other functional modifications is the basis by which makaluvamine F, discorhabdin B and discorhabdin C are formed. Evidence for the viability of this scheme was provided by a sponge tissue culture experiment performed by Munro and co-workers[127]. In this experiment tissue from the sponge *Latrunculia* species B was incubated in the presence of radioactive-labelled phenylalanine and it was found that the tissue used this radioactive-labelled phenylalanine in the biosynthesis of discorhabdin B. Munro *et al.* also considered the origin of the biosynthetic pathway taking into account the fact that there are three recognised means by which sponge cells can produce secondary metabolites. The production of these metabolites can be a property of the sponge cells themselves, can be generated exclusively by parasitic or commensal micro-organisms, or can be the result of a metabolic co-operation between the sponge cells and symbiotic micro-organisms. To address this problem the same tissue culture technique was used, however, in this experiment the sponge tissue was pre-treated with a broad spectrum antibiotic prior to exposure to radioactive-labelled phenylalanine and then, after exposure, the tissue was incubated in the presence of the antibiotic. Results of the experiment demonstrated that the radioactive-labelled phenylalanine was still incorporated into discorhabdin B by the sponge tissue and this suggested that participation of symbiotic bacteria in the biosynthesis of the discorhabdins is not required.

In the same paper by Munro and co-workers, describing their biosynthetic studies of discorhabdin B[127], it is reported that at least thirteen discorhabdins (A - M) have thus far been isolated by this research group. With the data of a number of these compounds (discorhabdin G - M) unpublished, it is obvious that care is needed in the naming of new discorhabdin compounds to avoid confusion. Overlap has already occurred with the paper of Baker *et al.* describing the isolation of discorhabdin G [89][128], a previously unreported pigment from the Antarctic sponge *Latrunculia apicalis*. The structure of Baker's discorhabdin G differs substantially to that of Munro's as yet unpublished discorhabdin G[†].

---

† Dr Murray Munro - personal communication.
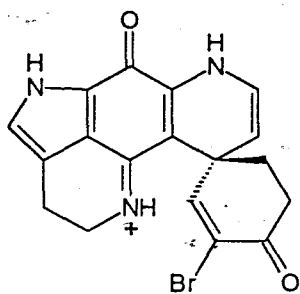
[89]

The considerable biological activity and unique nature of the highly fused skeleton in these marine alkaloids has inspired several groups to undertake synthetic studies on these compounds. Examples include reports of the total syntheses of discorhabdin C [63][129] and the makaluvamines A [81] - E [85][130]. In order to explore the structural features responsible for the biological activities of the discorhabdins, Munro and co-workers made a series of derivatives of discorhabdin C. These were evaluated in a wide range of screens for selective cytotoxicity, antifungal and antimicrobial activity[131].
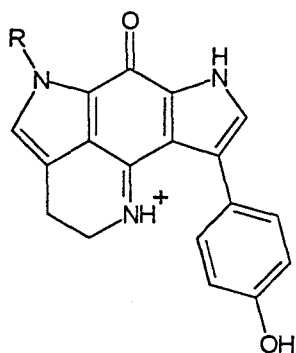
[90] R = H
[91] R = Me
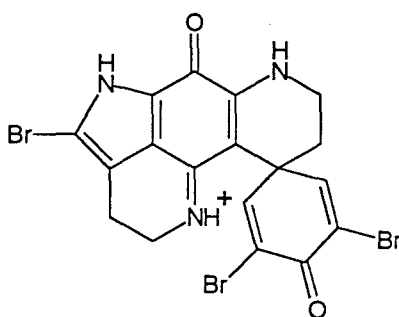
[92]
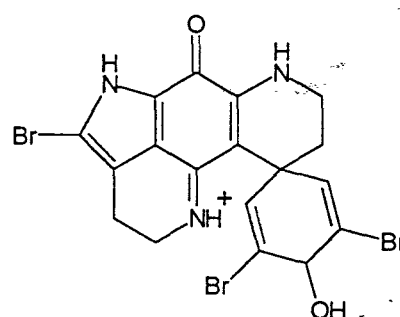
[93]

Section 3.2 and 3.3 of this chapter describe the isolation and structure determination of four new pyrroloiminoquinone alkaloids, the tsitsikammamines A [90] - D [93], from a South African Latrunculid sponge. Initial taxonomic studies indicate that this sponge is a new genus of Latrunculid sponge with morphological characteristics intermediate between those of *Zyzzya* and *Latrunculia* species[‡].

---

‡ Michelle Kelly-Borges - personal communication.

48

3.2 COLLECTION, EXTRACTION AND ISOLATION
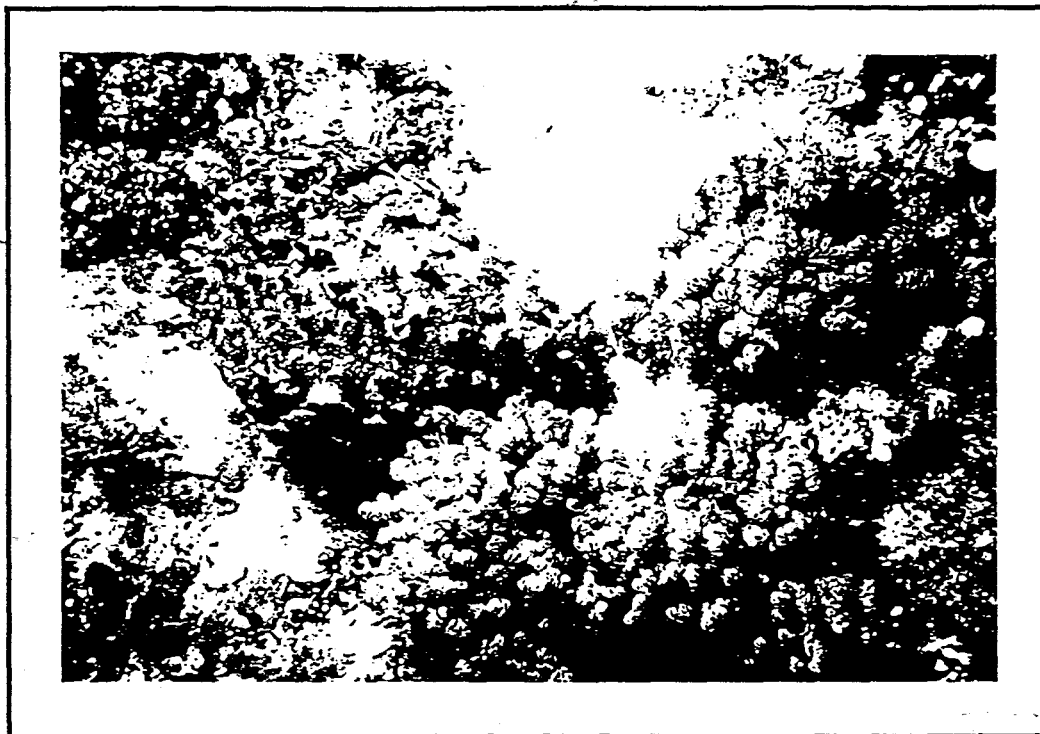
FIGURE 3.1 A new genus of Latrunculid sponge collected from Rheerders Reef in the Tsitsikamma Marine Reserve.

A brown nodular Latrunculid sponge (FIGURE 3.1) was collected in the Tsitsikamma Marine Reserve using SCUBA from a depth of -20 m in May 1993. A second collection of this sponge was made in May 1995. Several frozen sponges from the former collection were freeze-dried (dry weight 287.5 g) and steeped in MeOH/CHCl$_3$ (1:1) to yield 15.5 g of extract. The crude extract (tested at 250 µg disc⁻¹) displayed potent antimicrobial activity against Bacillus subitilis, Staphylococcus aureus and Escherichia coli equal to and exceeding that of the gentamycin standard (10 µg disc⁻¹). Unfortunately, the dark sponge extract showed extremely unfavourable solubility properties in both aqueous and organic solvents, however, aqueous solubility improved dramatically on the addition of a few drops of trifluoroacetic acid (TFA). The

improved aqueous solubility is attributed to protonation of the imine functionality. The bioassay directed fractionation of the crude extract (**SCHEME 3.2** and **SCHEME 3.3**) led to the isolation of the strongly antimicrobial pigments, tsitsikammamine A **[90]** - D **[93]** as their TFA salts.

Initial Sep-Pak cartridge column chromatography of the crude sponge extract (**SCHEME 3.2**) revealed that approximately one third of the extract was NaCl and one fifth was undesirable fatty acids and sterols, leaving only a small fraction containing the potent antimicrobial compounds. Thus, to improve yields, the fractionation procedure was repeated with a larger portion of crude extract and a partitioning step to remove the undesirable lipophilic constituents (**SCHEME 3.3**). Although several different HPLC columns under varying conditions were used in **SCHEME 3.3** to optimise the separation of compounds **[90]** - **[93]** it was generally found that these compounds show poor resolution and peak shape with HPLC separation.
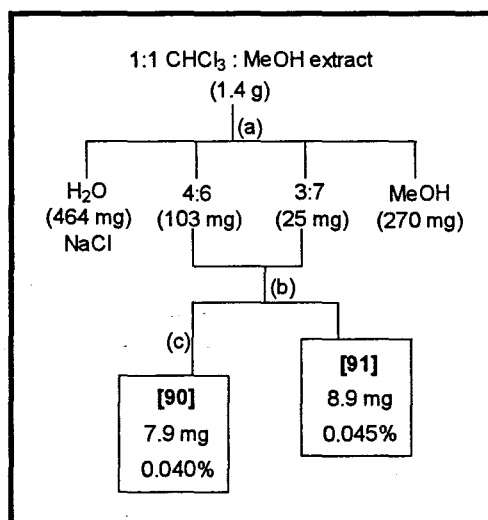
SCHEME 3.2 Chromatographic procedure for the isolation of compounds **[90]** and **[91]**. (a) cartridge column (C18, 10 g, Waters Sep-Pak, gradient elution: H_2O to MeOH / 0.05% TFA). (b) HPLC (C8, 10 μ, Phenomenex, RI detection, 25% $MeCN/H_2O$ / 0.05% TFA, 4 mL min^{-1}). (c) HPLC (C8, 10 μ, Phenomenex, RI detection, 22.5% $MeCN/H_2O$ / 0.05% TFA, 4 mL min^{-1}).

1:1 CHCl₃ : MeOH extract
(8.0 g)

(a)
(b)

| H₂O (1.8 g) NaCl | 6:4 (650 mg) | 4:6 (82 mg) | 2:8 (23 mg) | MeOH (37 mg) |

(c)

H₂O 7.5:2.5 (257 mg)   7:3 (227 mg)   6.5:3.5 (63 mg) MeOH

(d)
(e)

[92]
63.2 mg
0.056%

[90] (7.5 mg)   **[91]** (26.7 mg)   **[93]** 5.7 mg 0.019%   **[92]** (16.2 mg)
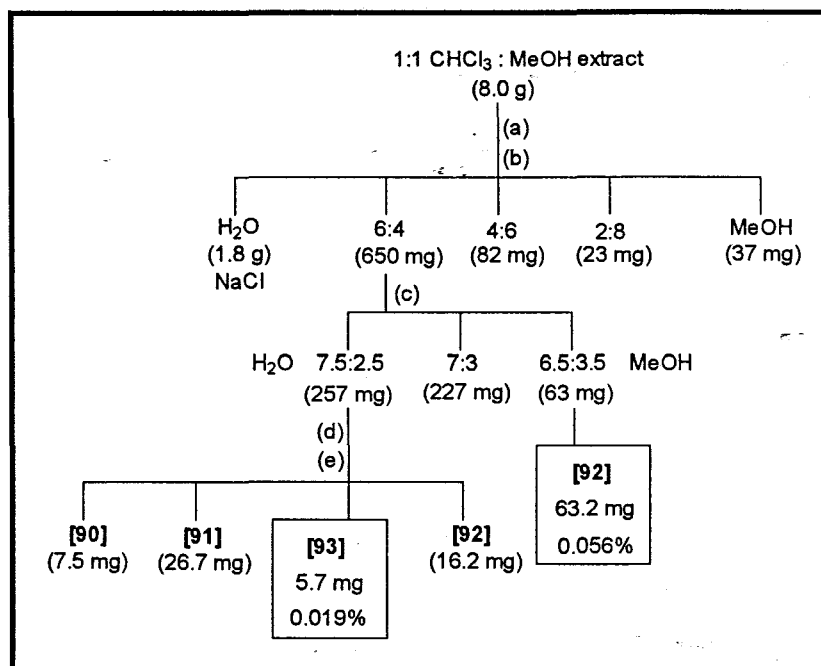
SCHEME 3.3 Chromatographic procedure for the isolation of compounds **[92]** and **[93]**. (a) solvent partioning (organic layer: 33% EtOAc/hexane and aqueous layer: H₂O / 0.1% TFA. Aqueous layer was concentrated and fractionated further). (b) cartridge column (C18, 10 g, Waters Sep-Pak, gradient elution: H₂O to MeOH / 0.05% TFA). (c) cartridge column (C18, 10 g, Waters Sep-Pak, gradient elution: 5% MeOH/H₂O to 50% MeOH/H₂O / 0.05% TFA). (d) various combinations of analytical and pilot-scale semi-prep. HPLC in order to optimise further fractionation (C18, C8 and CN columns; RI and UV detection; varying solvent combinations of MeCN, MeOH and H₂O at pH 3 with TFA; varying flow rates). (e) HPLC (C8, 10 µ, Phenomenex, UV detection 356 nm, 20% MeCN/H₂O at pH 3 with TFA, 4-6 mL min⁻¹).

3.3 STRUCTURE DETERMINATION

3.3.1 Tsitsikammamine A [90].

[90]
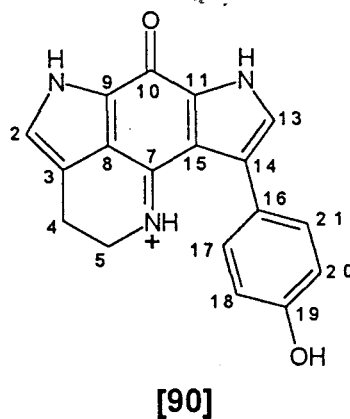
HRFAB mass spectral analysis indicated a protonated molecular ion at m/z 304.1087 (Δ -0.1 mDa) thus establishing the molecular formula of [90] as $C_{18}H_{14}N_3O_2$. The ^{13}C NMR spectrum of [90] revealed sixteen distinct carbon resonances. The molecular formula implied that two resonance pairs overlapped while the absence of ten ^{13}C signals from the DEPT NMR spectrum of [90] supported the presence of ten quarternary carbons (TABLE 3.1). Compound [90] gave a very simple ^1H NMR spectrum (FIGURE 3.2) which belied the complexity of its molecular structure.
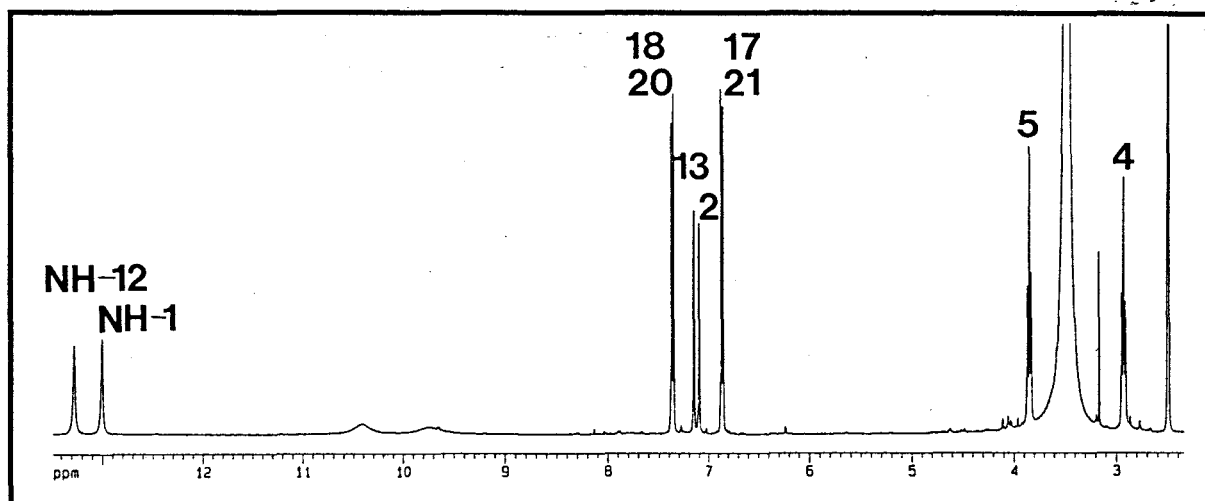
FIGURE 3.2 ^1H NMR (400 MHz, DMSO-d_6) spectrum of [90].

TABLE 3.1   NMR spectral data for tsitsikammamine A [90].

	^{13}C	^1H	COSY to	HMBC to
NH-1		13.01 br s	H-2,*H-4	C3, C8
2	123.1 d	7.10 d (1.8)	NH-1, *H-4	C3, C8, C9
3	119.2 s			
4	17.6 t	2.93 t (7.8)	H-5,*H-2,*NH-1	C2, C3, C8
5	45.0 t	3.85 t (7.8)	H-4	C3, C7
7	156.8 s			
8	120.7 s			
9	127.8 s			
10	166.3 s			
11	134.6 s			
NH-12		13.28 br s	H-13	
13	125.0 d	7.16 d (2.5)	NH-12	C11, C15, C16
14	122.4 s			
15	113.5 s			
16	127.2 s			
17	116.2 d	6.87 d (8.4)	H-18	C14, C19, C21
18	128.9 d	7.37 d (8.4)	H-17	C16, C19, C20
19	157.6 s			
20	128.9 d	7.37 d (8.4)	H-21	C16, C18, C19
21	116.2 d	6.87 d (8.4)	H-20	C14, C17, C19

* Long-range COSY coupling.

Spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C in DMSO-d_6 with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.

The spin systems comprising δ 13.01 (1H, br s, NH-1) coupled to δ 7.10 (1H, d, J = 1.8 Hz, H-2) and the mutually coupled methylene signals at δ 2.93 (2H, t, J = 7.8 Hz, H-4) and δ 3.85 (2H, t, J = 7.8 Hz, H-5) were established by a COSY NMR experiment. These systems, together with the pyrroloiminoquinone ^{13}C NMR

resonances at δ 166 (C-10), 157 (C-7), 128 (C-9) and 121 (C-8), are characteristic of the discorhabdin compounds A - D[113,114,116]. The pyrroloiminoquinone substructure of [90] therefore accounted for eight of the fourteen degrees of unsaturation implied in the molecular formula.

Two mutually coupled two-proton doublets at δ 6.87 (2H, d, J = 8.4 Hz, H-17 and H-21) and δ 7.37 (2H, d, J = 8.4 Hz, H-18 and H-20) each attached to carbons resonating at δ 116 and δ 129, respectively, in addition to two quarternary carbon signals at δ 127 (C-16) and δ 158 (C-19), suggested that [90] contained a *para*-substituted phenol[125]. The phenolic substituent accounted for a further four degrees of unsaturation. The two remaining degrees of unsaturation are therefore represented by an olefinic moiety (δ 125 and 122) and a fifth cyclic system. The COSY spectrum of [90] revealed a further spin system comprising δ 13.28 (1H, br s, NH-12) coupled to δ 7.16 (1H, d, J = 2.5 Hz, H-13) which suggested the possibility of a second 2,3,4-trisubstituted pyrrole ring system. This bispyrroloiminoquinone substructure was supported by the two and three bond correlations observed in the HMBC NMR experiment of [90] (FIGURE 3.3). The three bond correlations between the H-13 vinylic proton and the C-11 and C-15 quinone carbon atoms (δ 135 and 114 respectively) in the HMBC spectrum (FIGURE 3.4) proved crucial in placing the second pyrrole ring in a fused postion at C-11, C-15. Three bond correlations between the H-17 and H-21 aromatic protons and δ 122.4 (C-14) and between the H-13 pyrrole proton and δ 127.2 (C-16) (FIGURE 3.4) clearly placed the *para*-substituted phenol at C-14.
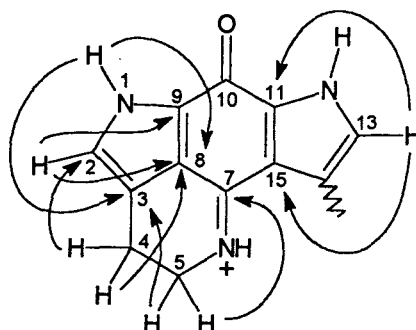
FIGURE 3.3 HMBC NMR correlations in the bispyrroloiminoquinone substructure.
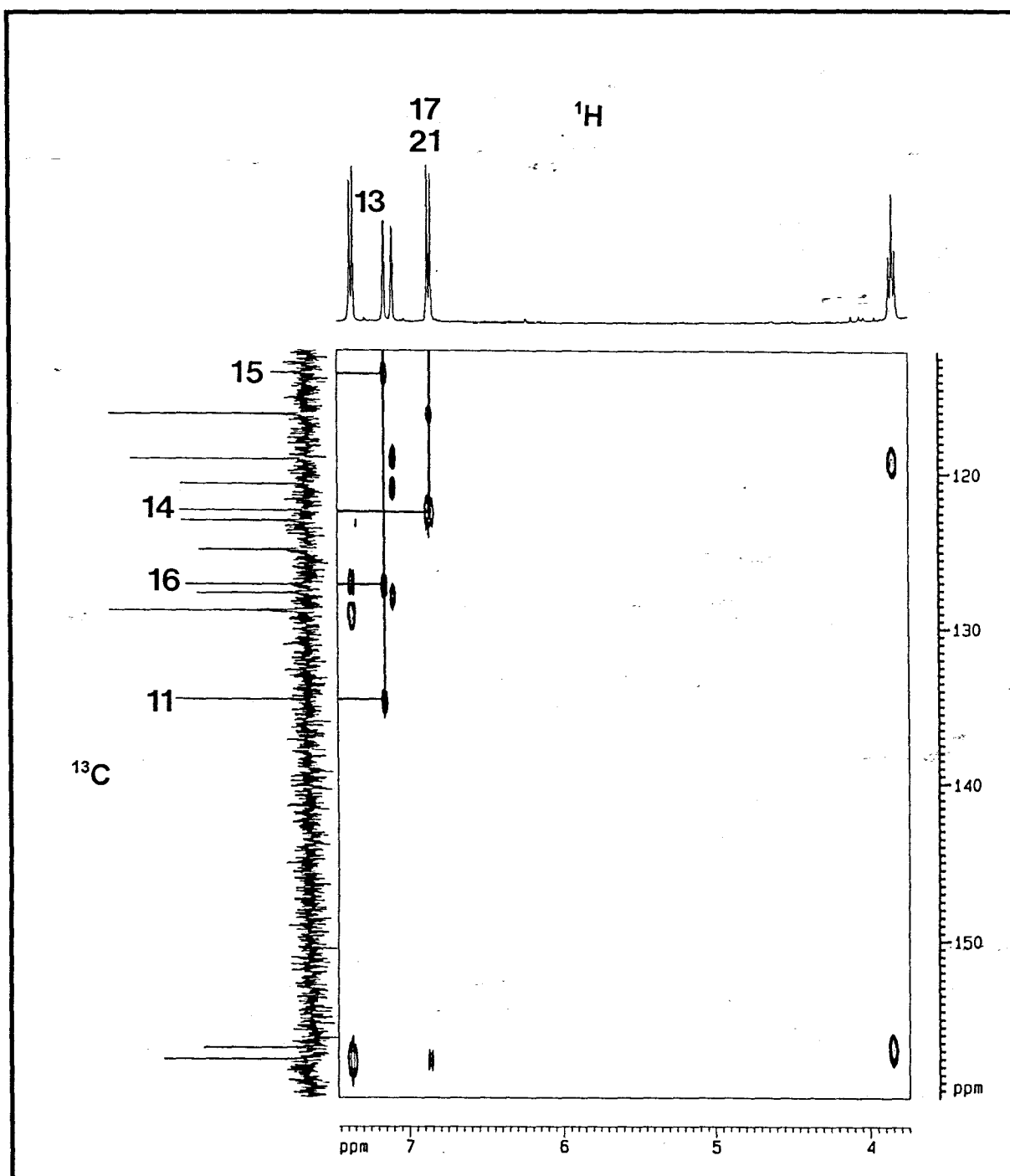
FIGURE 3.4 HMBC NMR (400 MHz, DMSO-d_6) spectrum of tsitsikammamine A [90].

It is interesting to note that the makaluvamines D [84], E [85] and G [88] all contain a *para*-substituted phenol attached to a pyrroloiminoquinone moiety. However, [90] is the first example from the Porifera where ring closure has occurred to create a

bispyrroloiminoquinone system. This ring closure is not unique and wakayin [80], isolated from the ascidian *Clavelina* species[123], contains a bispyrroloiminoquinone substructure with an indole substituent. The existence of [80] in an ascidian, and the isolation of [90] from a sponge, possibly questions Munro's suggestion[127] that the pyrroloiminoquinone compounds are exclusively products of sponge cell secondary metabolism rather than that of a symbiotic organism which might occur in selected sponges and ascidians.

3.3.2 Tsitsikammamine B [91].
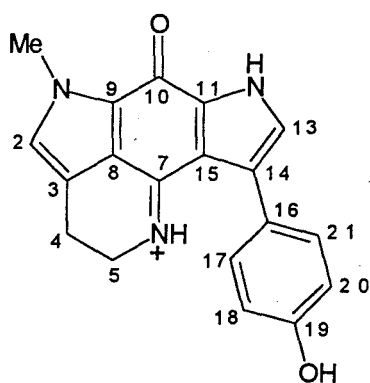
[91]

Tsitsikammamine B [91] gave a protonated molecular ion at *m/z* 318.1252 (Δ - 0.9 mDa) in its HRFAB mass spectrum, which is consistent with the molecular formula $C_{19}H_{16}N_3O_2$. The same major chromophores in the UV spectrum of [90] were evident in the UV spectrum of [91] (FIGURE 3.5) suggesting a common skeletal structure. The coupled methylene signals at δ 2.91 (2H, t, J = 7.8 Hz, H-4) and δ 3.82 (2H, t, J = 7.8 Hz, H-5), the iminoquinone ^{13}C resonances at δ 166.8 (C-10) and δ 156.2 (C-7) (TABLE 3.2), the proton signals at δ 13.28 (1H, br s, NH-12) and δ 7.16 (1H, s, H-13), and the two mutually coupled two-proton doublets at δ 6.86 (2H, d, J = 8.1 Hz, H-17 and H-21) and δ 7.36 (2H, d, J = 8.1 Hz, H-18 and H-20) in [91] were also consistent with a *para*-phenoxy substituted bispyrroloiminoquinone structure.
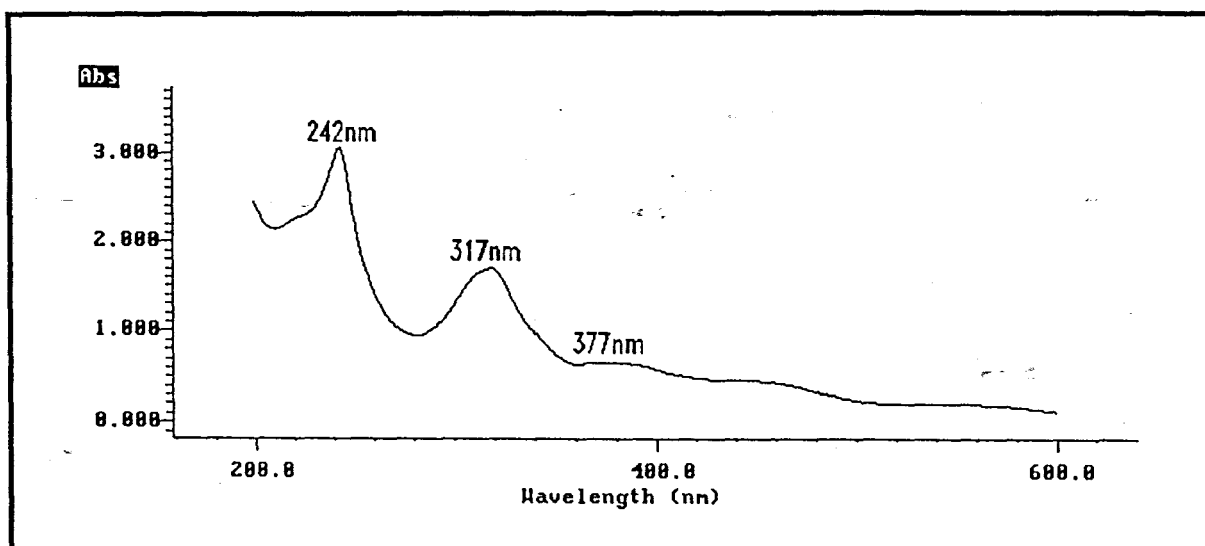
[90]
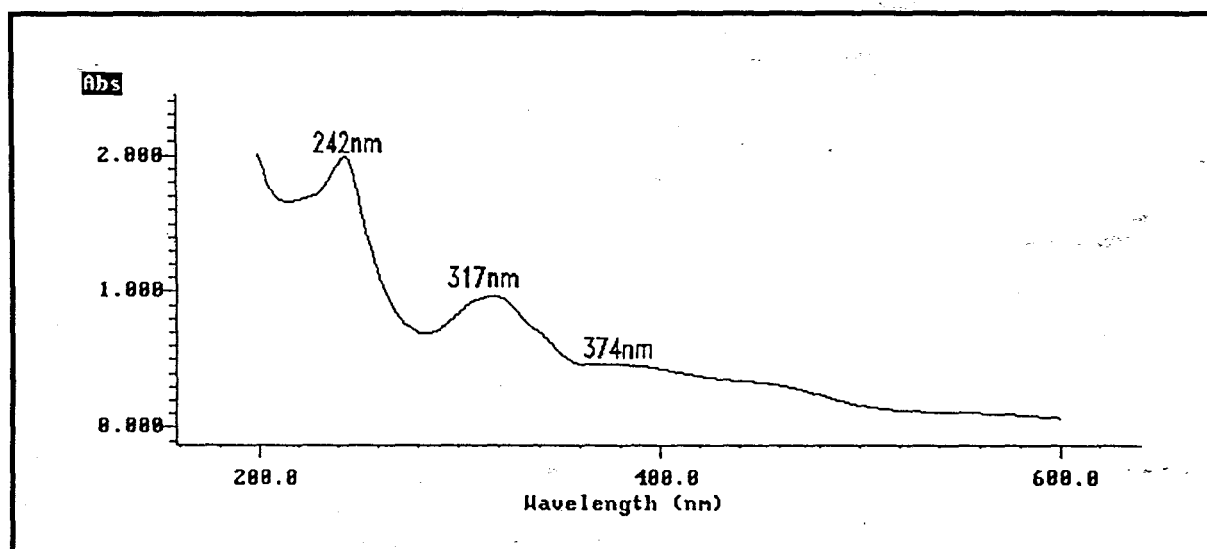
[91]

FIGURE 3.5 UV spectra (in MeOH) of [90] and [91].

The absence, however, of the NH-1 resonance in the ^{1}H NMR spectrum of [91], together with the collapse of the H-2 vinylic doublet in [90] into a one proton singlet at δ 7.11 (1H, s, H-2) in [91], and the additional methyl resonance at δ 3.92 (3H, s, N-Me), placed a methyl substituent at N-1 in this compound.

TABLE 3.2   NMR spectral data for tsitsikammamine B **[91]**.

	^{13}C	^1H	HMBC to
2	128.0 d	7.11 s	C3, C8, C9, N-Me
3	118.7 s		
4	17.6 t	2.91 t (7.8)	C3, C5, C8
5	44.7 t	3.82 t (7.8)	C3, C4, C7
7	156.2 s		
8	120.7 s		
9	126.4 s		
10	166.8 s		
11	134.6 s		
NH-12		13.28 br s	
13	125.2 d	7.16 s	C11, C15, C16
14	122.4 s		
15	113.3 s		
16	127.1 s		
17	116.3 d	6.86 d (8.1)	C14, C19, C21
18	129.0 d	7.36 d (8.1)	C16, C19, C20
19	157.6 s		
20	129.0 d	7.36 d (8.1)	C16, C18, C19
21	116.3 d	6.86 d (8.1)	C14, C17, C19
N-Me	35.8 q	3.92 s	C2, C9

Spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C in DMSO-d_6 with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.
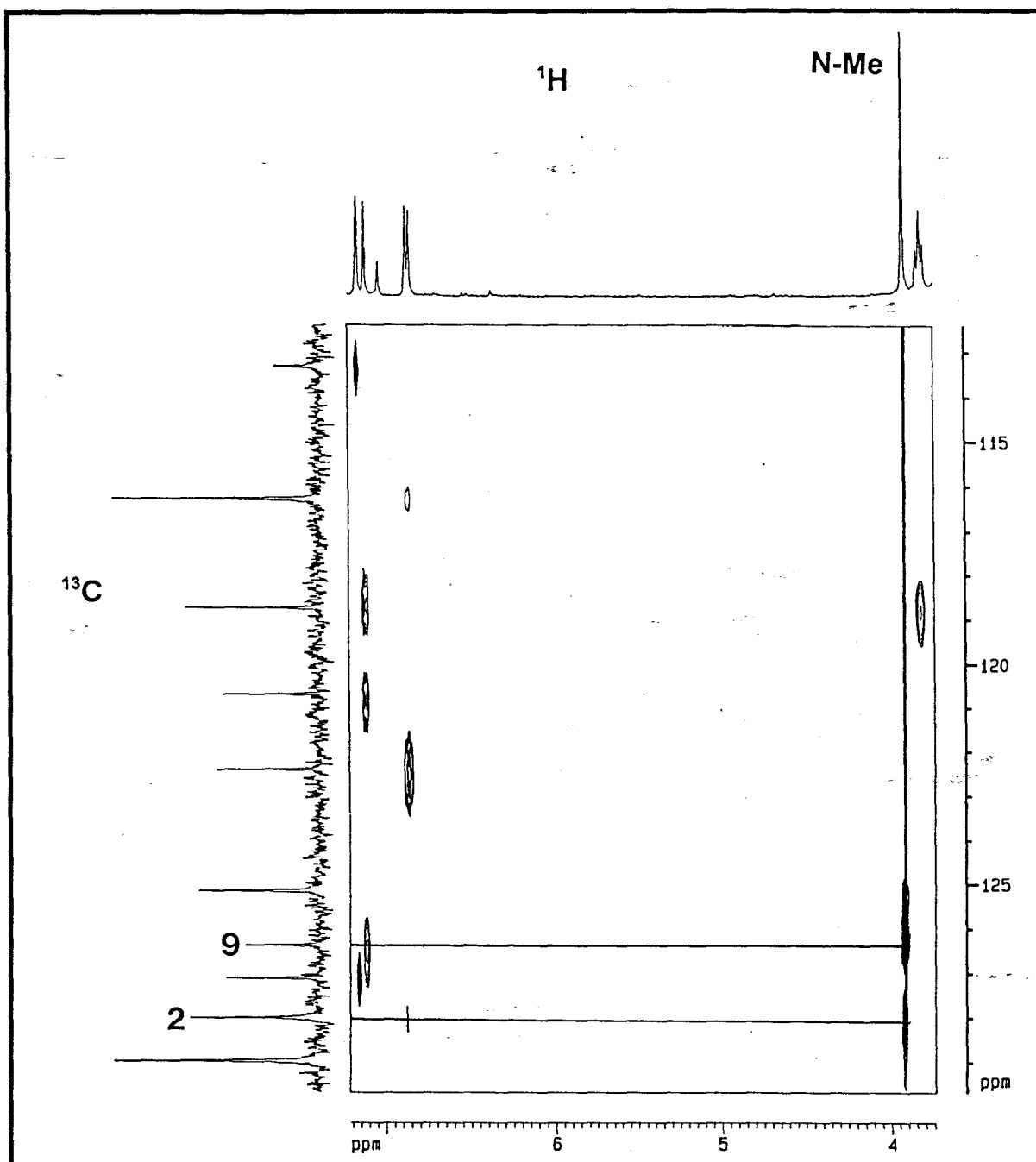
FIGURE 3.6 HMBC NMR (400 MHz, DMSO-d_6) spectrum of tsitsikammamine B [91].

Three bond correlations observed in the HMBC NMR spectrum of [91] (FIGURE 3.6), from the N-methyl protons to C-2 and C-9 in the pyrrole ring, confirmed the position of the methyl group at N-1. The occurrence of a methyl group at N-1 is a structural feature consistent with several of the makaluvamine compounds[125,126].

3.3.3 Tsitsikammamine C [92].
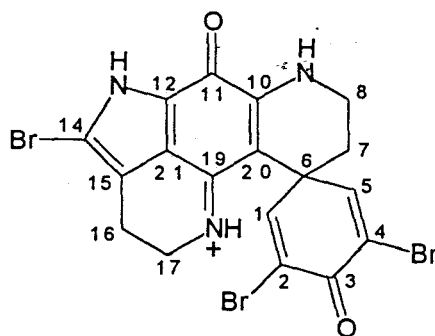
[92]

Tsitsikammamine C [92] showed a protonated molecular ion at m/z 539.8544 in its HRFAB mass spectrum, consistent with the molecular formula $C_{18}H_{13}N_3O_2Br_3$ (Δ +1.4 mDa). The ^{13}C NMR spectrum of [92] (FIGURE 3.7) in conjunction with a DEPT NMR experiment revealed the presence of four methylene (δ 17.5 - 44.5), two overlapping methine (δ 151) and twelve quarternery carbons (TABLE 3.3). Based on the previous observations for pyrroloiminoquinone structures, the spin system comprising δ 2.71 (2H, t, J = 7.4 Hz, H-16) coupled to δ 3.71 (2H, t, J = 7.4 Hz, H-17) further coupled to δ 8.22 (1H, br s, NH-18) together with the ^{13}C NMR resonances at δ 164 (C-11), 152 (C-19), 124 (C-12) and 124 (C-21) supported the presence of a pyrroloiminoquinone substructure in [92].
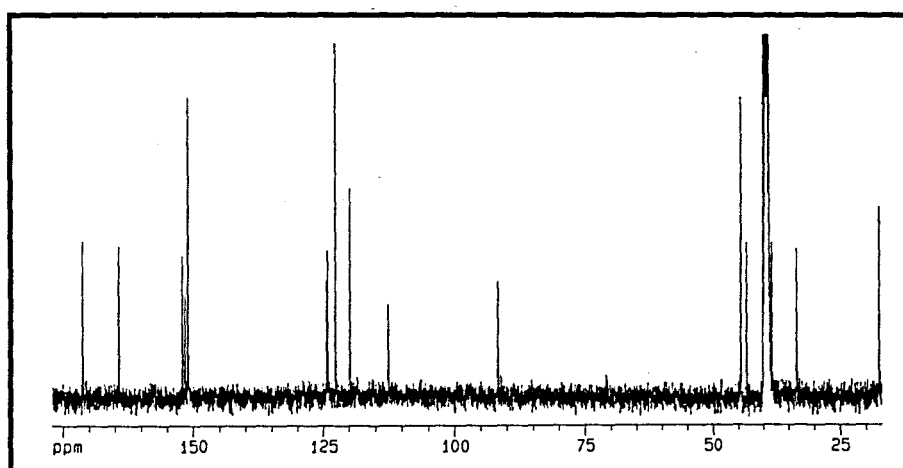
FIGURE 3.7 ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of [92].

TABLE 3.3 NMR spectral data for tsitsikammamine C **[92]**.

	^{13}C	^1H	COSY to	HMBC to
1	150.9 d	7.70 s		C2, C3, C5, C6, C7, C20
2	122.6 s			
3	171.2 s			
4	122.6 s			
5	150.9 d	7.70 s		C1, C2, C3, C6, C7, C20
6	44.5 s			
7	33.5 t	2.02 br s	H-8	C1, C5, C6, C20
8	38.3 t	3.63 br s	H-7, NH-9	
NH-9		10.27 br s	H-8	
10	151.5 s			
11	164.3 s			
12	124.1 s			
14	112.5 s			
15	119.8 s			
16	17.5 t	2.71 t (7.4)	H-17	C14, C15, C17, C21
17	43.4 t	3.71 t (7.4)	H-16, NH-18	C15, C19
NH-18		8.22 br s	H-17	
19	152.0 s			
20	91.7 s			
21	124.2 s			

Spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C in DMSO-d_6 with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.

However, the presence of an additional fused piperidine ring spin system with δ 2.02 (2H, br s, H-7) coupled to δ 3.63 (2H, br s, H-8) and further coupled to δ 10.27 (1H, br s, NH-9) in the COSY NMR spectrum of **[92]** suggested that the iminoquinone

existed within a phenanthroline ring system which is a structural feature common to all the discorhabdins. Therefore, based on this association, further structural elucidation was achieved by comparison of the spectral data of **[92]** with those of discorhabdin C **[63]**, the structure of which was determined by X-ray analysis[113]. A sharp two-proton vinylic singlet resonating at δ 7.70 (2H, s, H-1 and H-5), which gave a three bond HMBC correlation to a carbonyl ^{13}C NMR resonance at δ 171 (C-3) in the HMBC NMR spectrum of **[92]**, pointed to the existence of a symmetrical unsaturated spiro-cyclohexanone system in this compound. Comparison of the ^{1}H and ^{13}C NMR chemical shifts of this system with those of discorhabdin C[113] revealed that **[92]** had an equivalent 2,4-dibromocyclohex-1,4-dien-3-one moiety attached at C-6. Further comparison of the NMR and HRFAB mass spectral data with those for discorhabdin C clearly showed that the only difference between the two compounds was an additional bromine substituent in **[92]** at the expense of a proton. A three bond correlation from the H-16 methylene protons to the quaternary C-14 carbon signal in the HMBC spectrum, the absence of an olefinic proton singlet at δ 7.22 (H-14) in the ^{1}H NMR spectrum of **[92]**, and the upfield shift of the C-14 ^{13}C NMR signal from δ 128 for discorhabdin C to δ 113 for **[92]**, placed the bromine at the C-14 position and completed the assignment of tsitsikammamine C. This is the first example of a discorhabdin compound with a substituent at position-14.

3.3.4 Tsitsikammamine D **[93]**.
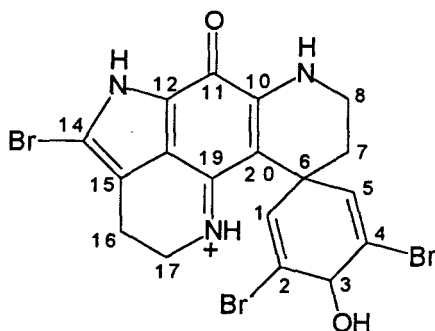
[93]

A protonated molecular ion at *m/z* 541.8710 in the HRFAB mass spectrum of **[93]** indicated a molecular formula of $C_{18}H_{15}N_3O_2Br_3$ (Δ +0.4 mDa). Comparison with the

molecular formula of compound **[92]** revealed that the only difference between the two compounds was an additional two protons in **[93]**. Comparison of the ^{13}C NMR data of **[93]** **(TABLE 3.4)** with those for **[92]** showed the absence of a carbonyl resonance at δ 171.2 (C-3) and the presence of an oxymethine signal at δ 70.7 which correlated to a one proton doublet at δ 4.67 (1H, d, J = 8.2 Hz, H-3) in the HMQC NMR spectrum of **[93]**. This proton doublet was in turn strongly coupled to an exchangeable single proton doublet at δ 6.20 (1H, d, J = 8.9 Hz, OH-3) in the COSY NMR spectrum of **[93]**. Addition of D$_2$O to the NMR sample caused the doublet at δ 6.20 to disappear and the doublet at δ 4.47 to coalesce into a sharp singlet **(FIGURE 3.8)**. The above implied the presence of a 2,4-dibromo-3-hydroxy-cyclohex-1,4-diene moiety in **[93]** and this was confirmed by the two bond correlations from H-3 to C-2 and C-4 in the HMBC spectrum.
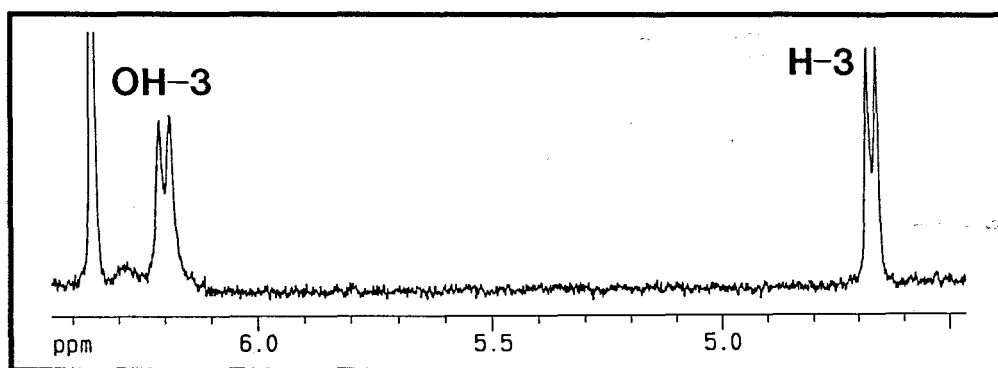
(a)
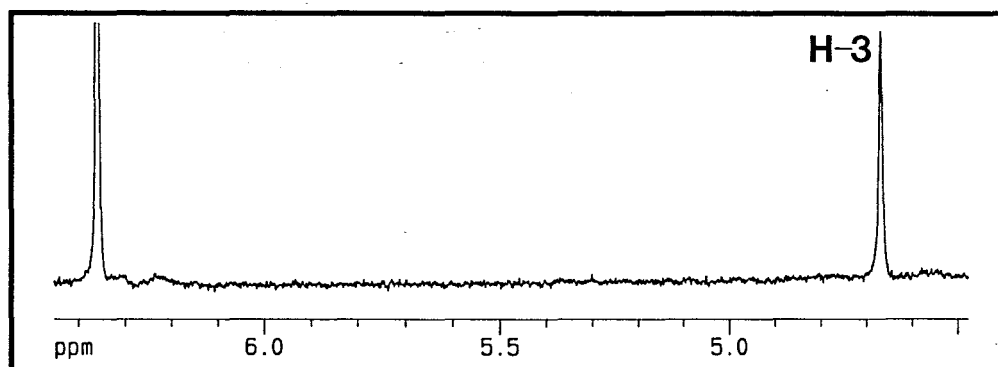
(b)

FIGURE 3.8 Comparative ^{1}H NMR (400 MHz, DMSO-d_6) spectra of **[93]**. (a) before addition of D$_2$O. (b) after addition of D$_2$O.

TABLE 3.4 NMR spectral data for tsitsikammamine D **[93]**.

	^{13}C	^{1}H	COSY to	HMBC to
1	134.0 d	6.36 s	H-3	C2, C3, C4, C5, C6, C20
2	124.4 s			
3	70.7 d	4.67 d (8.2)	H-1, H-5, OH-3	C2, C4
4	124.4 s			
5	134.0 d	6.36 s	H-3	C1, C2, C3, C4, C6, C20
6	41.9 s			
7	34.7 t	1.87 br s	H-8	C1, C5, C6, C20
8	38.1 t	3.51 br s	H-7, NH-9	
NH-9		10.07 br s	H-8	
10	151.5 s			
11	164.3 s			
12	124.1 s			
14	112.5 s			
15	119.8 s			
16	17.7 t	2.71 t (7.4)	H-17	C14, C15, C17, C21
17	43.6 t	3.78 t (7.0)	H-16, NH-18	C15, C19
NH-18		7.76 br s	H-17	
19	152.0 s			
20	96.0 s			
21	124.2 s			
OH-3		6.20 d (8.9)	H-3	

Spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C in DMSO-d_6 with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.

The presence of the makaluvamine-like compounds [90] and [91] together with the discorhabdin-like compounds [92] and [93] in this Latrunculid sponge supports Ireland's contention[125] that the less functionalised makaluvamines provide a logical biosynthetic pool for interrelation with the more highly elaborated discorhabdin family of compounds. The co-occurence of the new compounds [90] - [93] in this South African Latrunculid sponge has provided support for the sponge's classification as a new genus.

3.4 BIOLOGICAL ACTIVITY

The pyrroloiminoquinone alkaloids exhibit a wide range of biological activities including antiviral activity[114], antimicrobial activity[114,123,128], induced Ca^{2+} release from the sarcoplasmic reticulum[117], inhibition of the topoisomerase II enzyme[123,125,126], and *in vitro* cytotoxicity against a number of cell lines including murine leukaemia[114,115,126], human epidermoid carcinoma[117,126], human colon tumour[123,125,126], human lung cancer[126] and human breast cancer[126] cell lines.

Of all the crude extracts of the 350 different marine invertebrates collected by the research group at Rhodes University between 1992 and 1995 that were screened for antimicrobial activity, the crude extract of the Latrunculid sponge collected from the Tsitsikamma Marine Reserve exhibited the greatest antimicrobial activity. The crude extract ($MeOH/CHCl_3$) of this Latrunculid sponge displayed potent activity against the bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* equal to and exceeding that of the gentamycin standard (**TABLE 3.5**). Fractionation of this extract led to the isolation of tsitsikammamines A [90] - C [92] as pure compounds. Antimicrobial testing of these pure compounds indicated that they contributed largely to the potent antimicrobial activity of the crude extract against the Gram-positive bacteria *B. subtilis* and *S. aureus* (**TABLE 3.5**). However, these compounds exhibited only a slight activity against the Gram-negative bacterium *E. coli* suggesting either that other compounds in the crude extract are responsible for this activity or that this activity

is synergistic and only observed when a combination of compounds **[90]** - **[92]** is present.

TABLE 3.5   Zone of inhibition diameters measured in the antimicrobial disc assay of the crude extract of the Latrunculid sponge and of the tsitsikammamines A **[90]** - C **[92]**.

SAMPLE	B. subtilis	S. aureus	E. coli
Testing at 250 µg disc⁻¹:			
crude extract	23.0*	25.0*	21.0*
standard	23.0	23.5	19.0
Testing at 100 µg disc⁻¹:			
[90]	18.0	22.5	sl
[91]	18.0	23.0	sl
[92]	18.5	27.0	sl
standard	20.0	24.5	22.0
Testing at 50 µg disc⁻¹:			
[90]	17.0	19.0	sl
[91]	17.5	19.0	sl
[92]	18.0	25.0	sl
standard	22.0	24.0	21.0
Testing at 10 µg disc⁻¹:			
[90]	12.0	11.0	-
[91]	11.0	10.5	-
[92]	14.0	23.5	-
standard	20.5	24.0	20.5

*Inhibition zone diameter in mm, disc diameter = 8.0 mm and "sl" indicates slight activity (8.0 - 9.0 mm). Standard = gentamycin 10 µg disc⁻¹.

Tumour cells often have a defective ability to repair damage to DNA compared to normal cells, therefore, compounds with selective toxicity towards repair deficient cells might be potential anticancer agents[132]. A mechanism-based yeast bioassay which can selectively detect agents causing DNA damage in the repair deficient cells is employed by SKB in the search for potential anticancer agents from natural sources[132]. With the deletion of the DNA topoisomerase I gene, the assay can be used to detect agents which produce DNA damage specifically by interacting with DNA topoisomerases and can even differentiate between a DNA topoisomerase I and a DNA topoisomerase II inhibitor.

In previous communications[123,125] Ireland has reported the ability of pyrroloiminoquinones, specifically wakayin [80] and the makaluvamine-type compounds [81] - [87], to inhibit the topoisomerase II enzyme. This prompted an exploration of this activity in the tsitsikammamines. Interestingly, screening of the tsitsikammamines A [90] - D [93] in SKB's mechanism-based yeast bioassay showed no evidence that these compounds produce DNA damage and, by inference, these compounds, therefore, are not topoisomerase inhibitors. In this assay the tsitsikammamines were shown to be antifungal. Deletion of the topoisomerase I gene in the assay had no effect on the antifungal activity which suggests that the antifungal activity of these compounds is not by interaction with topoisomerase I but by an unknown mechanism. Work is presently in progress at SKB to investigate the mechanism of antifungal activity of compounds [90] - [93].

CHAPTER FOUR

NEW METABOLITES FROM THE SOUTH AFRICAN SOFT CORAL
CAPNELLA THYRSOIDEA

4.1 INTRODUCTION

Advances in underwater diving technology in the latter half of this century have enabled man to closely study and survey the marine reef environment. In early investigations of reef communities several important observations concerning the alcyonaceans (soft corals) were made and these have directly influenced the marine chemist. The fact that alcyonaceans seemed to be the largest single contributor to the biomass of many Indo-Pacific reefs, that they had a notable ability to discourage algal and microbial growth and prevent the settlement of larvae, and that they experience an extremely low incidence of predation[133], inspired marine chemists to target the soft corals as one of the first marine invertebrate groups for systematic chemical scrutiny. It was soon discovered that the alcyonaceans constitute a rich source of terpenoids and polyhydroxysterols[133].

In 1992 Coll reviewed the chemistry and chemical ecology of the Alcyonacea[83] (true soft corals), of which there are three large multigeneric families: the Alcyoniidae, Nephtheiidae and Xeniidae. In reviewing the distribution of terpenes within the different soft coral genera and families, Coll reported that the distribution of terpenes in the soft coral genus *Capnella* (Family: Nephtheiidae) was limited to sesquiterpenes. This conclusion was reached from reviewing the chemical investigations conducted on a single species of *Capnella* soft coral, *i.e. C. imbricata*.

[94]   **[95]**   **[96]**
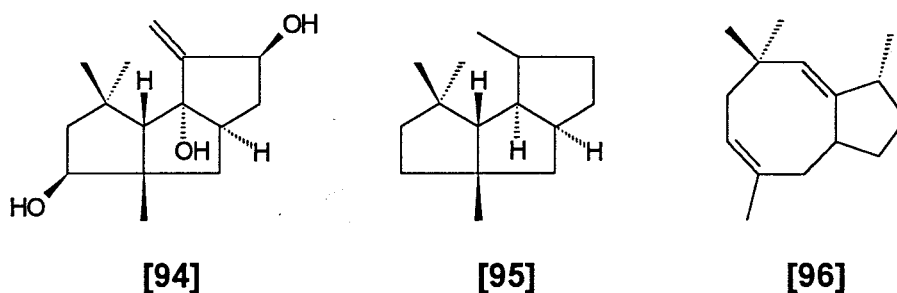
In 1974 a new tricyclic sesquiterpene **[94]**, was isolated from the soft coral *Capnella imbricata*[134]. This compound is a trihydroxy derivative of what is now termed

the capnellane skeleton [95]. Further research of the same species of soft coral yielded five additional sesquiterpenes[135-137] all containing the three 5-membered fused ring (capnellane) skeleton, and precapnelladiene [96][138], a possible biosynthetic precursor of the capnellane skeleton. Paradoxically, in 1985 it was reported that the major compounds in living colonies of *Capnella imbricata* were acetylated capnellenes[139] and it was thus surmised that the previously reported capnellene polyols were artifacts from hydrolysis during the sun-drying of the animals.

During the course of a marine invertebrate sampling expedition in the Tsitsikamma Marine Reserve in May 1993, the soft coral *Capnella thyrsoidea* was collected. This soft coral is apparently endemic to South Africa and is one of the most common soft corals found off the South African coast[59]. The soft coral exists as several phenotypic colour variants[t]. Section 4.2 and 4.3 of this chapter details the chemical analysis of extracts of two clearly distinguishable bright yellow and grey colour variants of *Capnella thyrsoidea*[84]. The study resulted in the isolation and identification of three pregnadiene steroids and four xenicane diterpene metabolites.

[97] R = H
[98] R = OAc

[99] R = OAc
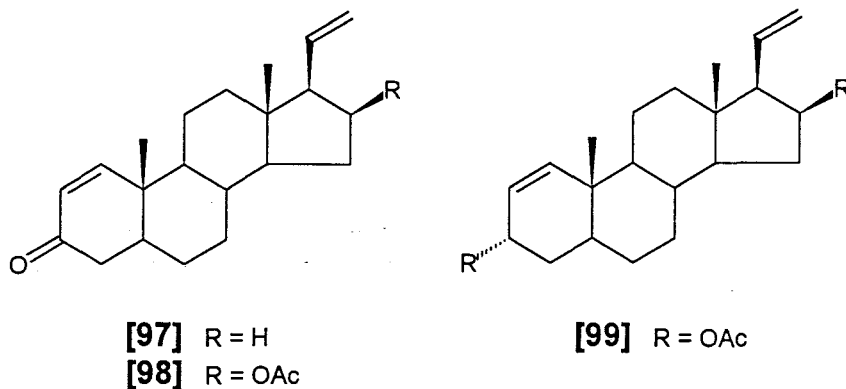
The pregnadiene compound [97], isolated from the yellow colour variant, was found to be identical with a previously isolated pregnadiene steroid from an Australian *Capnella* species (*C. erecta*)[140] and from an unidentified soft coral from Canton Island[141]. Two new pregnadiene steroids [98] and [99] were isolated and identified from the grey colour variant of *C. thyrsoidea*. Naturally occurring pregnane-derived steroids from the marine environment[141-144] are reported to be rare[140].

---

† Personal communication with Dr Gary Williams, California Academy of Sciences.
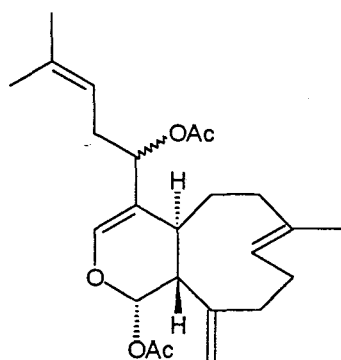
[100]
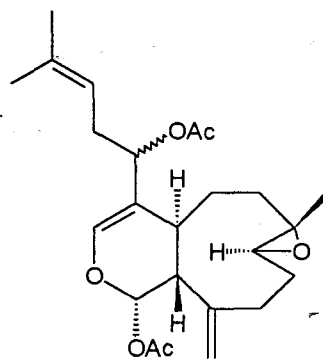
[101]
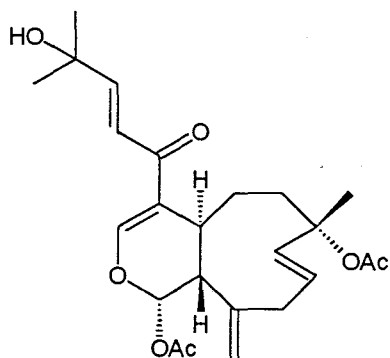
[102]
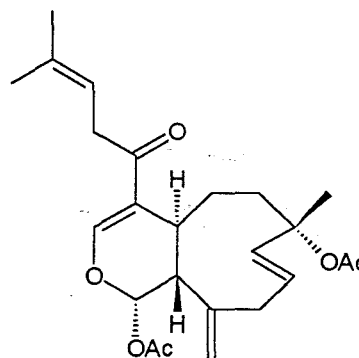
[103]

The crude extracts of the yellow and grey colour variants of C. thyrsoidea also yielded a series of xenicane diterpene compounds, the tsitsixenicins A [100], B [101], C [102] and D [103]. Xenicane diterpenes are predominantly found in soft corals of the genera Xenia[145-159] and Anthelia[63,64,160] (Family: Xeniidae) and at one stage it was believed that the xenicane diterpenes were produced exclusively by the family Xeniidae. More recently, however, xenicane diterpenes have been isolated from the soft coral families Helioporiidae[161] and Alcyoniidae[63], and also from a number of gorgonians of the genus Acalycigorgia[162-164].

The xenicane diterpenes isolated from the above marine invertebrates have been divided into three structural groups: the xenicins and xeniolides (numbering approximately fifty-two and eighteen respectively) both of which contain a 2-oxabicyclo[7.4.0]tridecane ring system, and the xeniaphyllanes (numbering

71

approximately fifteen) which possess a bicyclo[7.2.0]undecane structure. Xenicin [104][145], xeniolide A [105][147] and xeniaphyllenol [106][146] provide an example of each group respectively.

AcO OAc

OAc

OAc

OAc

[104]

OH

O

O

OH

[105]

HO
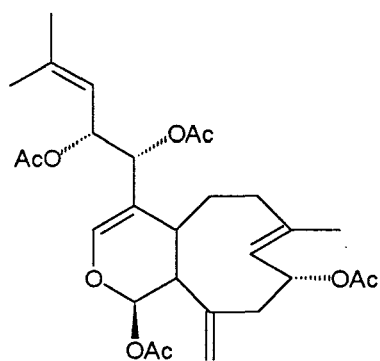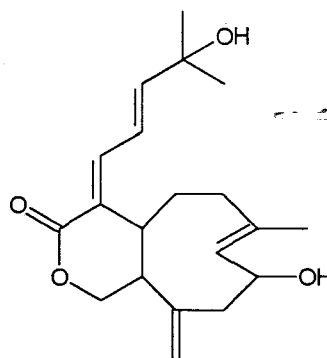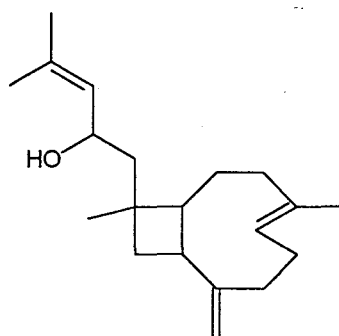
[106]

Xenicane diterpene compounds have never before been reported from the soft coral family Nephtheiidae and so the tsitsixenicins represent the first examples from this soft coral family. The discovery of these diterpenes is at variance with Coll's generalisation[83] that the distribution of terpenes in *Capnella* species is limited to sesquiterpenes and a revision of the chemotaxonomy of this genus is required.

4.2   COLLECTION, EXTRACTION AND ISOLATION
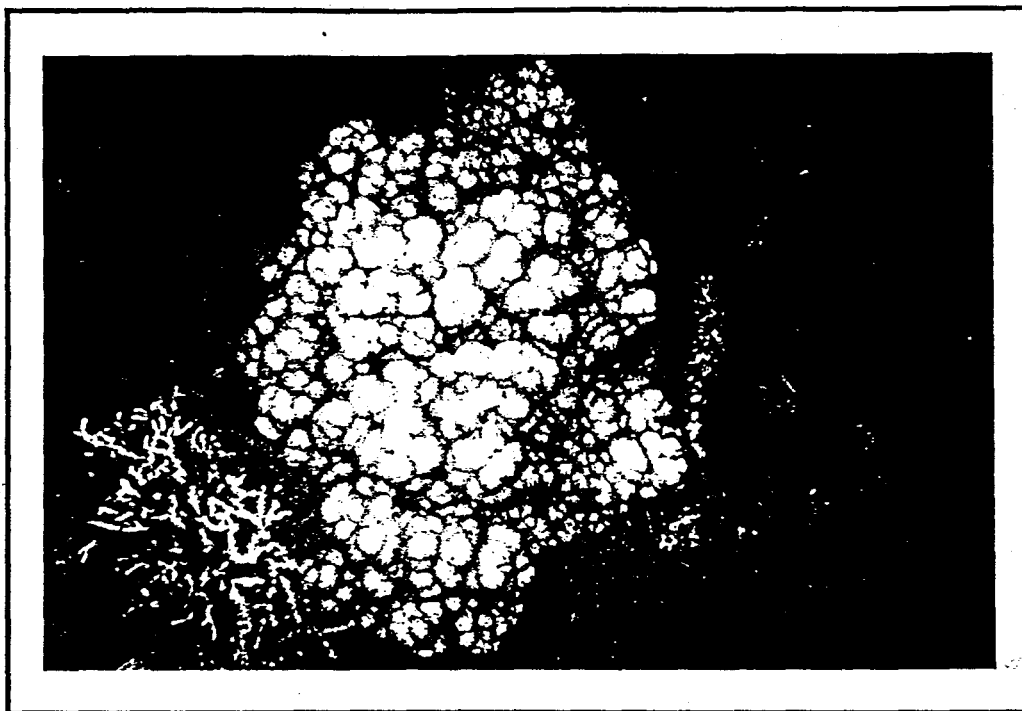
FIGURE 4.1 Yellow colour variant of the soft coral *Capnella thyrsoidea* collected from
Rheerders Reef in the Tsitsikamma Marine Reserve.

The clearly distinguishable bright yellow (**FIGURE 4.1**) and grey colourmorphs
of the soft coral *Capnella thyrsoidea* were collected using SCUBA at a depth of -30 m
in the Tsitsikamma Marine Reserve in May 1993 and recollected in May 1994. The
frozen yellow and grey colour variants were freeze-dried (dry weights 618 g and 207
g respectively) and steeped in EtOAc. The separate EtOAc extracts were concentrated
under reduced pressure to give dark brown oils (16.0 g and 4.7 g respectively). These
were screened by ^{1}H NMR spectroscopy and although the ^{1}H NMR spectra were
similar, significant differences in the series of peaks occurring between δ 4.8 and 7.2
precluded the combining of the two extracts. Fractionation of each extract was
conducted according to **SCHEME 4.1** and **SCHEME 4.2** respectively.

YELLOW COLOURMORPH

EtOAc extract
(2.10 g)

(956.7 mg)

(a)

20% EtOAc / hexane   50% EtOAc / hexane   EtOAc
(139.4 mg)

(b)

[97]
80.2 mg
0.14%

AFTER RECOLLECTION

EtOAc extract
(13.9 g)

(6.0 g)

(c)

20% EtOAc / hexane   50% EtOAc / hexane   EtOAc
(1.26 g)

(d)

mother liquor   [97]
(767.3 mg)   crystalline steroid
                           (492.4 mg)
(134.3 mg)

(e)

(65.6 mg)

(f)

(36.3 mg)

(g)

[100]
23.3 mg
0.06%
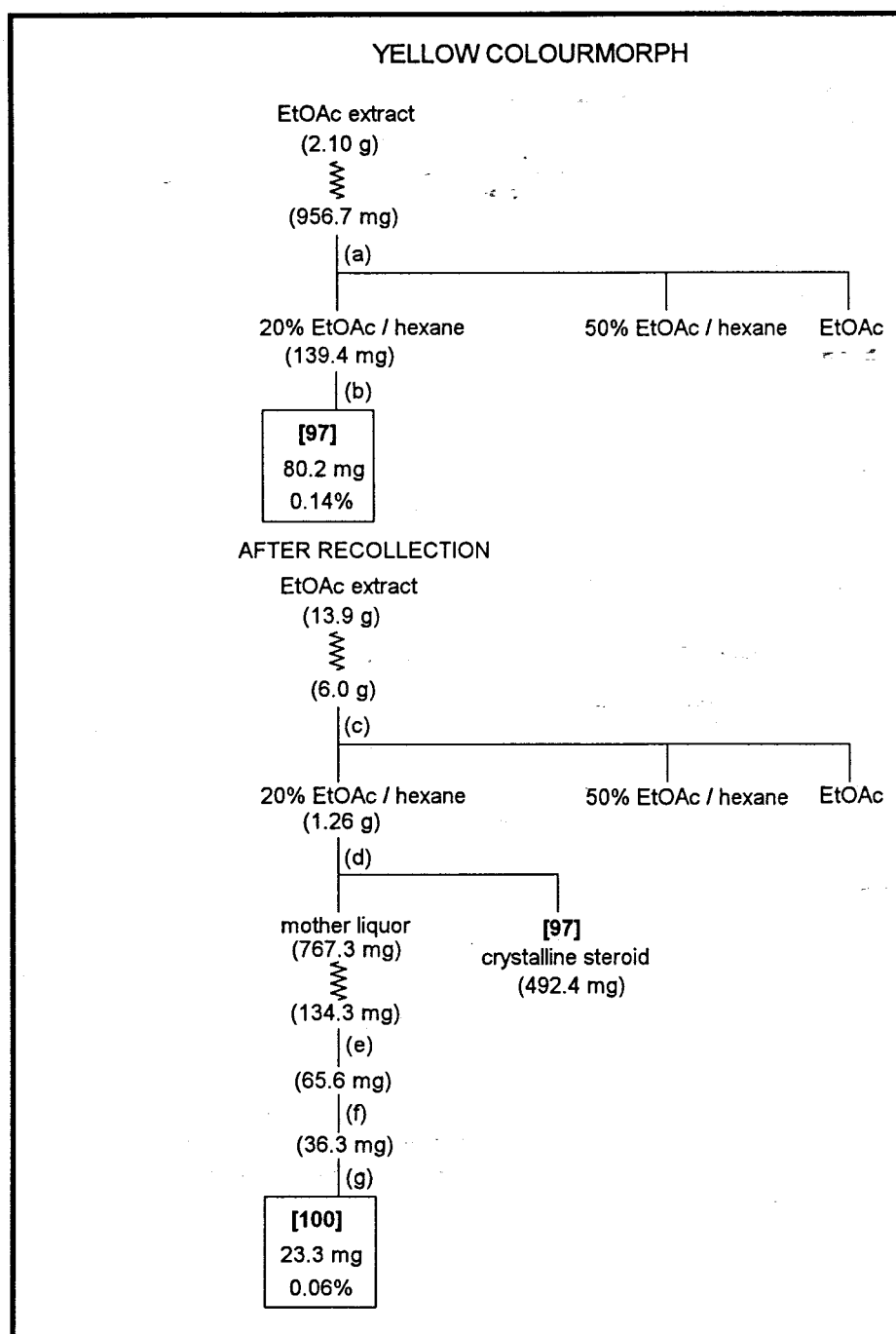
SCHEME 4.1 Chromatographic procedure for the isolation of compounds **[97]** and **[100]** from the yellow colour variant. (a) flash chromatography (silica, 230-400 mesh, 150 g, elution sequence: 20% EtOAc/hexane; 50% EtOAc/hexane; EtOAc). (b) HPLC (silica, 10 μ, Whatman, 7.5% EtOAc/hexane, 4 mL min^{-1}). (c) repeat of (a). (d) recrystallisation from hexane. (e) HPLC (C18, 10 μ, Phenomenex, 15% H$_2$O/MeOH, 4 mL min^{-1}). (f) HPLC (silica, 10 μ, Whatman, 5% EtOAc/hexane, 4 mL min^{-1}). (g) HPLC (silica, 10 μ, Whatman, 15% ether/isooctane, 4 mL min^{-1}).

GREY COLOURMORPH

EtOAc extract
(4.72 g)
(2.0 g)
(a)

(870 mg)
(208 mg)
(e)

(291 mg)
(b)
(196 mg)
(c)

(75 mg)
(f)

(88 mg)
(g)

[98] 7.5 mg 0.009%

(20.7 mg)
(d)

cholesterol
(132.0 mg)

[99] 4.8 mg 0.02%	[100] 67.8 mg 0.3%	[101] 5.6 mg 0.03%

[102] 5.9 mg 0.006%	[103] 8.3 mg 0.009%
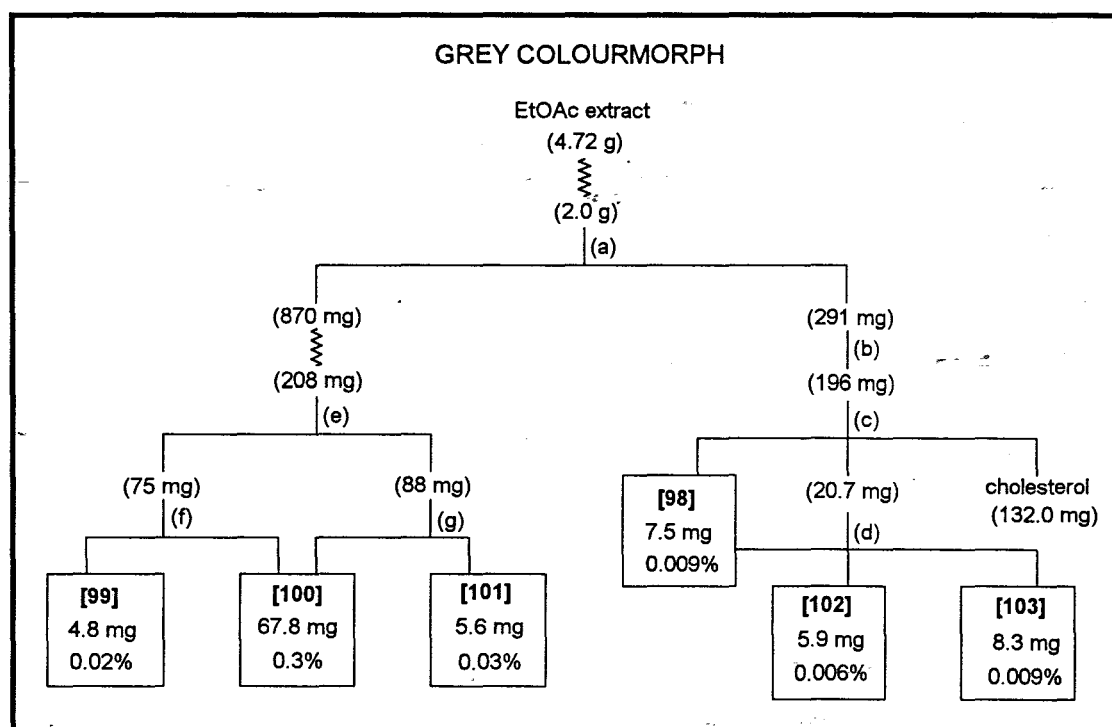
SCHEME 4.2 Chromatographic procedure for the isolation of compounds **[98]** - **[103]** from the grey colour variant. (a) flash chromatography (silica, 230-400 mesh, 150 g, 20% EtOAc/hexane). (b) repeat of (a). (c) HPLC (silica, 10 μ, Whatman, 15% EtOAc /hexane, 4 mL min^{-1}). (d) HPLC (C18, 10 μ, Phenomenex, 30% H$_2$O/MeCN, 4 mL min^{-1}). (e) HPLC (silica, 10 μ, Whatman, 8% EtOAc/hexane, 4 mL min^{-1}). (f) HPLC (C18, 10 μ, Phenomenex, 25% H$_2$O/MeCN, 4 mL min^{-1}). (g) HPLC (C18, 10 μ, Phenomenex, 27.5% H$_2$O/MeCN, 4 mL min^{-1}).

4.3 STRUCTURE DETERMINATION AND STEREOCHEMISTRY

4.3.1 Structure determination of the pregnadiene steroids [97], [98] and [99].
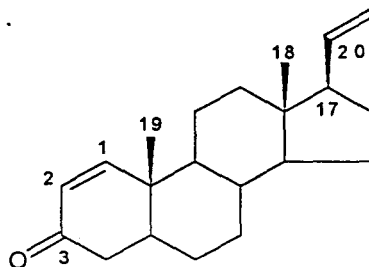
[97]

HREIMS data established the molecular formula of [97] as $C_{21}H_{30}O$ (m/z 298.2313, Δmmu +17). The absorbance at 1674 cm^{-1} in the IR spectrum of [97], together with two olefinic proton doublets at δ 7.13 (1H, d, $J_{1,2}$ = 10.2 Hz, H-1) and δ 5.83 (1H, d, $J_{1,2}$ = 10.0 Hz, H-2) situated at low field in the ^{1}H NMR spectrum of [97] (**FIGURE 4.2**), suggested the presence of an α,β-unsaturated ketone characteristic of 1-en-3-one steroids. A complex series of signals at δ 5.74 (1H, ddd, J = 8.8, 10.8, 16.5 Hz, H-20), 4.95 (2H, m, H-21) and 1.95 (1H, m, H-17) were assigned to the protons of an exocyclic vinylic group at C-17. Two three proton singlets at δ 0.61 (3H, s, H_3-18) and 1.00 (3H, s, H_3-19) were ascribed to the C-18 and C-19 methyl groups respectively. The above data, together with a combination of COSY, HMQC and HMBC NMR experiments, enabled complete structural elucidation of [97] which was identical, by comparison of ^{13}C NMR data (**TABLE 4.1**), melting point and optical rotation, with 5α-pregna-1,20-dien-3-one previously isolated from *C. erecta*[140] and an unidentified soft coral[141].

The distinctive pair of doublets at δ 7.13 (H-1) and 5.83 (H-2), triplet of doublets at δ 5.74 (H-20) and two proton multiplet at δ 4.95 (H_2-21) can be used to identify pregna-1,20-dien-3-one type compounds in crude extracts or chromatography fractions. Although no additional pregna-1,20-dien-3-one or similar compounds were evident in the ^{1}H NMR spectra of the remaining yellow colour variant chromatography fractions,

the ^{1}H NMR spectra of two fractions from the flash chromatography of the grey colour variant revealed the presence of these compounds. Both fractions were chromatographed further to yield the new compounds [98] and [99].

[98]
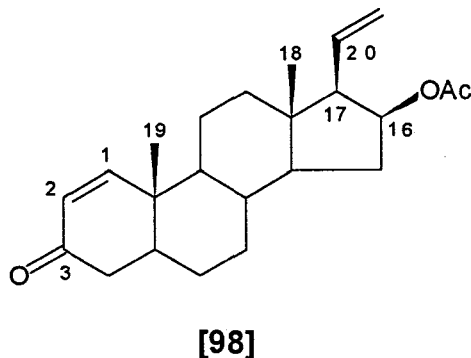
A molecular formula of $C_{23}H_{32}O_3$ (m/z 356.2338, Δmmu -13) from HREIMS data and an acetate methyl singlet at δ 2.02 in the ^{1}H NMR spectrum of [98] (FIGURE 4.3) confirmed that this compound was a monoacetoxy derivative of [97]. The position of the acetoxy group at C-16 followed from the coupling observed between H-16 (δ 5.11, 1H, m) and H-17 (δ 2.15, 1H, dd, $J_{16,17}$ = 4.1Hz, $J_{17,20}$ = 7.7 Hz) in the COSY NMR spectrum of [98] and established the structure of [98] as 16β-hydroxy-5α-pregna-1,20-dien-3-one 16-acetate. The methodology used to establish the stereochemistry of the acetoxy functionality at C-16 is described in section 4.3.2.

[99]
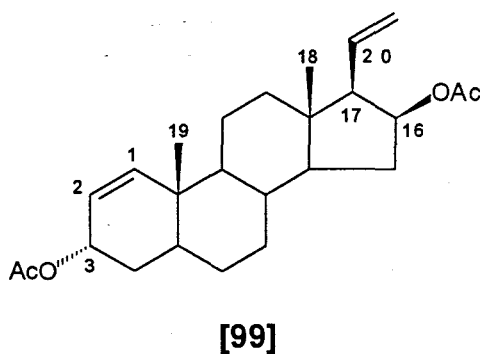
HREIMS established the molecular formula of the third pregnadiene compound [99] as $C_{25}H_{36}O_4$ (m/z 400.2609, Δmmu -4). The two acetate methyl proton singlets at δ 2.02 and 2.04 in the ^{1}H NMR spectrum of [99] (FIGURE 4.4) and the similarities observed between the spectral data of [99] and compounds [97] and [98] supported

a diacetoxy 5α-pregna-1,20-diene structure for this compound with one of the acetoxy groups at C-16 as in [98]. The second acetoxy group was unequivocally placed at C-3 based on the absence of the characteristic α,β-unsaturated carbonyl signal at δ 200 in the ^{13}C NMR spectrum of [99] and the coupling observed between the H-3 oxymethine proton at δ 5.15 (1H, m, H-3), the olefinic proton at δ 5.60 (1H, ddd, $J_{2,4}$ = 1.2 Hz, $J_{2,3}$ = 5.5 Hz, $J_{1,2}$ = 10.0 Hz, H-2) and the methylene protons at δ 1.53 and 1.81 (2H, m, H_a-4 and H_e-4) in the COSY NMR spectrum of this compound. These data verified 3α,16β-dihydroxy-5α-pregna-1,20-diene 3,16-diacetate as the structure for [99]. The methodology used to establish the stereochemistry of the acetoxy functionalities at C-3 and C-16 is discussed in section 4.3.2.

Although there was no evidence of [97] in any of the chromatography fractions from the grey colour variant of *C. thyrsoidea*, biosynthetic arguments and comparible spectral data exhibited by compounds [97] - [99] suggest a common 5α-pregnadiene parent structure for [98] and [99].
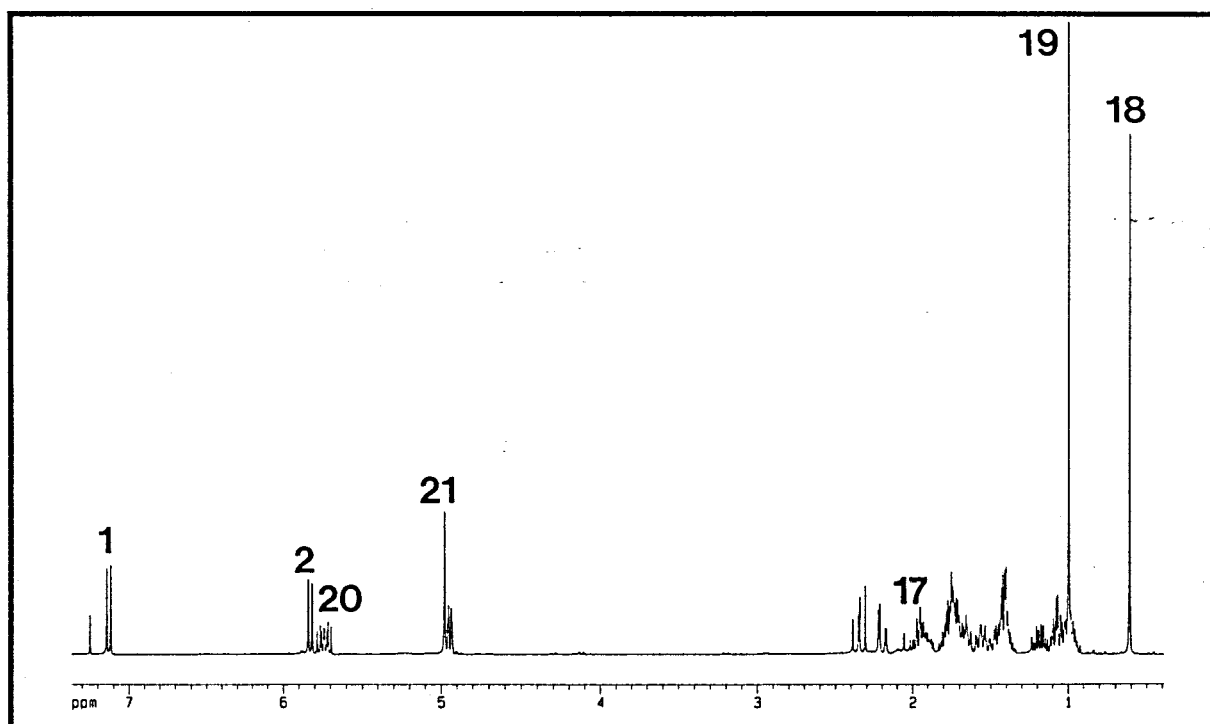
FIGURE 4.2 ^1H NMR (400 MHz, $CDCl_3$) spectrum of [97].
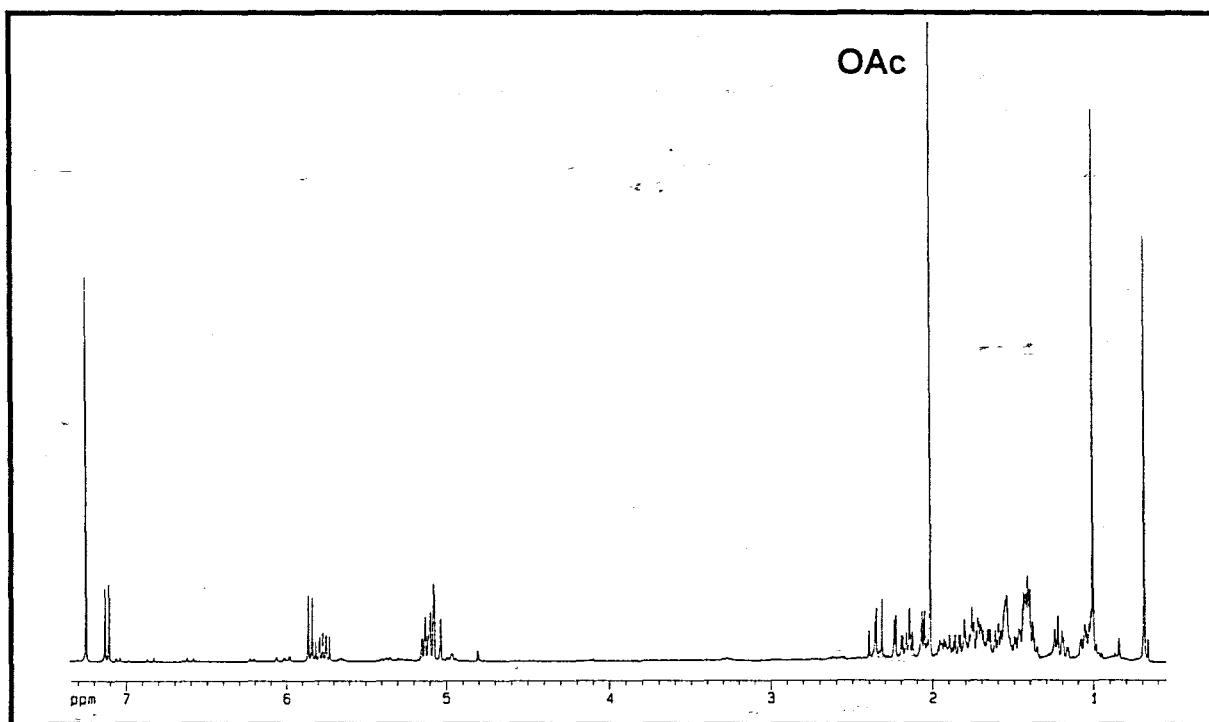
FIGURE 4.3 ^{1}H NMR (400 MHz, CDCl$_3$) spectrum of [98].
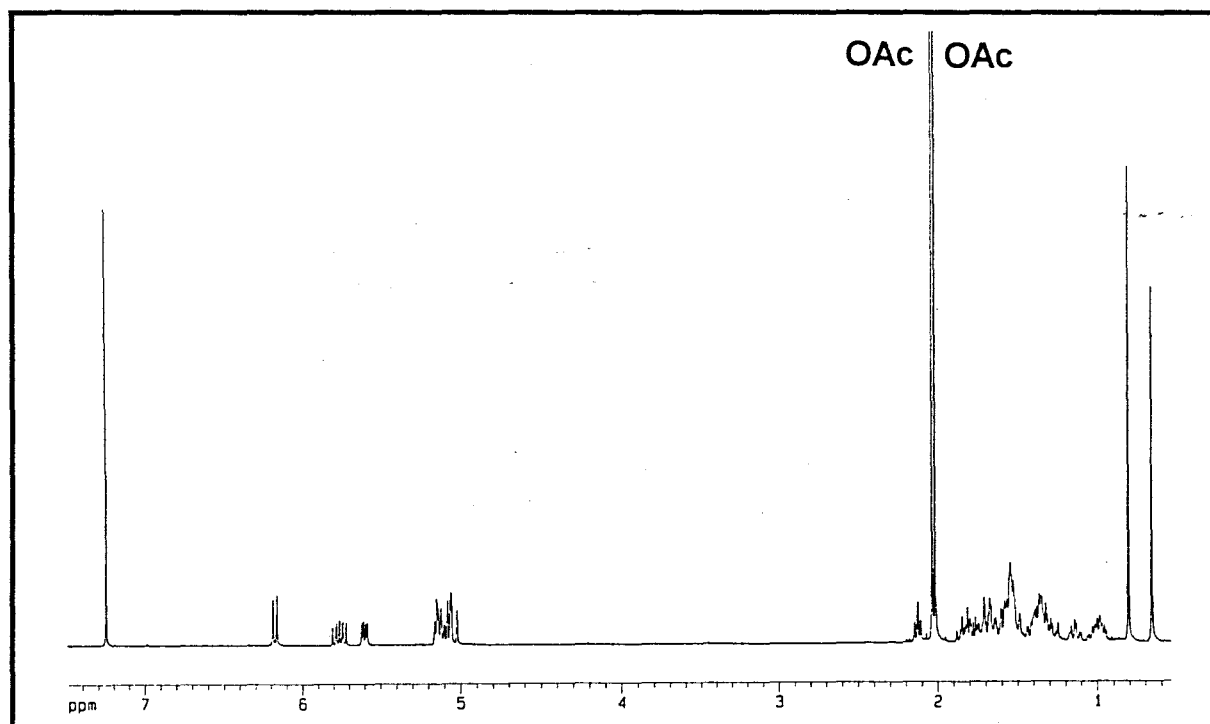
FIGURE 4.4 ^{1}H NMR (400 MHz, CDCl$_3$) spectrum of [99].

TABLE 4.1 ^{13}C NMR (100 MHz, $CDCl_3$) data for **[97]** - **[99]** and **[107]** - **[108]**.

	[97]	[98]	[99]	[107]	[108]
1	158.5 d	158.0 d	142.0 d	158.2 d	140.3 d
2	127.4 d	127.5 d	122.5 d	127.5 d	126.2 d
3	200.1 s	200.0 s	67.3 d	200.1 s	64.4 d
4	40.1 t	40.1 t	31.8 t	41.0 t	31.9 t
5	44.4 d	44.4 d	39.6 d	44.4 d	39.0 s
6	27.2 t	27.5 t	27.6 t	27.6 t	27.9 t
7	31.4 t	31.2 t	31.7 t	31.3 t	29.7 t
8	35.8 d	35.3 d	35.3 d	35.6 d	35.4 d
9	50.3 d	50.3 d	51.0 d	50.3 d	51.3 d
10	39.1 s	39.1 s	37.9 s	39.9 s	38.3 s
11	27.6 t	33.8 t	33.9 t	35.3 t	34.8 t
12	37.4 t	37.2 t	37.3 t	37.3 t	35.7 t
13	43.7 s	44.2 s	44.2 s	44.9 s	45.0 s
14	55.6 d	53.3 d	53.4 d	53.2 d	53.4 d
15	20.8 t	20.4 t	20.2 t	20.4 t	20.3 t
16	24.7 t	78.4 d	78.5 d	76.5 d	76.6 d
17	55.3 d	61.6 d	61.6 d	66.2 d	66.3 d
18	13.0 q	14.2 q	14.2 q	14.4 q	14.4 q
19	13.0 q	13.1 q	13.9 q	13.1 q	13.9 q
20	139.5 d	135.9 d	136.1 d	136.8 d	136.9 d
21	114.7 t	117.0 t	166.8 t	117.2 t	116.9 t
$COCH_3$		21.2 q	21.3 q		
			21.5 q		
$COCH_3$		171.1 s	171.1 s		
			170.1 s		

All assignments are supported by DEPT, COSY, HMQC and HMBC NMR experiments.

4.3.2 Stereochemistry of the pregnadiene steroids [98] and [99].

[97] R = H
[98] R = OAc
[107] R = OH

[99] R = OAc
[108] R = OH
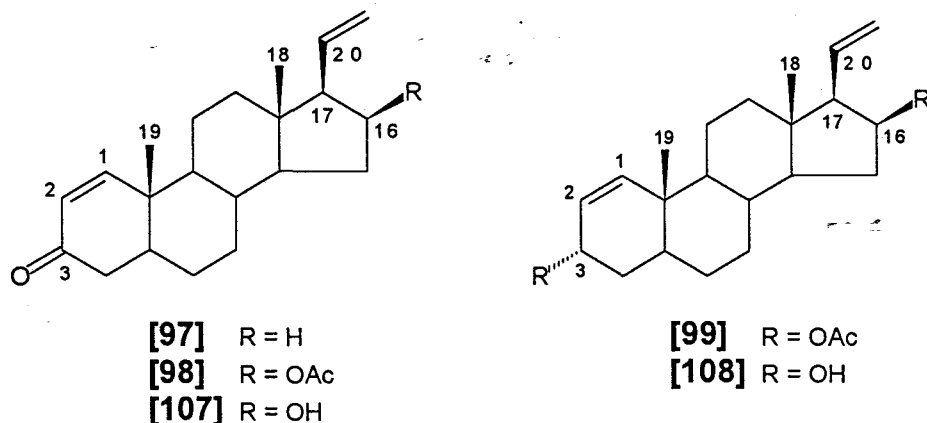
The 16β stereochemistry of the acetoxy in [98] was suggested from selected NOEDS NMR experiments. Irradiation of the H-17 signal resulted in significant enhancement of the H-16 signal while irradiation of H-16 caused enhancement of H-17 and H-15α. Confirmation of this stereochemistry was obtained by saponifying [98] to give 16β-hydroxy-5α-pregna-1,20-dien-3-one [107]. The γ-*gauche* effect of the hydroxy group(s) in hydroxylated steroid molecules is a useful tool for the stereochemical elucidation of these compounds[165]. A γ-hydroxy substituent effect, due to the 16β-hydroxyl functionality, causes an upfield shift (-3 ppm) of the C-20 signal in the ^{13}C NMR spectrum of [107] relative to [97]. In addition the ^{13}C chemical shift of the C-18 methyl, which is nearly 1,3-*syn*periplanar to the 16β-hydroxy group, is affected by a small δ-hydroxy substituent effect and is shifted marginally downfield (+1 ppm)[165] (TABLE 4.1).

LiAlH$_4$ reduction of [99] gave the diol [108]. The ^{1}H NMR spectrum of [108] contained a broad triplet at δ 4.1 ($W_{\frac{1}{2}}$ = 10 Hz) assigned to a 3β proton[141] (a 3α proton requires $W_{\frac{1}{2}}$ = 25 Hz)[166] thus confirming the α-configuration of the oxygen functional group at C-3. The 16β orientation of the second acetoxy group in [99] was established using the same techniques and associated arguments presented for [98].

4.3.3 Structure determination of the tsitsixenicins A [100] - D [103].

[100]
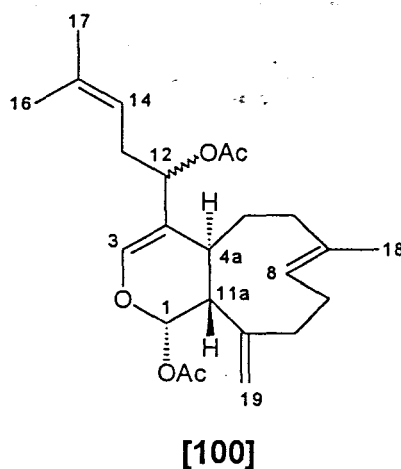
HREIMS data established the molecular formula of tsitsixenicin A [100] as $C_{24}H_{34}O_5$ (*m/z* 402.2398, Δmmu -8). The two acetate carbonyl (δ 169 and 170) and eight olefinic (δ 113, 116, 119, 124, 134, 136, 143 and 149) resonances in the ^{13}C NMR spectrum of [100] (FIGURE 4.5) accounted for six of the eight degrees of unsaturation implied by the molecular formula. The remaining two degrees of unsaturation therefore required a bicyclic diterpene skeleton for this compound.
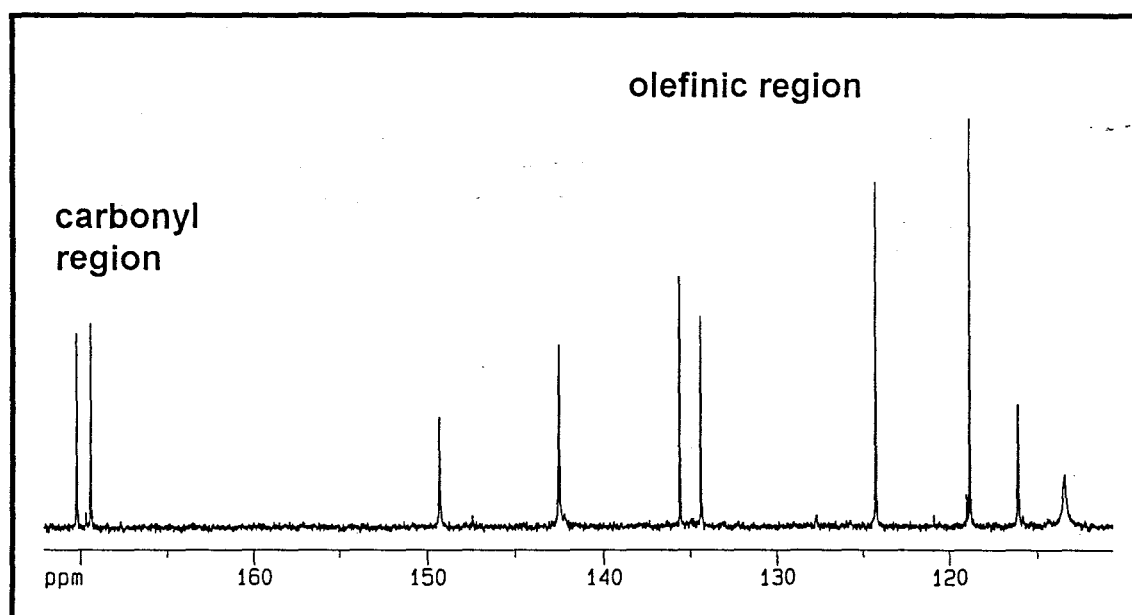
FIGURE 4.5 Partial ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of tsitsixenicin A [100].

The ^{1}H and ^{13}C NMR data of **[100]** (**TABLE 4.2**), supported by an HMQC NMR experiment (**FIGURE 4.6**), revealed the presence of one acetylated hemi-acetal at δ_H 5.75 (1H, d, $J_{1,11a}$ = 3.6 Hz, H-1) and δ_C 92, an olefinic methylene group at δ_H 4.91 and 4.78 (2H, br s, H-19 and H-19') and δ_C 113, three vinylic methyl groups at δ_H 1.64 (3H, br s, H-16), 1.67 (6H, br s, H-17 and H-18) and δ_C 17, 18 and 25 and an enol ether methine proton at δ_H 6.52 (1H, d, $J_{3,4a}$ = 1.9 Hz, H-3) and δ_C 143.
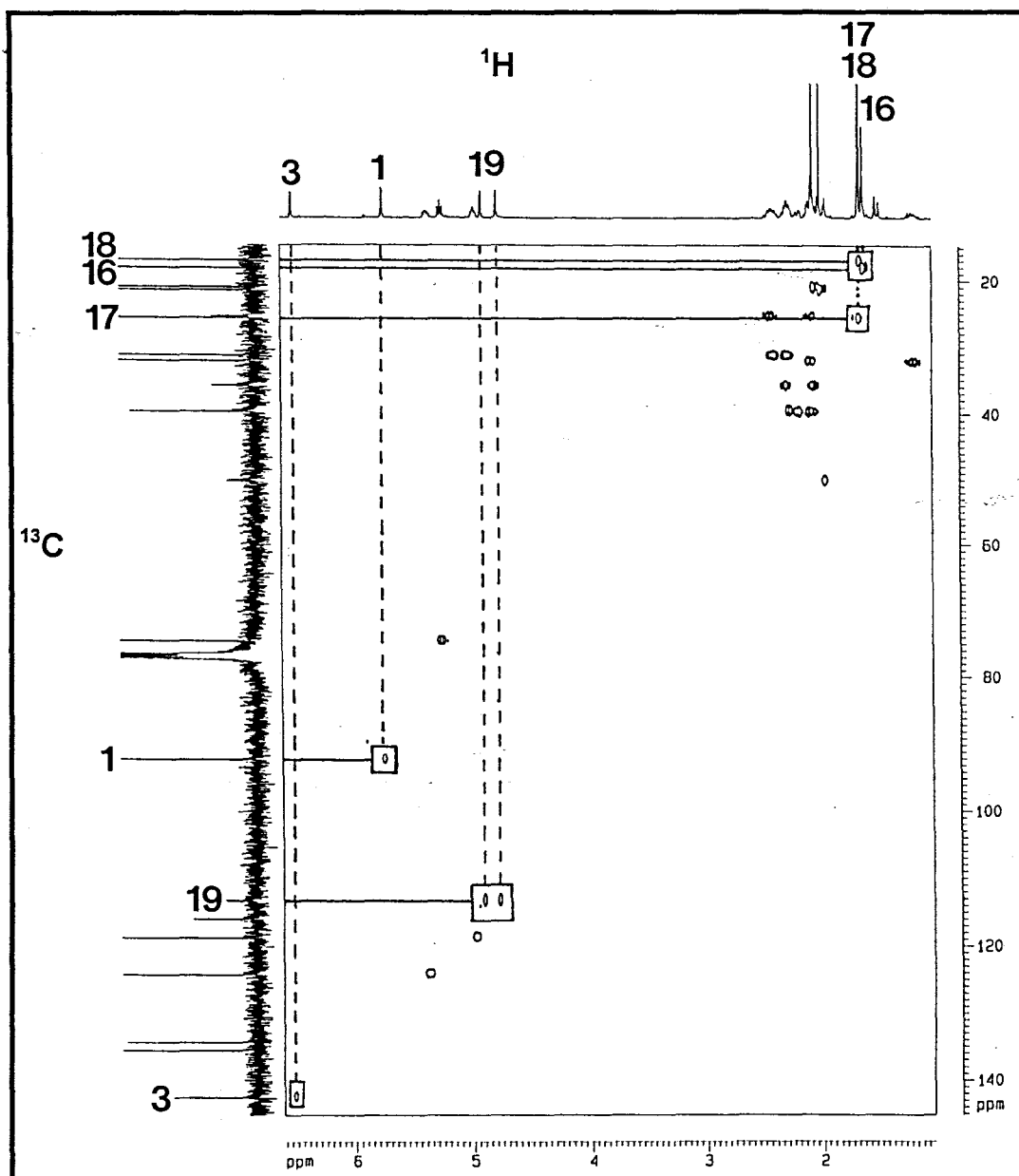
FIGURE 4.6 HMQC NMR (400 MHz, CDCl$_3$) spectrum of tsitsixenicin A **[100]**.

TABLE 4.2 NMR spectral data for tsitsixenicin A **[100]**.

	^{13}C	^1H
1	92.3 d	5.75 d ($J_{1,11a}$ 3.6)
3	142.6 d	6.52 d ($J_{3,4a}$ 1.9)
4	116.1 s	
4a	39.7 d	2.29 m
5, 5'	32.3 t	2.08 m, 1.22 m
6, 6'	39.7 t	2.19 m, 2.07 m
7	134.4 s	
8	124.3 d	5.37 dd (8.0, 9.5)
9, 9'	25.5 t	2.43 m, 2.07 m
10, 10'	35.9 t	2.31 m, 2.07 m
11	149.3 s	
11a	50.1 d	1.96 br s
12	74.7 d	5.25 t ($J_{12,13}$ 7.6)
13, 13'	31.4 t	2.43 m, 2.31 m
14	118.9 d	4.97 t (6.9)
15	135.6 s	
16	18.1 q	1.64 br s
17	25.7 q	1.67 br s
18	17.0 q	1.67 br s
19, 19'	113.4 t	4.91 br s, 4.78 br s
COCH_3	21.5 q	2.07 s
	21.0 q	2.01 s
$COCH_3$	169.5 s	
	170.2 s	

Spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C in $CDCl_3$ with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.

The contiguous coupling sequence observed in the COSY NMR spectrum of [100] from the hemi-acetal proton at δ 5.75 (1H, d, J = 3.6 Hz, H-1) through the methine protons at δ 1.96 (1H, br s, H-11a) and δ 2.29 (1H, m, H-4a) and finally through weak allylic coupling to the enol ether proton at δ 6.52 (1H, d, $J_{3,4a}$ = 1.9 Hz, H-3) suggested that one of the rings in the bicyclic structure was a 1-acetoxydihydropyran moiety. Prominent correlations in the HMBC NMR spectrum of [100] from H-3 to C-1, C-4 and C-4a (FIGURE 4.7A) further supported this structural assignment.

A   B
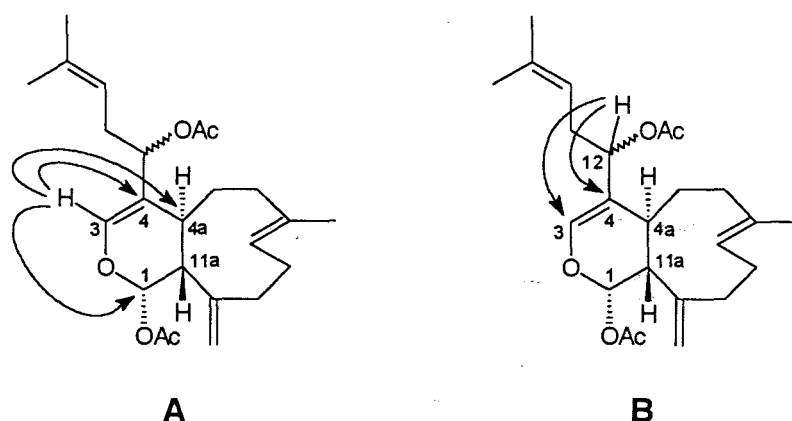
FIGURE 4.7 Selected HMBC NMR correlations in compound [100].

2D-NMR spectral analysis of [100] also indicated the presence of a 1-acetoxy-4-methyl-pent-3-ene fragment which was placed at C-4 on account of HMBC correlations from the acetoxy methine proton at δ 5.25 (1H, t, $J_{12,13}$ = 7 Hz, H-12) to carbons at δ 143 (C-3) and 116 (C-4) (FIGURE 4.7B). The 1-acetoxydihydropyran ring system and associated six carbon side chain is characteristic of xenicane diterpenes containing a 2-oxabicyclo[7.4.0]tridecane structure. The remaining protons and carbon atoms could thus be assigned to a nine-membered ring with an exocyclic methylene functionality and a vinylic methyl group from the 2D-NMR data of [100] and by comparison of the ^{1}H and ^{13}C NMR spectral data of [100] with published values for these compounds[149,157,160,162-164]. Tsitsixenicin A was also isolated from the grey colour variant and this was found to be identical (NMR, [α]$_D$) with [100] obtained from the yellow colour variant.

[101]   [109]
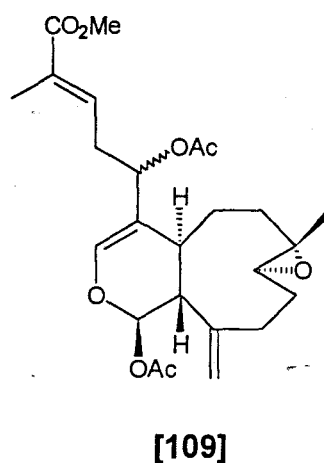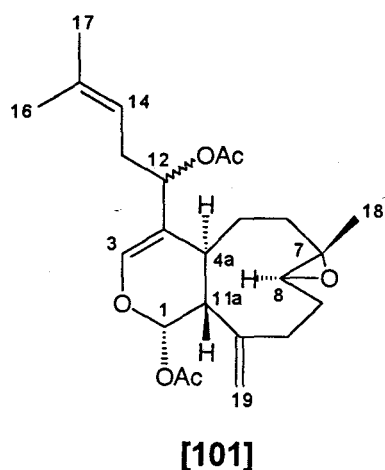
The molecular ion at *m/z* 418.2378 (Δmmu +23) in the HREIMS spectrum of tsitsixenicin B **[101]** is compatible with the molecular formula $C_{24}H_{34}O_6$ and this corresponds to the addition of a single oxygen atom to **[100]**. The ^{13}C NMR data of **[101]** (**TABLE 4.3**) were consistent with that of **[100]** except for the replacement of the Δ^7-trisubstituted double bond with an oxirane ring. A DEPT NMR experiment confirmed the quartenary character of the epoxy carbon atom at δ 59 (C-7) while an HMQC NMR experiment assigned the prominent oxymethine doublet of doublets at δ 2.98 (1H, dd, J = 3.2, 10.8 Hz, H-8) in the ^1H NMR spectrum to the carbon atom at δ 62 (C-8). The position of the epoxy methine proton at C-8 was confirmed by comparison of the spectral data of **[101]** with that reported for acalycigorgan B **[109]**[164]. Two and three bond correlations between the C-18 methyl protons and C-7 and C-8 respectively in the HMBC NMR spectrum of tsitsixenicin B **[101]** provided further evidence for the position of the epoxide functionality.

[102]   [103]
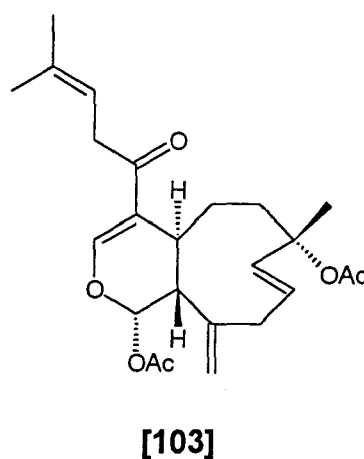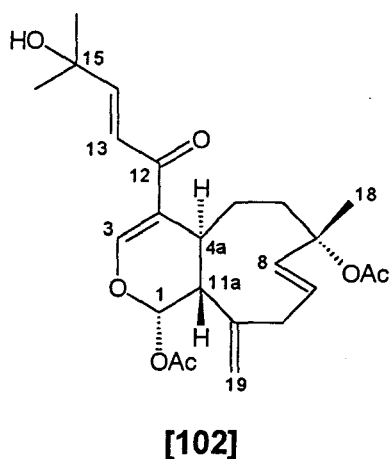
HREIMS established the molecular formulae of tsitsixenicins C **[102]** and D **[103]** as $C_{24}H_{32}O_7$ (Δmmu +13) and as $C_{24}H_{32}O_6$ (Δmmu -25) respectively. The ^{13}C NMR data of compounds **[102]** and **[103]** (**TABLE 4.3**) indicated the same basic xenicane diterpene skeleton established for compounds **[100]** and **[101]** with the differences confined to the six carbon side-chain at C-4 and the substitution pattern around the nine-membered ring. One of the two acetoxy groups, evident from the ^{1}H and ^{13}C NMR spectra of compounds **[102]** and **[103]**, was situated at δ 93 (C-1) while the second acetoxy was placed at C-7 from an HMBC correlation (**FIGURE 4.8**) between the C-18 methyl protons at δ 1.61 (3H, s, H-18) and the quarternary oxygenated carbon atom δ 84 (C-7).
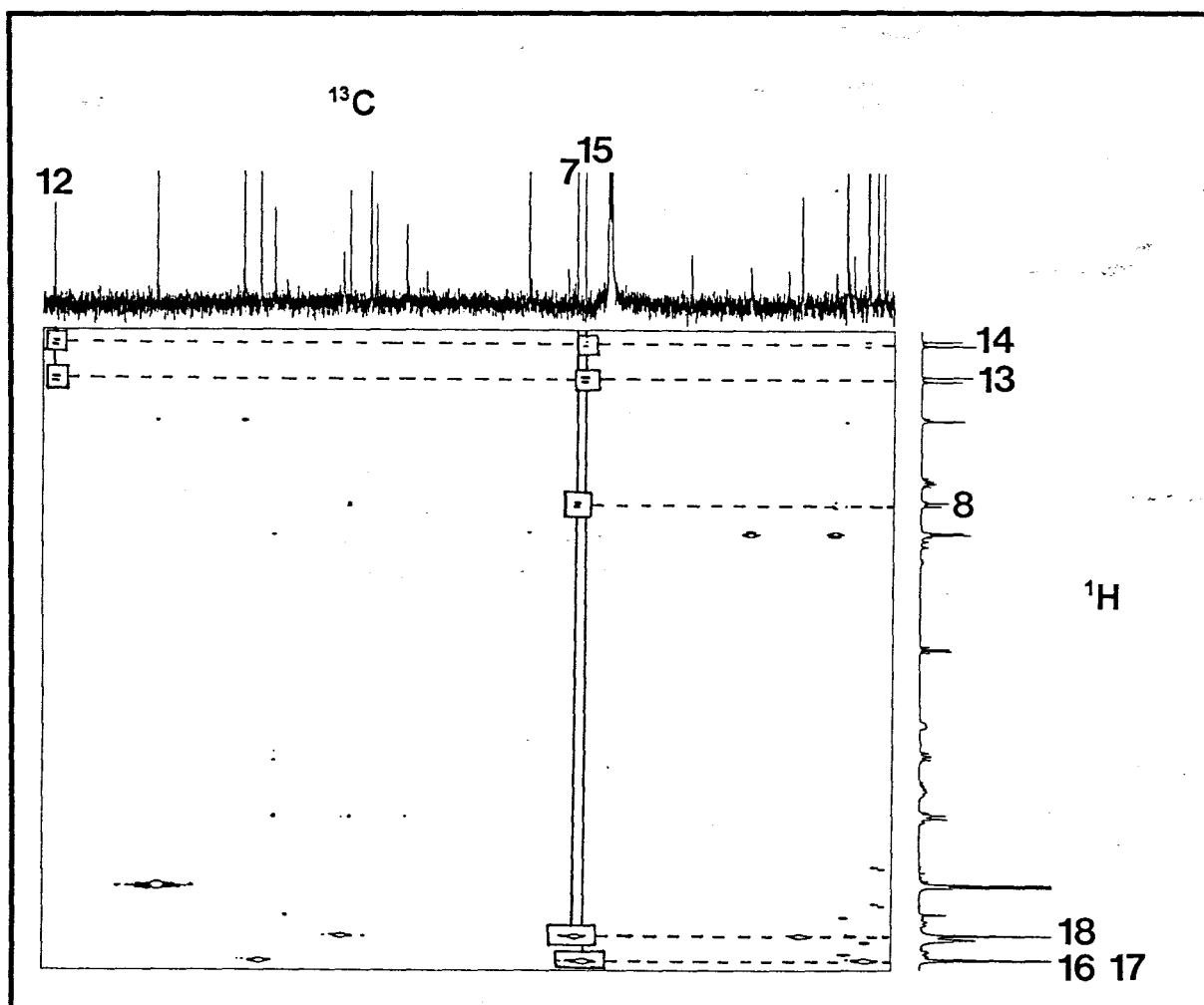
FIGURE 4.8 HMBC NMR (400 MHz, CDCl₃) spectrum of tsitsixenicin C **[102]**.

The Δ^8-position of the endocyclic olefin in **[102]** and **[103]** followed from a two bond HMBC correlation between the vinylic proton at δ 5.40 (1H, d, $J_{8,9}$ = 11.9 Hz, H-8) and C-7. The spectral data of tsitsixenicin C and D suggested that the differences between these two compounds were limited to the side chain at C-4. The ^{13}C NMR resonance at δ 82 and a hydroxyl absorbance (3620 cm^{-1}) in the IR spectrum of **[102]** indicated the presence of a tertiary hydroxyl group in this compound. This functionality was placed at C-15 in **[102]** from the two bond HMBC correlations observed between the C-16 and C-17 methyl group protons at δ 1.40 (3H, s, H-16) and δ 1.39 (3H, s, H-17) respectively and the carboxy signal at δ 82 (C-15) (**FIGURE 4.8**). Other two bond HMBC correlations from the two vinylic proton signals at δ 6.82 (1H, d, $J_{13,14}$ = 15.8 Hz, H-14) and δ 6.51 (1H, d, $J_{13,14}$ = 15.8 Hz, H-13) to the two carbon resonances at δ 82 (C-15) and δ 190 (C-12) respectively (**FIGURE 4.8**), established the 1-keto-4-hydroxy-4-methyl-pent-2-ene structure of the C-4 side chain in **[102]**.

The 1-keto-4-methyl-pent-3-ene structure of the C-4 side chain in **[103]** was similarly determined from 2D NMR data. A prominent three bond correlation between H-3 and C12 in the HMBC spectra of **[102]** and **[103]** further supported the position of the ketone functionality in the side chain in both these compounds and linked the side chain to the 1-acetoxy-dihydropyran ring at C-4 as expected.

TABLE 4.3 ^{13}C NMR (100 MHz, $CDCl_3$) data for tsitsixenicin B [101], C [102] and D [103].

	[101]	[102]	[103]
1	92.0 d	93.4 d	93.3 d
3	142.4 d	151.7 d	151.2 d
4	116.2 s	124.3 s	123.5 s
4a	37.0 d	28.2 d	28.6 d
5	31.2 t	27.0 t	26.9 t
6	39.2 t	37.5 t	37.8 t
7	59.8 s	83.7 s	83.5 s
8	62.3 d	131.2 d	131.4 d
9	25.3 t	129.8 d	129.7 d
10	31.2 t	30.7 t	30.8 t
11	146.8 s	145.5 s	145.6 s
11a	49.3 d	48.1 d	48.0 d
12	74.3 d	190.2 s	197.7 s
13	31.3 t	125.6 d	38.6 t
14	118.6 d	148.4 d	117.0 d
15	134.7 s	82.1 s	135.0 s
16	18.1 q	24.2 q	25.8 q
17	25.7 q	24.1 q	18.1 q
18	17.2 q	28.5 q	28.5 q
19	116.2 t	118.1 t	117.9 t
$COCH_3$	21.4 q	22.3 q	22.2 q
	21.0 q	20.9 q	20.9 q
$COCH_3$	170.2 s	169.2 s	169.3 s
	169.3 s	169.2 s	169.1 s

All assignments are supported by DEPT, COSY, HMQC and HMBC NMR experiments.

4.3.4 Stereochemistry of the tsitsixenicins A [100] - D [103].

[100]   [110]
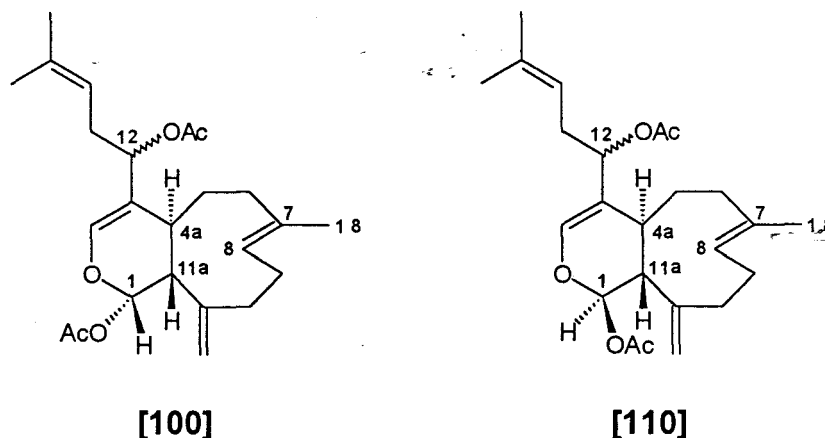
In tsitsixenicin A [100] an E configuration could be assigned to the Δ^7 double bond based on the Me-18 chemical shift at δ 17.0[149] (a Z configuration requires δ 22-25). The slightly broadened H-11a singlet at δ 1.96 in the ^{1}H NMR spectrum of [100] indicated that the coupling to H-4a is very small (a dihedral angle of approximately 90°) and is in accordance with the *trans*-fused bicyclic ring system found in other naturally occurring xenicane diterpenes. Although an α stereochemistry for the proton at C-1 is prevalent in 1-acetoxy-xenicane compounds[63,145,146,148-150,160,163,164], NOEDS experiments tentatively suggested a β configuration for H-1 in tsitsixenicin A [100]. Irradiation of the H-11a signal only caused significant enhancement (12%) of the H-1 signal, while irradiation of H-1 gave enhancement of H-11a (5%) and no enhancement of the H-4a signal. An enhancement of the H-4a signal is expected in NOEDS experiments with xenicane compounds containing a H-1α relative stereochemistry[164]. A close look at the coupling constants measured between H-1 and H-11a in analogous 1-acetoxy-xenicane compounds (TABLE 4.4) indicates a disparity between the coupling of $J_{1,11a}$ = 3.6 Hz in [100] and the average coupling of $J_{1,11a}$ = 2.0 Hz in these compounds supporting the proposed difference in stereochemistry.

9-Deacetoxy-14,15-deepoxyxeniculin [110] isolated from the Red Sea soft coral *Xenia obscuronata*[149] is diastereomeric with [100]. Unfortunately no optical rotation was reported for [110] where the relative stereochemistries at C-1, C-4a and C-11a were

90

TABLE 4.4 $J_{1,11a}$ coupling constants (Hz) in 1-acetoxy-xenicane compounds.

REFERENCE	$J_{1,11a}$ COUPLING	R	X
145	2	OAc	H
146	2.6	H	H
149	1.8	H	H
149	2.2	H (7,8-epoxide)	H
148	2	OH	H
150	2	H	H
160	1.6	H	H
160	1.6	H	OH
163	1.8	H	H
63	1.9	OAc	OH
63	2.3	H	H
63	2.3	H	OH
164	1.8	H	H
164	2.1	H (7,8-epoxide)	H
AVERAGE	**2.0**		
RANGE	**1.6 - 2.6**		

assigned after comparison of ^{13}C chemical shifts with analogous compounds of known stereochemistry. However, the recent isolation of **[110]** from an *Alcyonium* species of soft coral collected from Aliwal Shoal in South Africa (see chapter five) lends support to the H-1β configuration in **[100]** based on a comparison of NOEs, $J_{1,11a}$ coupling constants and optical rotations of **[100]** and **[110]** (**TABLE 4.5**).

TABLE 4.5 Comparison of $J_{1,11a}$ coupling, NOEs and $[\alpha]_D^{21}$ in **[100]** and **[110]** .

	[100]	[110]
$J_{1,11a}$ (Hz)	3.6	2.2
NOEs Irradiation of : H-1 H-11a H-4a	Enhancement of : H-11a (5%) H-1 (12%) no effect on H-1	Enhancement of : H-11a (2%) H-1 (7%) H-1 (3%)
$[\alpha]_D^{21}$	-63.8°	+126.9°

The comparatively stronger enhancements of H-11a and H-1 in **[100]** after irradiation of H-1 and H-11a, respectively, not only suggests a close spatial arrangement but also a β orientation for both protons. The enhancement of H-1 in **[110]** from the irradiation of H-4a and the $J_{1,11a}$ = 2.2 Hz coupling are in accordance with 1-acetoxy-xenicane compounds reported in the literature having an H-1α configuration. Although the considerable difference in optical rotations lends credence to the assigned configuration at C-1, this does not provide unequivocal proof as the stereochemistry at C-12 in **[100]** and **[110]** remains unassigned. The C-1 ^{13}C chemical shift in **[100]** (δ 92.3) differs marginally from that reported for **[110]** (δ 92.0) and the assignment of stereochemistry from ^{13}C chemical shift comparisons must therefore be considered tenuous in these compounds.
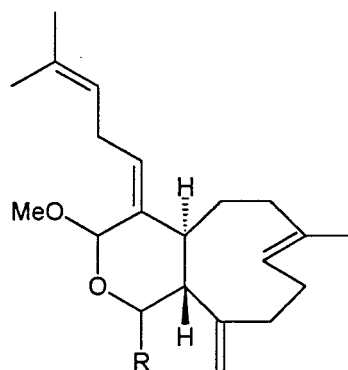
[111] R = α - OAc
[112] R = β - OAc

Gentle refluxing of a methanolic solution of **[100]** gave compound **[111]** in almost quantitative yield. A similar compound, acalycigorgin D **[112]**, isolated from the gorgonian *Acalycigorgia* species[164], was assumed to be an artifact arising from **[110]** via a S_N2' *syn* reaction with the MeOH extraction solvent. The above observation supports this contention and it was fortuitous that only EtOAc was used to extract both colour variants of *C. thyrsoidea*.
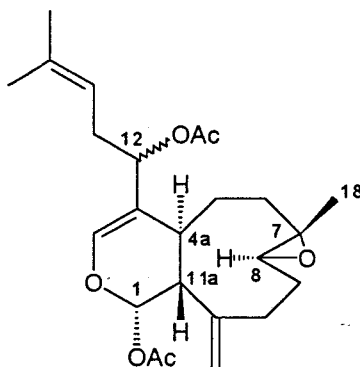
[101]

The observed NOEs in tsitixenicin B **[101]** between 8-H and the α-proton at C-4a, between the C-18 methyl protons and the β-proton at C-11a, and the apparent lack of enhancement of H-8 with the irradiation of the C-18 methyl protons and *vice versa*, in a series of NOEDS experiments, are in accordance with observations reported for a 7(8)-*E*-epoxy configuration[157]. There seems to be a disparity in literature[157,164] concerning the stereochemistry of the 7(8)-oxirane ring in xenicane compounds and this is discussed in greater detail in section 5.3.4. The stereochemistry at C-12 in tsitsixenicin B remains unassigned. Previous attempts to define the stereochemistry of an acetoxy group at C-12 in xenicane compounds by chemical transformations and spectroscopy have proven unproductive[157,160]. Reactions with mild acid, mild base, $NaBH_4$ or $LiAlH_4$ have all led to decomposition of the parent compound.

In tsitsixenicin C **[102]** an *E* stereochemistry could be assigned to the exocyclic olefin based on the $J_{13,14}$ = 16 Hz coupling constant for the vinylic protons in the side chain. Irradiation of the Me-18 protons in NOEDS experiments on **[102]** and **[103]**

induced significant enhancement of H-8 and marginal enhancement of H-11a suggesting a tentative α-configuration for the acetoxy group at C-7.
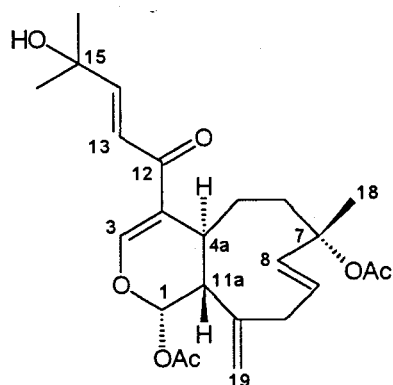
[102]
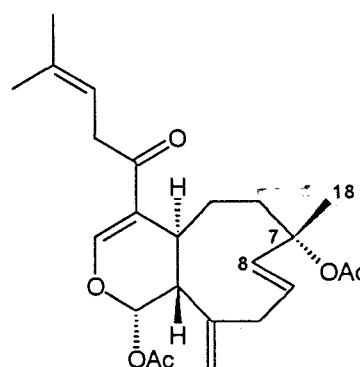
[103]

The significant enhancement of the H-11a signal on irradiation of H-1 in the NOEDS experiments on [101] (12%), [102] (14%) and [103] (9%) confirmed that the configuration at C-1 in these compounds was consistent with that proposed for tsitsixenicin A [100]. In addition, no enhancement of H-4a was observed on irradiation of either H-1 or H-11a in any of these three compounds.

Structural elucidation of the xenicane diterpenes, particularly the stereochemistry, is difficult due to the inherent instability of the xenicane ring system[150] as reflected by the degradation of a number of the pure compounds in air and in solution (particularly $CHCl_3$)[63,157,160]. It was observed that compounds [100] - [103] had all decomposed to some extent over several weeks storage at 0°C. Previous reports[157,160] suggest that [101] and [102] are oxidation products of [100] and [103], respectively. It is unclear, however, as to whether [101] and [102] exist as such in the soft coral or whether they are artifacts of the isolation procedures.

4.4 BIOLOGICAL ACTIVITY

Inspired by the isolation of new anti-inflammatory diterpenes from a South African soft coral[60], the pregnadiene steroids [97] - [99], the tsitsixenicins A [100] - D [103] and compound [111] were screened for anti-inflammatory activity based on their ability to inhibit superoxide production in neutrophils[t].

Neutrophils play an important role in the protection of the host from infections through the production of superoxide. This serves as a precursor for a series of lethal oxidants (H_2O_2, $^-$OH and HOCl) which are responsible for attacking the infectious bacteria. However, in abnormal environments such as ischaemia, trauma or the activation of the auto-immune system, neutrophils can produce superoxide in uncontrollable and excessive amounts which causes serious tissue injury and inflammation. To prevent this injury either the production of superoxide needs to be inhibited or excess superoxide must be rapidly neutralised by superoxide scavengers. Previously, research in this area has largely focussed on superoxide scavengers and it appears that no drugs have been developed with inhibition of superoxide production as their primary pharmacological function[167]. Thus, the screening of natural products for superoxide inhibition provides a significant opportunity for the discovery of new anti-inflammatory drugs.

The assay used to screen the pregnadiene and tsitsixenicin compounds for potential anti-inflammatory effects involved stimulating the release of superoxide from rabbit and human cell neutrophils using the chemotactic factor formyl-methyl-leucyl-phenylalanine (FMLP). FMLP causes a rapid increase in superoxide production over a five minute period and this is detected colourimetrically by the superoxide induced reduction of ferric cytochrome C to its ferrous form. By monitoring the change in absorbance of cytochrome C the ability of compounds to inhibit superoxide production can be assayed. The results of the assay are contained in **FIGURE 4.9** and **TABLE 4.6**.

---

† Assay courtesy of InflaZyme Pharmaceuticals Limited, Vancouver, Canada.

Sample No.	% Inhibition (rabbit)	% Inhibition (human)
[97]	34.1	-104.7*
[98]	29.9	-25.9*
[99]	75.6	39.9
[100]	100.0	-
[101]	99.4	-
[102]	66.5	34.2
[103]	80.5	19.2
[111]	100.0	33.6
DMSO	34.0	0
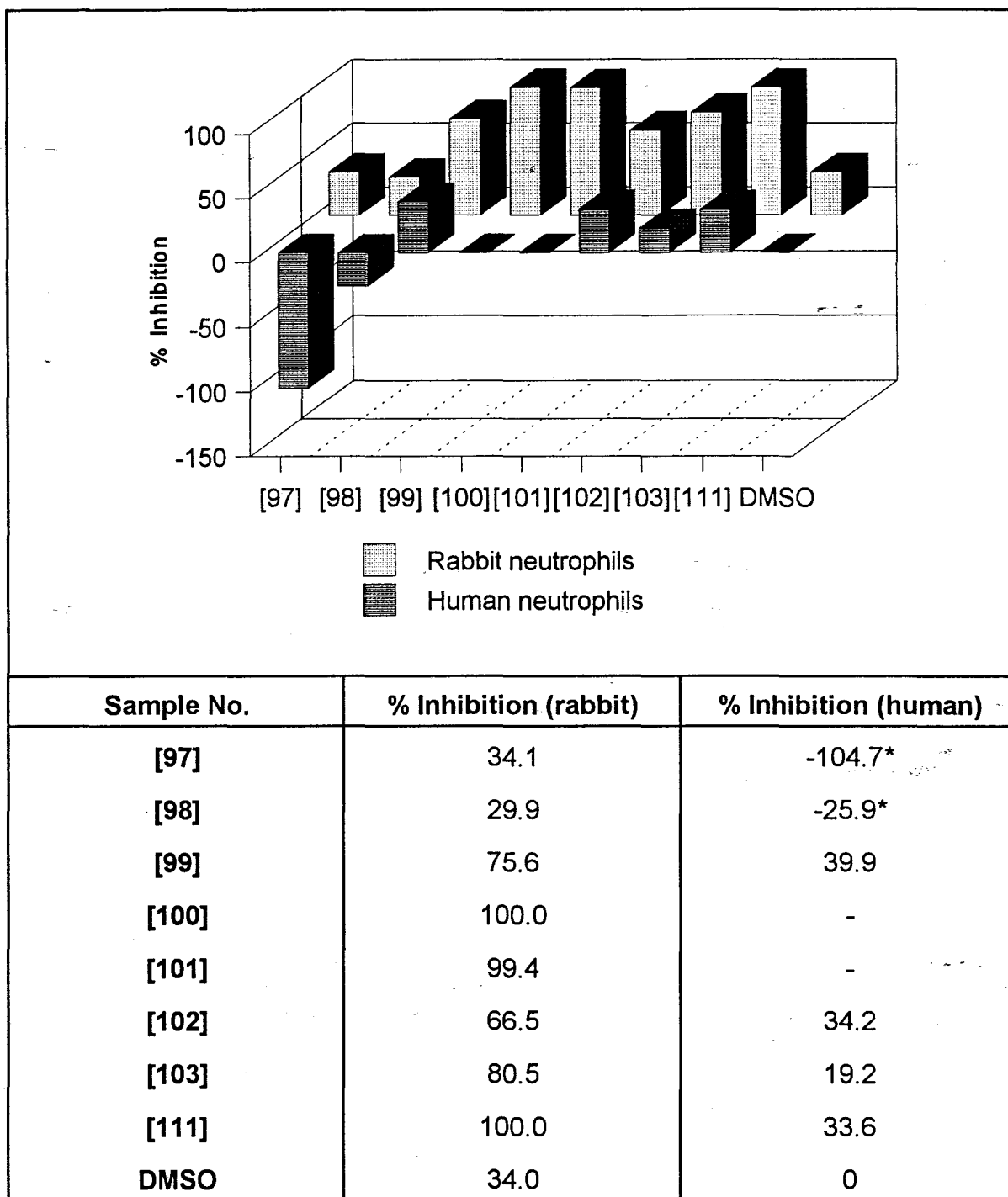
* indicates stimulation of superoxide production.

FIGURE 4.9 The effect of the pregnadiene steroids **[97]** - **[99]**, the tsitsixenicins A **[100]** - D **[103]**, and compound **[111]** on superoxide production in rabbit and human neutrophils at a concentration of 12.5 μg mL^{-1}.

TABLE 4.6 The effect of compounds [100], [101] and [111] on superoxide production in rabbit and human neutrophils at a concentration of 1.25 µg mL^{-1}.

Sample No.	% Inhibition (rabbit)	% Inhibition (human)
[100]	-	68.4
[101]	72.6	20.7
[111]	-	3.0
DMSO	34.0	0

Compounds [100], [101], [103] and [111] showed good inhibition (>80%) of superoxide production in rabbit neutrophils at a concentration of 12.5 µg mL^{-1} with only [101] retaining a high level of activity on ten fold dilution. It is generally observed that human cell neutrophils are less active than rabbit cell neutrophils in this assay and the superoxide inhibition by compounds in the former is generally reduced. Accordingly, only [100] and [101] showed good to moderate inhibition of superoxide production in the human neutrophil bioassay at low concentration (1.25 µg mL^{-1}). Unfortunately, the chemical instability of these two compounds precluded their further development as anti-inflammatory agents. Interestingly, the pregnadiene steroids [97] and [98] stimulated superoxide production in rabbit neutrophils and this observation is attributed to cytotoxicity in which cell lysis increases superoxide levels.

Recent reports have included other interesting biological activities for xenicane diterpenes including the inhibition of fertilised ascidian eggs[164], ichthyotoxicity[157], and cytotoxicity towards mouse leukaemia cell lines and several human carcinomata[63].

CHAPTER FIVE

NEW METABOLITES FROM TWO SOUTH AFRICAN SOFT CORALS
ALCYONIUM SPECIES A AND SPECIES B

5.1   INTRODUCTION

A continuation of the search for bioactive compounds from South African soft corals[84] led to the isolation of six metabolites from two *Alcyonium* species. The soft coral genus *Alcyonium* has been described by Williams[59] as a cosmopolitan genus of many species. The two soft corals in this study could only be identified to genus level and so have been designated *Alcyonium* species A and species B. In accordance with the knowledge that alcyonaceans constitute an abundant source of polyhydroxysterols and terpenoids[83,133], sections 5.2 and 5.3 of this chapter report on the isolation and structure determination of two new polyhydroxysterols from *Alcyonium* species A, and two new and two known xenicane diterpenes from *Alcyonium* species B.

Complex mixtures of monohydroxysterols such as cholesterol [113], 24-methylcholesterol [114] and 24-methylenecholesterol [115] as well dihydroxysterols and polyhydroxysterols have frequently been reported from soft corals of the family Alcyoniidae. Examples of these reports include the genera *Alcyonium*[168,169], *Sinularia*[170-176] and *Lobophytum*[177,178]. It is the genus *Alcyonium* which is the focus of this chapter.

[113]

[114]
[115] Δ^{24}
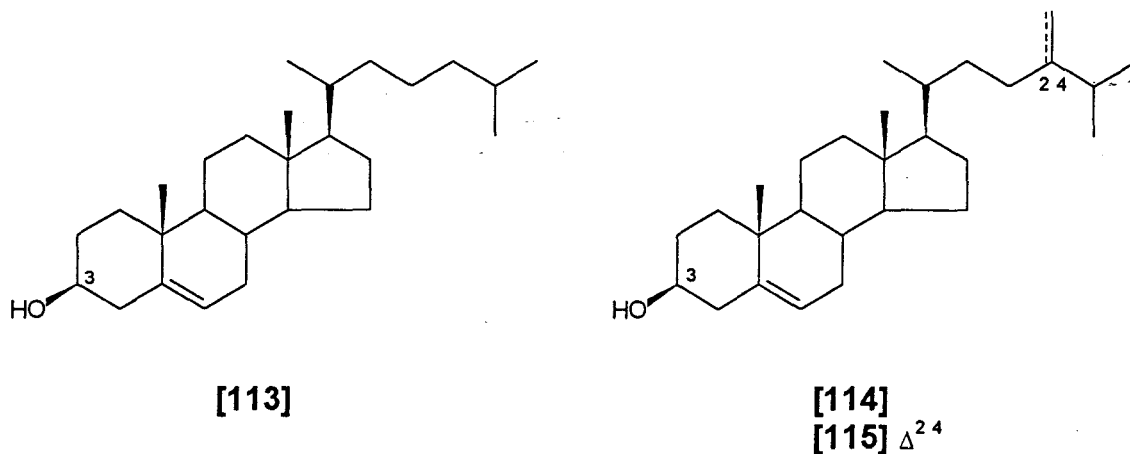
A chemical investigation of an *Alcyonium* species of soft coral by Kobayashi and co-workers[168], collected in the Andaman and Nicobar Islands, resulted in the isolation of the polyhydroxysterol 3β,7β-dihydroxy-24-methylenecholesterol [116], and three new

polyhydroxysterol glycosides [117], [118] and [119]. Compound [116] had previously been isolated from a finger sponge *Haliclona oculata*[179].

[116]

[117] R = H
[118] R = β - OH
[119] R = α - OH
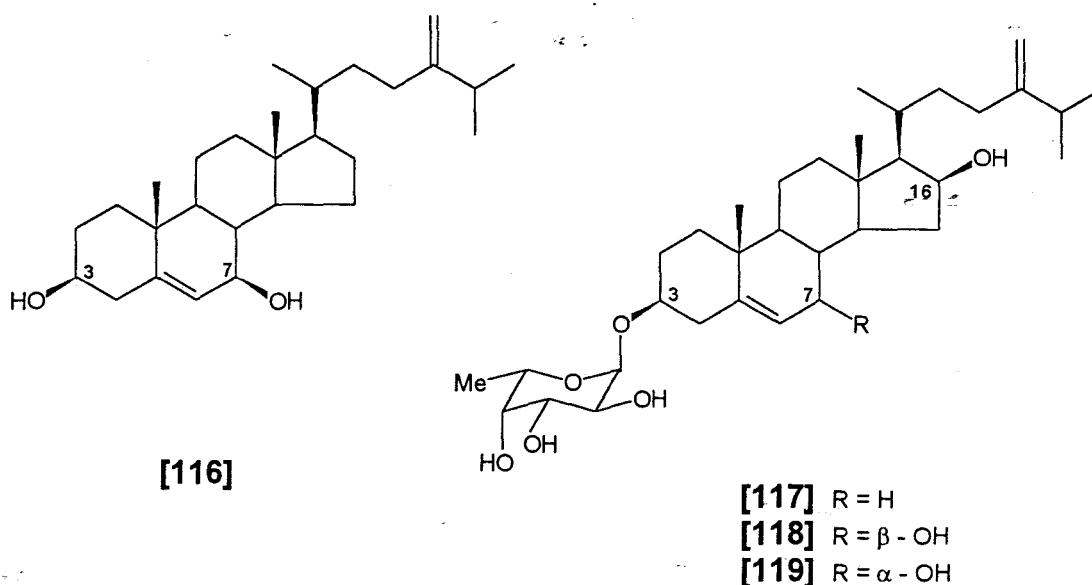
A recent study of the soft coral *Alcyonium patagonicum*, collected from the South China Sea, yielded the new sterol 24-methylenecholest-4-ene-3β,6β-diol [120][169]. The structure was determined by spectral analysis and compound [120] was found to be cytotoxic against the murine leukaemia cell line P-388.

[120]

[121]
[122] Δ²⁴
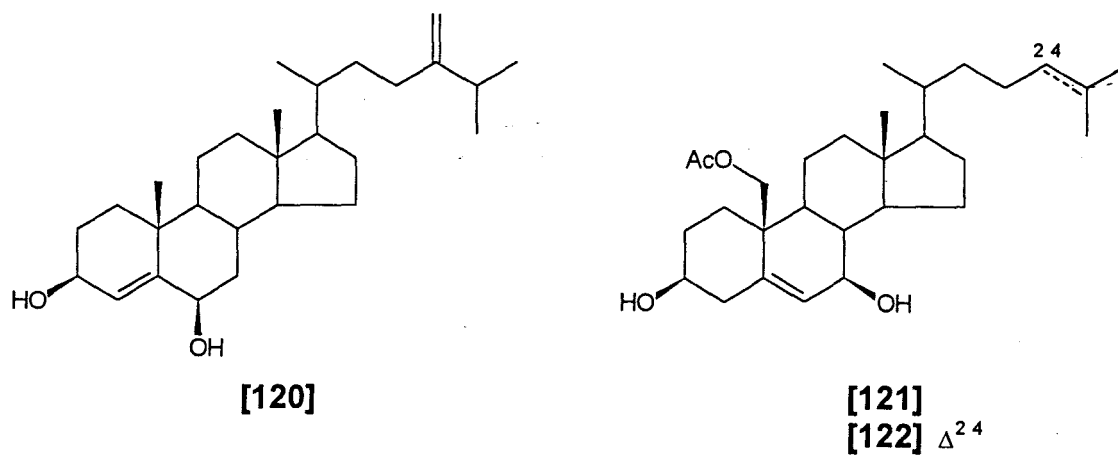
The first part of sections 5.2 and 5.3 describe the isolation and structure determination of two new polyhydroxysterols, cholest-5-ene-3β,7β,19-triol 19-acetate [121] and cholest-5,24-diene-3β,7β,19-triol 19-acetate [122], from the soft coral *Alcyonium* species A collected in the Tsitsikamma Marine Reserve.

As discussed in chapter four, xenicane diterpenes were originally believed to exist exclusively in the soft coral family Xeniidae, but were subsequently isolated from several other sources. The latter part of sections 5.2 and 5.3 report on the isolation and structure determination of two known xenicane diterpenes, 9-deacetoxy-14,15-deepoxyxeniculin [110] and zahavin A [16], and two new xenicane diterpenes, 7-epoxyzahavin A [123] and xeniolide C [124], from the South African soft coral *Alcyonium* species B collected from the Aliwal Shoal off the Natal coast.
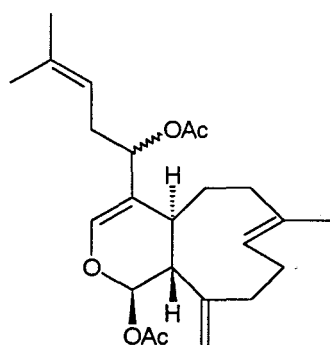
[110]
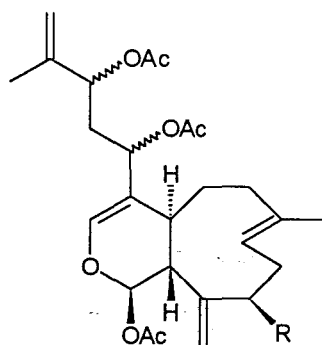
[16] R = H
[17] R = OH
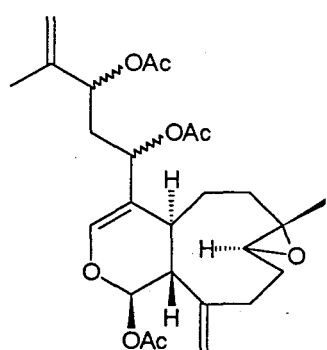
[123]
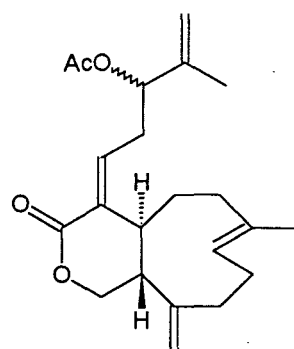
[124]

9-Deacetoxy-14,15-deepoxyxeniculin [110] has previously been reported by Kashman *et al.* from the soft coral *Xenia obscuronata* (Family: Xeniidae) collected in the Red Sea[149]. Recently, after completion of our chemical investigation of *Alcyonium* species B, a paper by Kashman and co-workers was published which detailed the isolation of zahavin A and B from a newly described soft coral *Alcyonium aureum* (Benayahu, 1995, Alcyoniidae) collected at Sodwana Bay in South Africa[63]. Sodwana Bay is approximately 500 km north of Aliwal Shoal. Spectroscopic similarities between

the metabolites of the soft coral *Alcyonium* species B and *A. aureum* indicated that *Alcyonium* species B also contained zahavin A [16]. Kashman reported that zahavin A [16] and B [17] were the first examples of the discovery of xenicane diterpenes in the genus *Alcyonium* (Family: Alcyoniidae). Because of the very recent taxonomic description of *A. aureum* and the relative proximity of the collection sites the possibility exists that *Alcyonium* species B and *A. aureum* are taxonomically the same soft coral. In addition to zahavin A, the new metabolite 7-epoxyzahavin A [123] was isolated from the soft coral *Alcyonium* species B.

[105]
[126] (Z) - Δ$^{4(12)}$
[127] 7, 8 - epoxide of [126]

[125] R = H
[128] R = Ac
[129] 7, 8 - epoxide of [125]

[130]
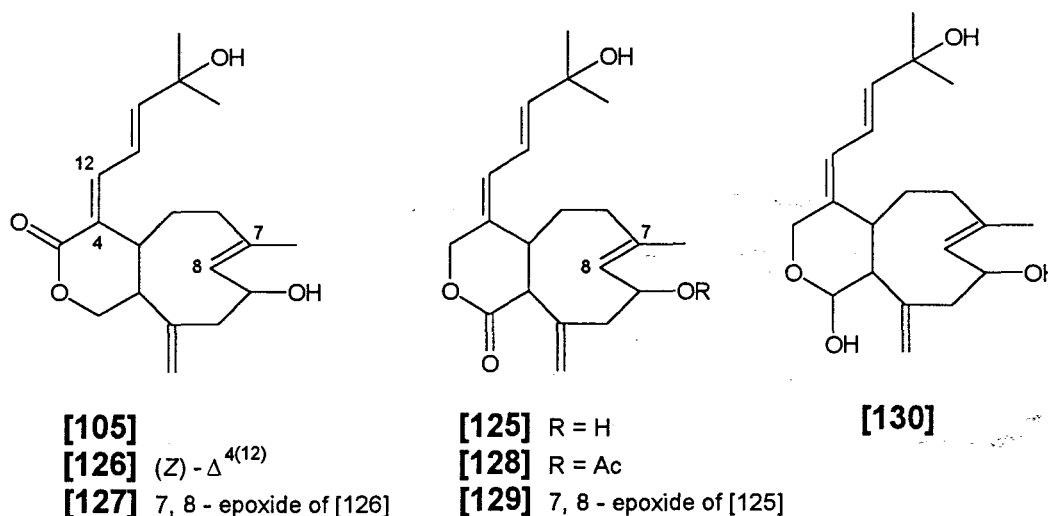
Interestingly, the second new compound isolated from the soft coral *Alcyonium* species B has a basic xeniolide skeleton and has been named xeniolide C [124]. Xeniolide type metabolites were not reported from *A. aureum*. The xeniolide skeleton was first described by Kashman and Groweiss[147] with the isolation of xeniolide A [105] and B [125] from the Red Sea soft coral *Xenia macrospiculata*. This was followed by the characterisation of isoxeniolide A [126] and 7,8-oxido-isoxeniolide A [127] from the Papua-New Guinea soft coral *Xenia novae-britanniae*[148] and xeniolide B 9-acetate [128], 7,8-epoxyxeniolide B [129] and xenialactol [130] from the Red Sea soft corals *Xenia macrospiculata* and *X. obscuronata*[149]. The difference between isoxeniolide A [126] and xeniolide A [105] lies in the geometry of the Δ$^{4(12)}$ double bond with an *E*-configuration proposed for [105] as opposed to a *Z*-configuration for [126].

Recently an investigation of the soft coral *Xenia elongata* has yielded the ichthyotoxic diterpenoid deoxyxeniolide B [131][157]. While compound [131] was reported to be stable in the solid state, in solution ($CHCl_3$, Me_2CO, H_2O) it is oxidised to the oxidation products [132] and [133].
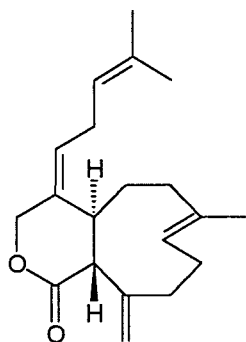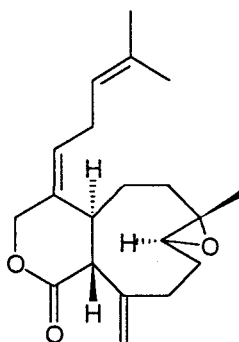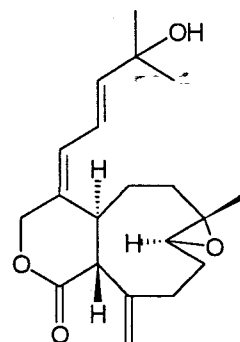
[131]   **[132]**   **[133]**

5.2 COLLECTION, EXTRACTION AND ISOLATION
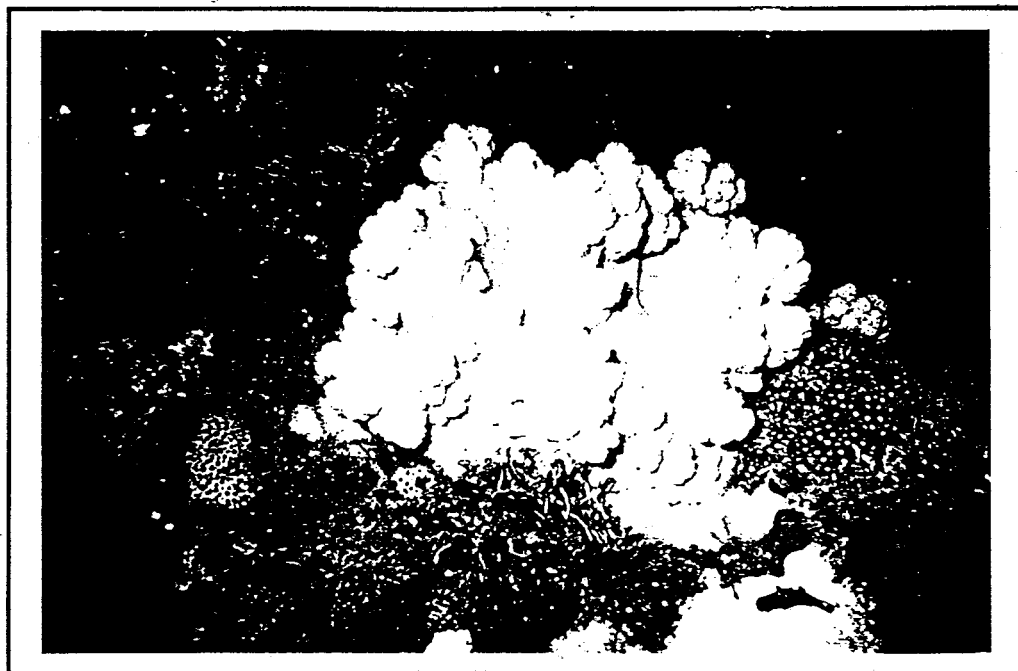
FIGURE 5.1 Soft coral, *Alcyonium* species A, from the Tsitsikamma Marine Reserve.
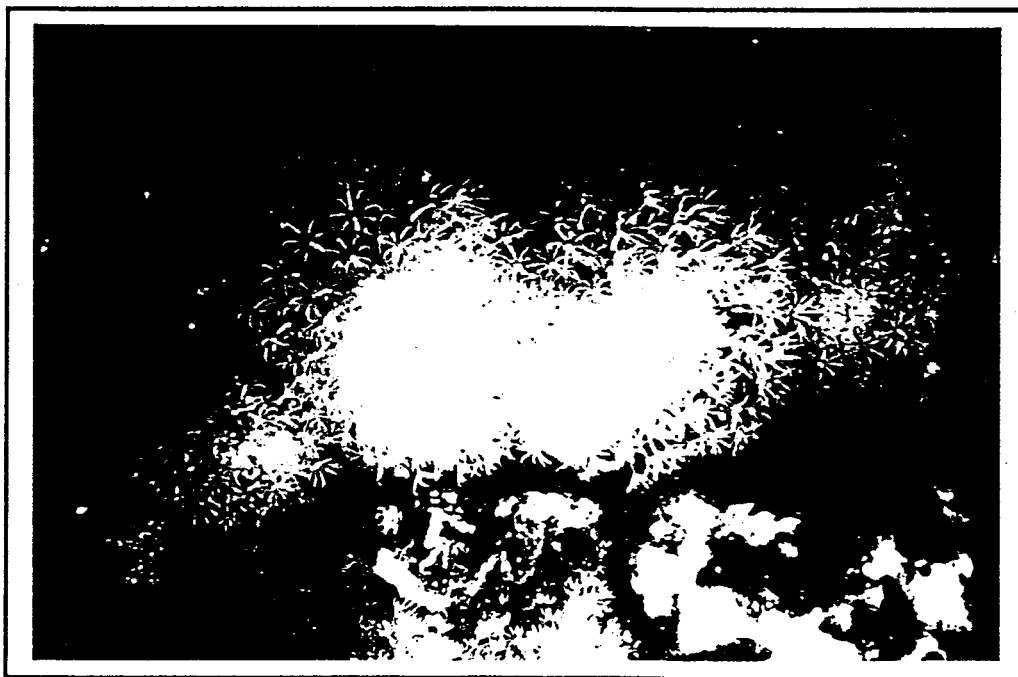
FIGURE 5.2 Soft coral, *Alcyonium* species B, collected off Aliwal Shoal, Umkomaas.

The slimy orange soft coral *Alcyonium* species A (**FIGURE 5.1**) was collected by SCUBA at a depth of -24 m in the Tsitsikamma Marine Reserve in May 1994. The soft coral was stored frozen until removal and freeze-drying. The EtOAc extract of the freeze-dried soft coral (dry weight 203 g) was subjected to silica gel chromatography followed by normal phase and reverse phase HPLC to yield the new polyhydroxysterols cholest-5-ene-3β,7β,19-triol 19-acetate [**121**] and cholest-5,24-diene-3β,7β,19-triol 19-acetate [**122**] in 0.02 and 0.04% yield respectively. The chromatographic procedure is shown in **SCHEME 5.1**.
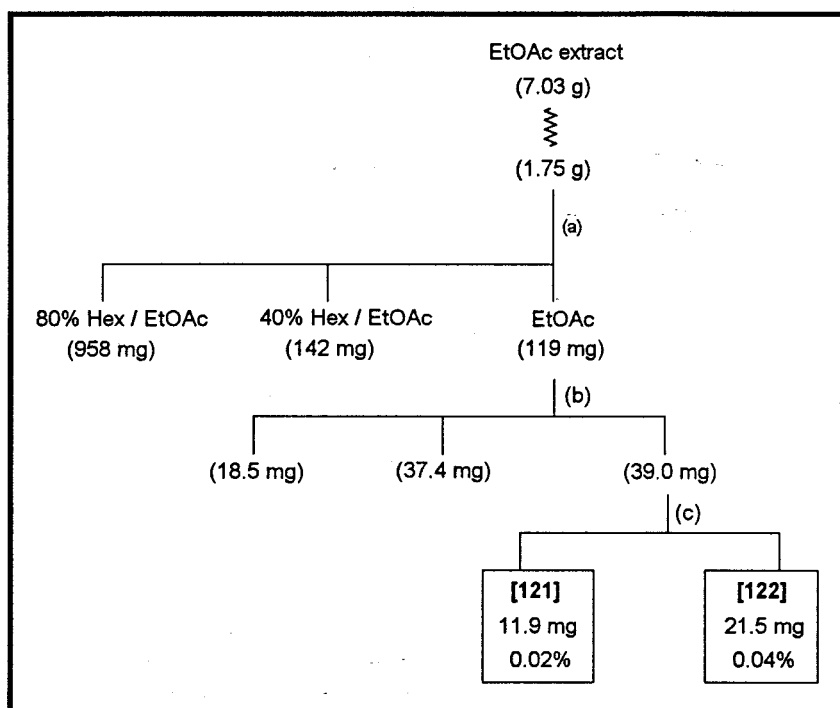
SCHEME 5.1 Chromatographic procedure for the isolation of compounds [**121**] and [**122**]. (a) flash chromatography (silica, 230-400 mesh, 150 g, elution sequence: 80% hexane/EtOAc; 40% hexane/EtOAc; EtOAc). (b) HPLC (silica, 10 μ, Whatman, 40% hexane/EtOAc, 4 mL min⁻¹). (c) HPLC (C18, 10 μ, Phenomenex, 7.5% H_2O/MeOH, 4 mL min⁻¹).

The yellow-white soft coral *Alcyonium* species B (**FIGURE 5.2**) was collected by SCUBA from the Aliwal Shoal on the Natal coast in June 1994. The frozen soft coral was freeze-dried (dry weight 210 g) and steeped in EtOAc. The crude EtOAc extract showed slight inhibition of the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* in the antimicrobial disc assay. Chromatography of two portions of the EtOAc extract was performed according to **SCHEME 5.2** and this yielded the known compounds 9-deacetoxy-14,15-deepoxyxeniculin [110] and zahavin A [16], and the new compounds 7-epoxyzahavin A [123] and xeniolide C [124].
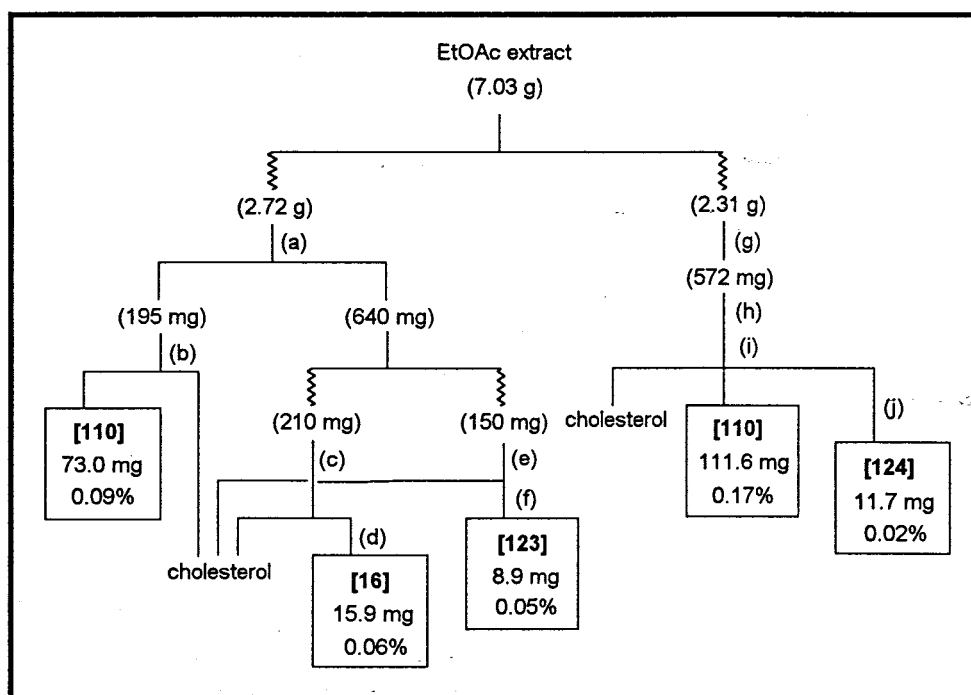
SCHEME 5.2 Chromatographic procedure for the isolation of compounds [110], [16], [123] and [124]. (a) flash chromatography (silica, 230-400 mesh, 150 g, elution sequence: 70% hexane/EtOAc; 50% hexane/EtOAc; EtOAc). (b) HPLC (silica, 10 μ, Whatman, 90% hexane/EtOAc, 4 mL min^{-1}). (c) HPLC (silica, 10 μ, Whatman, 85% hexane/EtOAc, 4 mL min^{-1}). (d) HPLC (C18, 10 μ, Phenomenex, 30% H$_2$O/MeCN, 4 mL min^{-1}). (e) HPLC (silica, 10 μ, Whatman, 85% hexane/EtOAc, 4 mL min^{-1}). (f) HPLC (C18, 10 μ, Phenomenex, 20% H$_2$O/MeOH, 4 mL min^{-1}). (g) repeat of (a). (h) recrystallisation and removal of cholesterol. (i) HPLC (silica, 10 μ, Whatman, 85% hexane/EtOAc, 4 mL min^{-1}). (j) repeat of (i).

5.3 STRUCTURE DETERMINATION AND STEREOCHEMISTRY

5.3.1 Structure determination of the hydroxysterols [121] and [122].
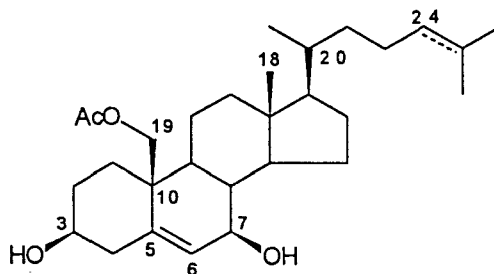
[121]
[122] Δ^{24}

HREIMS data established the molecular formula of compound [121] as $C_{29}H_{48}O_4$ (m/z 460.3541, Δmmu -11). The IR spectrum of [121] suggested the presence of hydroxyl (3380 cm^{-1}) and acetoxy (1740 cm^{-1}) functionalities. The ^{13}C NMR spectrum of [121] (FIGURE 5.3) showed twenty nine clearly resolved resonances including a carbonyl (δ 171), two oxymethine (δ 73 and 71), an oxymethylene (δ 64) and two vinylic (δ 138 and 130) signals.
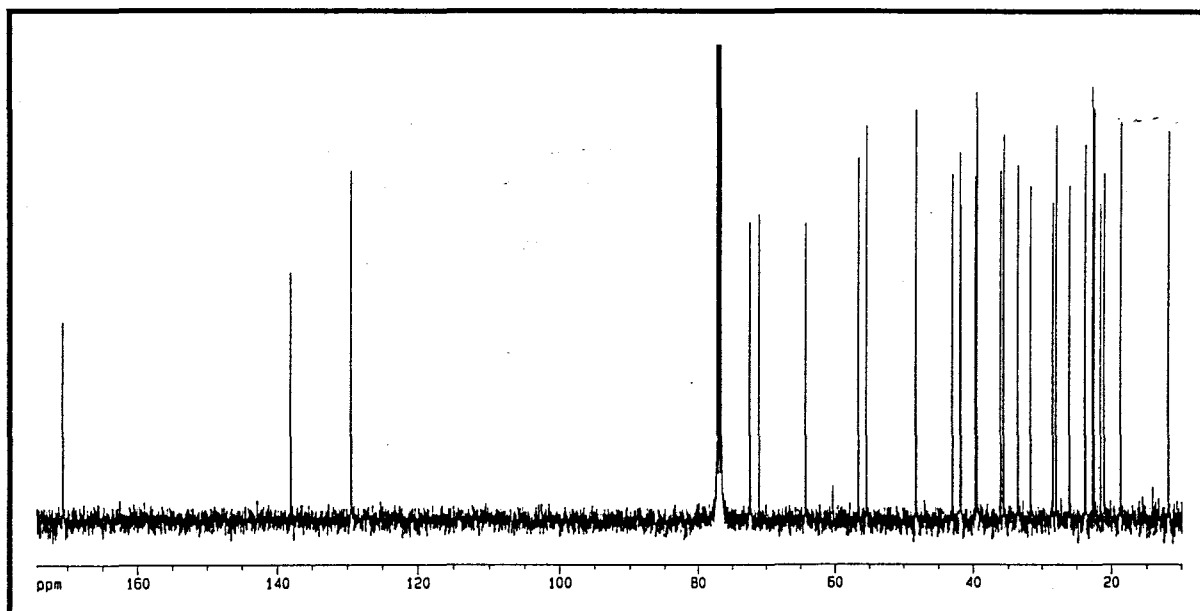
FIGURE 5.3 ^{13}C NMR (100 MHz, CDCl$_3$) spectrum of compound [121].

The molecular formula together with single carbonyl and olefinic moieties implied a tetracyclic skeleton for **[121]**. The oxymethine signal at δ 71 and the two vinylic signals in the ^{13}C NMR spectrum of **[121]**, along with a multiplet at δ 3.56 in the ^{1}H NMR spectrum of **[121]**, typical of a 3-hydroxysterol proton[173], suggested a 3-hydroxy-5-cholestene steroid skeleton[170,171,180] for compound **[121]**. The absence of the characteristic resonance for a steroidal C-19 methyl group in the ^{13}C NMR spectrum, the presence of a coupled AB system between the oxymethylene protons at δ 4.48 (1H, d, J = 11.9 Hz, H-19) and δ 3.98 (1H, d, J= 11.9 Hz, H-19') in the COSY NMR spectrum, and the presence of two and three bond correlations from H-19 and H-19' to C-1, C-5, C-9, C-10 and the acetate carbonyl **(FIGURE 5.4)** in the HMBC NMR spectrum **(FIGURE 5.5)** of **[121]** placed the acetate group at C-19.
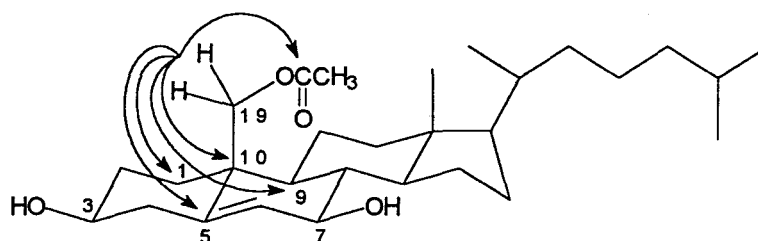
FIGURE 5.4 Selected two and three bond HMBC NMR correlations for compound **[121]**.

A broad single proton doublet at δ 3.80 (1H, br d, J = 7.8 Hz, H-7), strongly coupled in the COSY NMR spectrum of **[121]** with the Δ^5 vinylic proton at δ 5.52 (1H, br s, H-6), located the second hydroxyl at C-7. The proposed cholest-5-ene-3,7,19-triol 19-acetate structure for **[121]** is consistent with the coupling correlations from COSY, relay COSY and double relay COSY NMR experiments presented in **TABLE 5.1**, and also with the ^{13}C NMR data of **[121]** shown in **TABLE 5.2**. The stereochemistry of the C-3 and C-7 substituents is described in section 5.3.2.
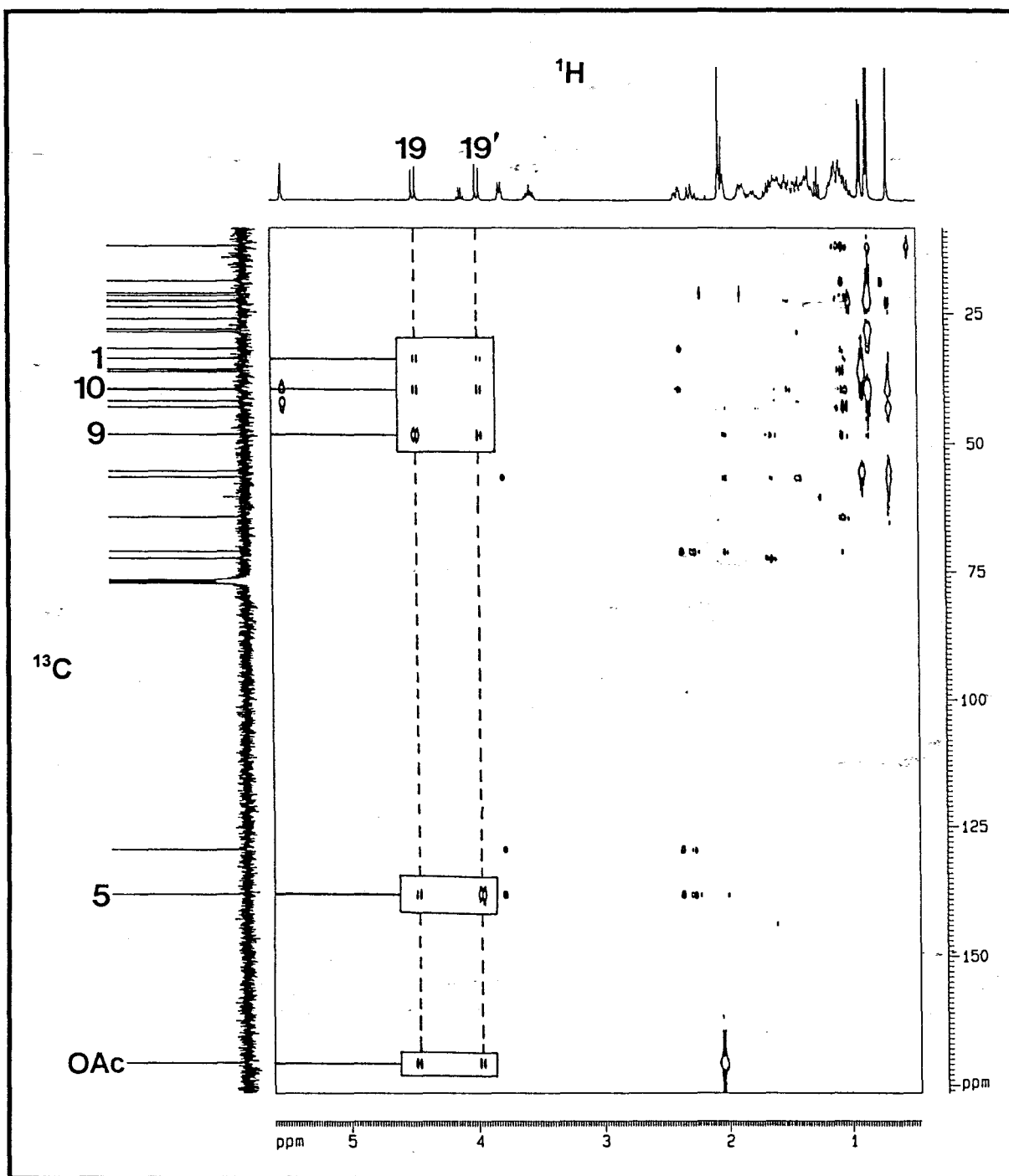
FIGURE 5.5 HMBC NMR (400 MHz, CDCl$_3$) spectrum of compound **[121]**.

TABLE 5.1 COSY, relay COSY and double relay COSY NMR (400 MHz, CDCl₃) correlations in compound [121].

	CHEMICAL SHIFT (δ)	COSY to ¹H	RELAY COSY to ¹H	DOUBLE RELAY COSY to ¹H
1ax	1.07	2	3	2,4
1eq	2.03	2		3,4,19,19'
2ax	1.44	1,3	3,4	1ax,3,4
2eq	1.87			
3	3.56	2,4	1,2,4,6,7	1eq,2,4,6,OAc
4ax	2.27	3,4eq,6,7	2,3,4eq,6,7	1,2,3,4eq,6,7,8,OAc
4eq	2.39	2,3,4ax	2,3,4ax,6,7	1,2,3,4ax,6,7,OAc
6	5.52	4ax,7	3,4,7,8	3,4,7,8,9,19,19'
7	3.80	4ax,6,8	3,4ax,6,8,9	4,6,8,9
8	1.65	7	6,7,9	4ax,6,7
9	1.03		7,8	6,7
12	2.03			18
18	0.70			12
19 / 19'	3.98 / 4.48		1eq,OAc	1eq,6,OAc
20	1.34	21	21	21
21	0.91	20	20	20
25	1.51	26,27	26,27	26,27
26	0.85	25	25	25
27	0.85	25	25	25
OAc	2.05		19,19'	3,4,19,19'

TABLE 5.2 ^{13}C NMR (100 MHz, CDCl$_3$) data for compounds **[121]**, **[122]** and **[135]**.

	[121]	**[122]**	**[135]**
1	33.6 t	33.6 t	33.2 t
2	31.8 t	31.8 t	31.9 t
3	71.2 d	71.1 d	71.1 d
4	41.8 t	41.8 t	41.8 t
5	138.2 s	138.1 s	138.2 s
6	129.5 d	129.5 d	130.7 d
7	72.5 d	72.5 d	72.5 d
8	42.0 d	42.0 d	42.3 d
9	48.3 d	48.3 d	48.7 d
10	39.6 s	39.6 s	43.2 s
11	21.6 t	21.6 t	21.7 t
12	39.8 t	39.8 t	39.9 t
13	43.1 s	43.1 s	41.5 s
14	56.6 d	56.6 d	56.9 d
15	26.1 t	26.1 t	26.1 t
16	28.5 t	28.5 t	28.6 t
17	55.5 d	55.4 d	55.5 d
18	11.9 q	11.9 q	12.2 q
19	64.3 t	64.3 t	62.6 t
20	35.7 d	35.5 d	35.7 d
21	18.8 q	18.7 q	18.8 q
22	36.2 t	36.1 t	36.2 t
23	23.8 t	24.7 t	23.9 t
24	39.5 t	125.1 d	39.5 t
25	28.0 d	131.0 s	28.0 d
26	22.8 q	17.6 q	22.8 q
27	22.5 q	25.7 q	22.6 q
COCH$_3$	170.7 s	170.7 s	
COCH$_3$	21.1 q	21.1 q	

All assignments are supported by DEPT, COSY, HMQC and HMBC NMR experiments.

The molecular formula of compound [122], established as $C_{29}H_{46}O_4$ (m/z 458.3391, Δmmu -5) from HREIMS data, indicated a molecular structure with two hydrogen atoms less than the molecular structure of [121]. The presence of m/z 251 (M^+-HOAc-2H$_2$O-side chain) and 209 (M^+-HOAc-2H$_2$O-ring D fission) peaks in the EI mass spectra of both [122] and [121] implied that they differ only in the side chain at C-17. Thus, two additional olefinic signals at δ 131 and 125 in the ^{13}C NMR spectrum of [122], absent from the ^{13}C NMR spectrum of [121], suggested an unsaturation in the side chain of [122]. Signals in the ^{1}H NMR spectrum of [122] (FIGURE 5.6) at δ 1.59 (3H, s, H-26) and δ 1.67 (3H, s, H-27), consistent with the presence of vinylic methyl groups, together with an olefinic triplet resonance at δ 5.08 (1H, t, J = 7.1 Hz, H-24) broadened by long range coupling to H-26 and H-27, confirmed the presence of a C-24 olefin. Three bond correlations between the C-26 and C-27 vinyl methyl group protons and the carbon atom at δ 125 (C-24) in the HMBC NMR spectrum of [122] placed this double bond in the Δ^{24} position. Consequently the structure of [122] was established as cholest-5,24-diene-3,7,19-triol 19-acetate. The stereochemistry of the C-3 and C-7 oxygen functionalities is discussed in section 5.3.2.
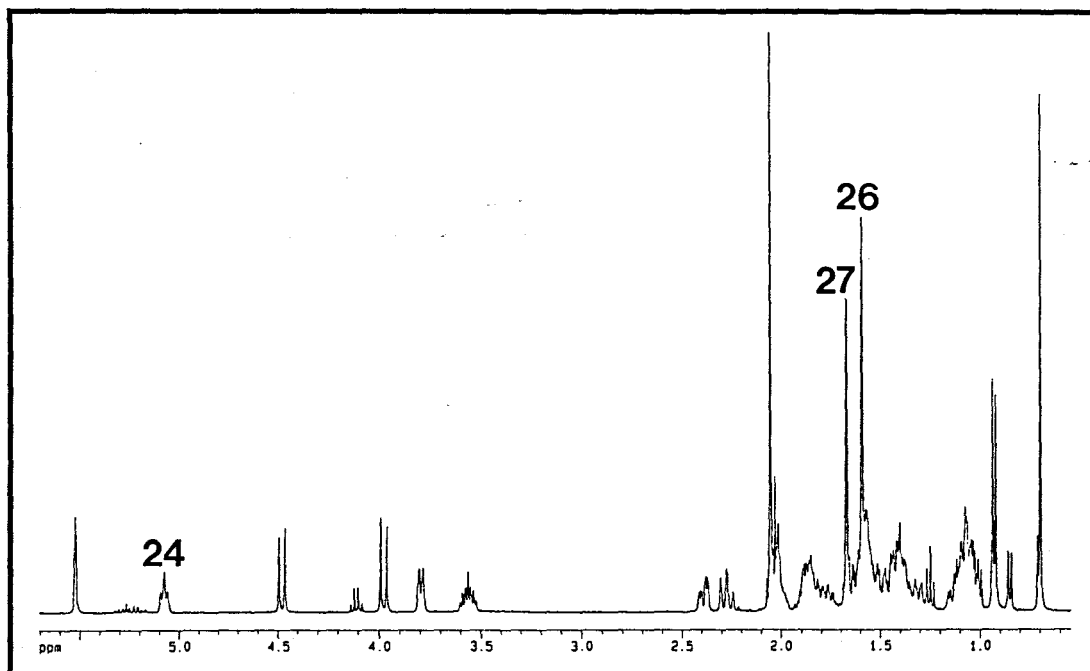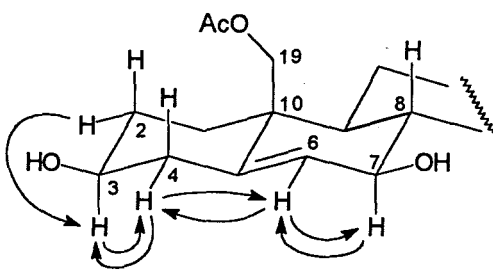
FIGURE 5.6 ^{1}H NMR (400 MHz, CDCl$_3$) spectrum of compound [122].

5.3.2 Stereochemistry of the hydroxysterols [121] and [122].

The complexity (seven-line multiplet), the $W_{\frac{1}{2}}$ = 22 Hz and the chemical shift of the carbinol methine signal resonating at δ 3.56 in the ^{1}H NMR spectrum of [121] and [122] are consistent with those normally seen in a 3β-hydroxysteroid for a 3α-carbinol proton[166,181]. The 3α-orientation of this carbinol proton was confirmed from enhancements observed in the NOE difference spectra of [121]. These enhancements are summarised in TABLE 5.3.

TABLE 5.3 Selected enhancements from the NOEDS (400 MHz, $CDCl_3$) spectra of compound [121].

H^1 IRRADIATED	H^1 ENHANCED	ENHANCEMENT
2 eq	3 ax	8%
3 ax	4 eq	8%
4 eq	3 ax, 6	4%, 12%
6	4 eq, 7 ax	9%, 7%
7 ax	6	7%

The large coupling observed between H-7 and H-8 (J = 7.8 Hz) in the ^{1}H NMR spectra of compounds [121] and [122], consistent with a diaxial relationship[173], established a 7β-OH configuration for the second hydroxy group in both these compounds. Support for the 7β-OH assignment was obtained by acetylating compound [121] to yield the triacetate [134]. The coupling observed between H-6 and H-7 (J = 2.1

113

Hz) in [134] was consistent with a dihedral angle of about 80° previously deduced from examination of molecular models of 7β-acetoxy-Δ^5-steroids[182-184] (the epimeric 7α-acetoxy isomers show larger coupling constant values of J = 5 Hz resulting from an H-6/H-7 bond angle of about 25°).
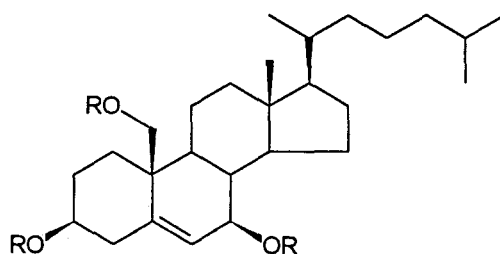
[134] R = Ac
[135] R = H

Final confirmation of the structure and stereochemistry of [121] was achieved by reducing [121] with LiAlH$_4$ to yield cholest-5-ene-3β,7β,19-triol [135]. The melting point and ^{13}C NMR data (TABLE 5.2) of this product compared favourably with that of cholest-5-ene-3β,7β,19-triol previously isolated from the black coral *Antipathes subpinnata*[185].

5.3.3 Structure determination of the xenicane diterpenes [110], [16], [123] and [124].

[110]   [100]
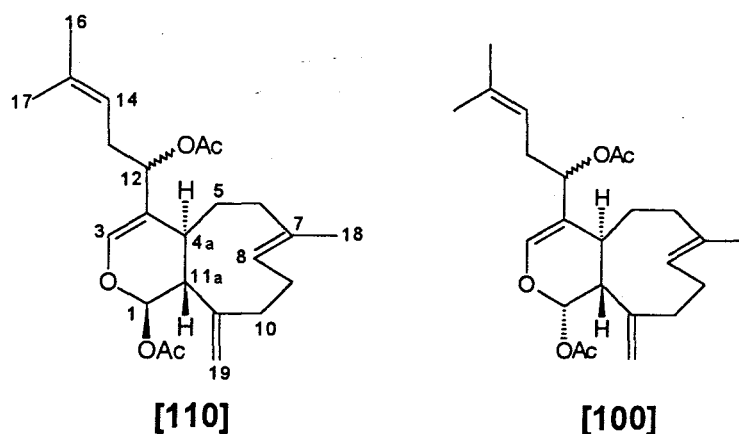
The molecular ion at *m/z* 402.2429 (Δmmu +13) in the HREIMS spectrum of compound [110] is compatible with the molecular formula $C_{24}H_{34}O_5$. Interestingly, this molecular formula was found to be identical to that of tsitsixenicin A [100] isolated from

114

the soft coral *Capnella thyrsoidea*[84] (chapter four). This naturally led to a comparison of the NMR spectral data of **[110]** and **[100]** which were very similar. The ^{13}C NMR spectrum of **[110]** also contained two acetate carbonyl (δ 169 and 170) and eight olefinic (δ 113, 117, 119, 124, 135, 136, 137 and 151) resonances and implied a bicyclic xenicane diterpene skeleton. Analogous coupling sequences and correlations in the COSY and HMBC NMR experiments of **[110]**, when compared to those in **[100]**, suggested that these compounds were identical. However, a difference in optical rotation, **[110]** ($[\alpha]_D^{21}$ +127°) and **[100]** ($[\alpha]_D^{21}$ -64°), and slight differences in the ^{13}C and ^{1}H NMR chemical shifts between the two compounds (**TABLE 5.4**) suggested that they were stereoisomers.

[16]   **[123]**
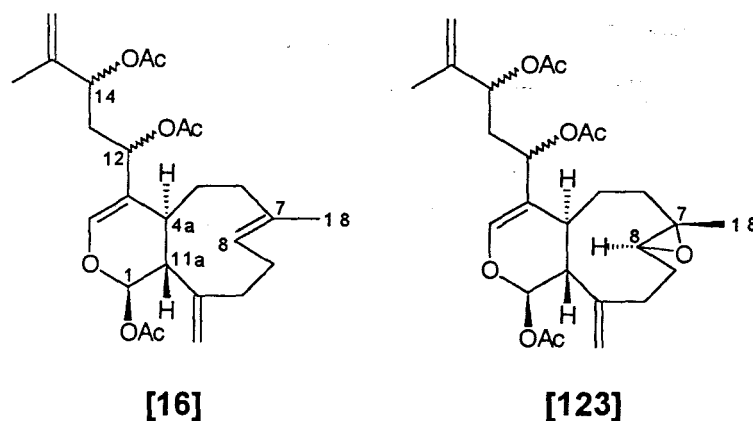
HREIMS data established the molecular formula of compound **[16]** as $C_{26}H_{36}O_7$ (*m/z* 460.2448, Δmmu -11). Three acetate carbonyl (δ 169.9, 169.8 and 169.7) and eight vinylic (δ 113, 114, 117, 125, 136, 137, 143 and 151) resonances in the ^{13}C NMR spectrum of **[16]** accounted for seven of the nine degrees of unsaturation implied by the molecular formula and again required a bicyclic diterpene skeleton. The ^{1}H and ^{13}C NMR data of compound **[16]** (**TABLE 5.5**), together with a two bond correlation from the enol ether proton at δ 6.38 (1H, s, H-3) to a quarternary vinylic carbon at δ 117 (C-4), and three bond correlations from the same enol ether proton to an acetylated hemi-acetal carbon at δ 92 (C-1) and to a methine carbon at δ 38 (C-4a), implied that one of the rings in the bicyclic structure of **[16]** had the same 1-acetoxydihydropyran ring structure as found in compound **[110]**.

115

TABLE 5.4 Comparison of ^{1}H and ^{13}C NMR data for compounds **[110]** and **[100]**.

	[110]		[100]	
	^{13}C	^{1}H	^{13}C	^{1}H
1	92.0 d	5.84 d (2.2)	92.3 d	5.75 d (3.6)
3	136.5 d	6.37 s	142.6 d	6.52 d (1.9)
4	116.9 s		116.1 s	
4a	37.8 d	2.00 m	39.7 d	2.29 m
5, 5'	30.0 t	1.87 m, 1.50 m	32.3 t	2.08 m, 1.22 m
6, 6'	40.2 t	2.22 m, 1.94 m	39.7 t	2.19 m, 2.07 m
7	135.6 s		134.4 s	
8	124.4 d	5.26 br d (5.9)	124.3 d	5.37 dd (8.0, 9.5)
9, 9'	25.2 t	2.45 m, 2.11 m	25.5 t	2.43 m, 2.07 m
10, 10'	35.5 t	2.26 m, 2.24 m	35.9 t	2.31 m, 2.07 m
11	151.1 s		149.3 s	
11a	49.5 d	1.96 m	50.1 d	1.96 br s
12	72.2 d	5.35 t (6.2)	74.7 d	5.25 t (7.6)
13, 13'	33.4 t	2.33 m	31.4 t	2.43 m, 2.31 m
14	118.8 d	5.07 t (6.6)	118.9 d	4.97 t (6.9)
15	134.5 s		135.6 s	
16	18.0 q	1.60 br s	18.1 q	1.64 br s
17	25.8 q	1.68 br s	25.7 q	1.67 br s
18	16.7 q	1.64 br s	17.0 q	1.67 br s
19, 19'	113.2 t	4.87 br s, 4.83 br s	113.4 t	4.91 br s, 4.78 br s
COCH_3	21.2 q	2.03 s	21.5 q	2.07 s
	21.0 q	2.05 s	21.0 q	2.01 s
COCH_3	169.7 s		169.5 s	
	170.0 s		170.2 s	

Spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C in CDCl$_3$ with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.

TABLE 5.5 ¹H and ¹³C NMR data for compounds **[16]** and **[123]**.

	[16]		[123]	
	¹³C	¹H	¹³C	¹H
1	92.1 d	5.84 d (2.3)	91.6 d	5.93 d (3.1)
3	137.1 d	6.38 s	137.5 d	6.40 s
4	117.3 s		116.9 s	
4a	37.6 d	1.98 m	37.1 d	2.06 m
5, 5'	30.2 t	1.90 m, 1.57 m	29.2 t	2.08 m, 1.67 m
6, 6'	40.2 t	2.23 m, 1.94 m	39.5 t	2.25 m, 1.08 m
7	135.7 s		59.8 s	
8	124.5 d	5.32 m	62.8 d	2.95 dd (3.7, 10.8)
9, 9'	25.2 t	2.45 m, 2.12 m	24.7 t	2.28 m, 1.49 m
10, 10'	35.5 t	2.27 m, 2.24 m	32.1 t	2.39 m
11	151.0 s		148.0 s	
11a	49.6 d	1.99 m	47.9 d	2.42 m
12	68.9 d	5.34 m	68.6 d	5.29 dd (4.7, 8.2)
13	38.6 t	1.94 m	38.9 t	1.90 m
14	73.3 d	5.21 dd (4.1, 9.3)	73.1 d	5.19 dd (5.3, 8.2)
15	143.0 s		142.8 s	
16, 16'	112.7 t	4.97 br s, 4.89 br s	112.8 t	4.95 br s, 4.89 br s
17	18.3 q	1.74 br s	18.3 q	1.72 br s
18	16.8 q	1.66 br s	16.9 q	1.30 br s
19, 19'	113.5 t	4.89 br s, 4.83 br s	115.2 t	5.04 br s, 4.95 br s
COCH_3	21.0 q	2.05 s	21.0 q	2.07 s
	21.0 q	2.05 s	21.0 q	2.05 s
	21.0 q	2.03 s	21.0 q	2.03 s
$COCH_3$	167.7 s		169.5 s	
	169.8 s		169.8 s	
	170.0 s		170.0 s	

Spectra were recorded at 400 MHz for ¹H and 100 MHz for ¹³C in CDCl₃ with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.

The comparison between **[16]** and **[110]** was extended to the nine-membered ring. Contiguous coupling in the COSY NMR spectra of **[16]** was evident from the methine proton at δ 1.98 (1H, m, H-4a) to protons at δ 1.90 and 1.57 (2H, m, H-5 and H-5') and to protons at δ 2.23 and 1.94 (2H, m, H-6 and H-6') and also from an olefinic proton at δ 5.32 (1H, m, H-8) to the protons at δ 2.45 and 2.12 (2H, m, H-9 and H-9') and to protons at δ 2.27 and 2.24 (2H, m, H-10 and H-10'). Correlations in the HMBC NMR spectrum of **[16]** (**FIGURE 5.7**) were observed from the vinylic methyl protons at δ 1.66 (3H, br s, H-18) to carbons at δ 40 (C-6) and 125 (C-8) and from the exocyclic methylene protons at δ 4.89 and 4.83 (2H, br s, H-19 and H-19') to carbons at δ 36 (C-10) and 50 (C-11a).
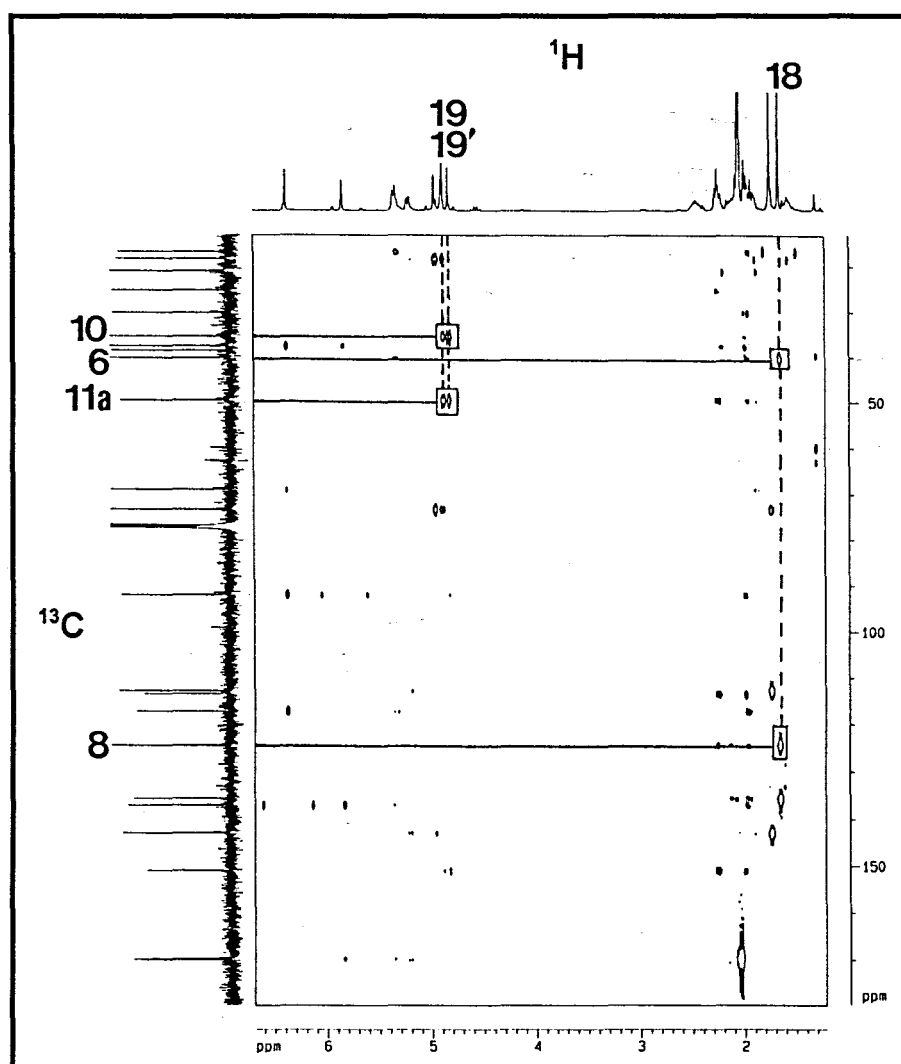
FIGURE 5.7 HMBC NMR (400 MHz, CDCl$_3$) spectrum of compound **[16]**.

The above 2-D NMR correlations, together with a comparison of the ^{13}C NMR data of [16] and [110], unequivocally showed that [16] also contained a 2-oxabicyclo[7.4.0]tridecane ring system with the same functional group distribution as established in [110]. The only difference between the two compounds, therefore, was in the composition of the side chain. Having accounted for the bicyclic ring system, 2-D NMR spectral analysis indicated the presence of a 1,3-diacetoxy-4-methyl-pent-4-ene side chain. This was shown to be positioned at C-4 by virtue of the two-bond correlation from the acetoxy methine proton at δ 5.34 (1H, m, H-12) to the carbon at δ 117 (C-4) and the three bond correlations from the same proton to carbons at δ 137 (C-3) and 38 (C-4a) (FIGURE 5.8) in the HMBC NMR spectrum of [16]. The assignment of a terminal olefin in the side chain was supported by three bond correlations from the methylene protons at δ 4.97 and 4.89 (2H, br s, H-16 and H-16') to the vinylic methyl carbon at δ 18 (C-17) and to the acetoxy carbon at δ 73 (C-14) (FIGURE 5.8). Irradiation of the H-14 signal resulted in no change in the splitting pattern of the H-12 signal and vice-versa in a ^{1}H-decoupling NMR experiment (400 MHz, C_6D_6) performed on compound [16]. This observation indicated that the acetoxy carbons were not adjacent to each other and confirmed the -CH(OAc)CH$_2$CH(OAc)- substructure of the side chain in [16].
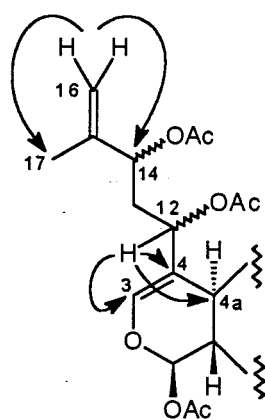
FIGURE 5.8 Selected HMBC NMR correlations in compound [16].

Subsequent to the assignment of [16], Kashman et al. published the structure of zahavin A[63]. Comparison of the NMR spectral data of [16] and zahavin A indicated that they were identical compounds. Although there is a difference in the optical

rotation measured for [16] ($[\alpha]_D^{21}$ +21.5°) and the published value for zahavin A ($[\alpha]_D^{21}$ +7.3°), this is possibly a consequence of the chemical instability of these compounds in $CHCl_3$ (the solvent used for both rotation measurements) rather than any stereochemical difference.

HREIMS data established the molecular formula of [123] as $C_{26}H_{36}O_8$ (m/z 476.2419, Δmmu +11). Comparison of this molecular formula with that of compound [16] indicated that the only difference was an additional oxygen atom in the molecular formula of [123]. Comparison of the ^1H and ^{13}C NMR data of [123] with that of [16] (TABLE 5.5) indicated that the vinylic proton resonance at δ 5.32 (1H, m, H-8) and the olefinic carbon resonances at δ 125 (C-8) and 136 (C-7) in the ^1H and ^{13}C (FIGURE 5.9) NMR spectra of [16], respectively, had been replaced by an oxymethine doublet of doublets at δ 2.95 (1H, dd, J = 3.7, 10.8 Hz, H-8) and two carboxy resonances at δ 63 (C-8) and 60 (C-7) in the ^1H and ^{13}C (FIGURE 5.10) NMR spectra of [123], respectively. The above data established that a 7,8-epoxide functionality in compound [123] had replaced the unsaturation at this position in [16].

[136]   [131]
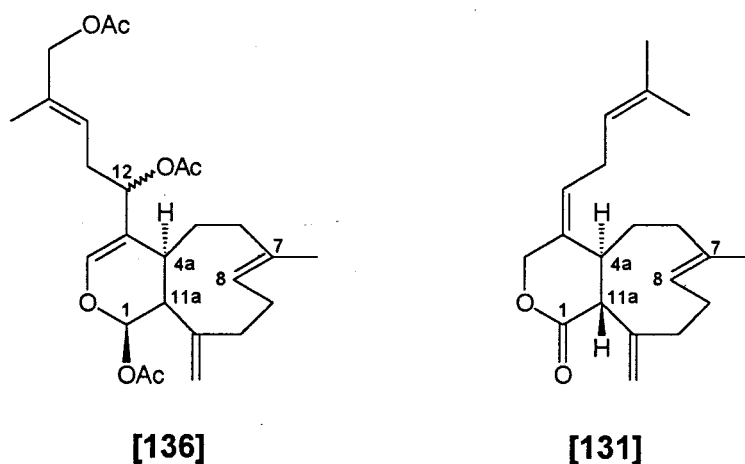
It is unclear as to whether the 7,8-epoxide is itself a natural product or an oxidation artifact of the isolation procedure. There are conflicting reports on the ease of oxidation of the Δ^7-double bond in xenicane diterpenes. Scheuer[160] suggested that 7,8-epoxidation of [136] occurred readily on exposure to air while Higuchi only obtained the 7,8-epoxide of [131] in thirteen per cent yield after stirring [131] in $CHCl_3$ solution

for two weeks. The fact that the 7,8-epoxide signal at δ 2.95 (1H, dd, J = 3.7, 10.8 Hz, H-8) in the ^{1}H NMR spectrum of [123] was evident in the initial ^{1}H NMR spectrum of the crude EtOAc extract of the *Alcyonium* species B soft coral suggests that compound [123] may itself be present in the soft coral. However, further work is needed to qualify this observation.
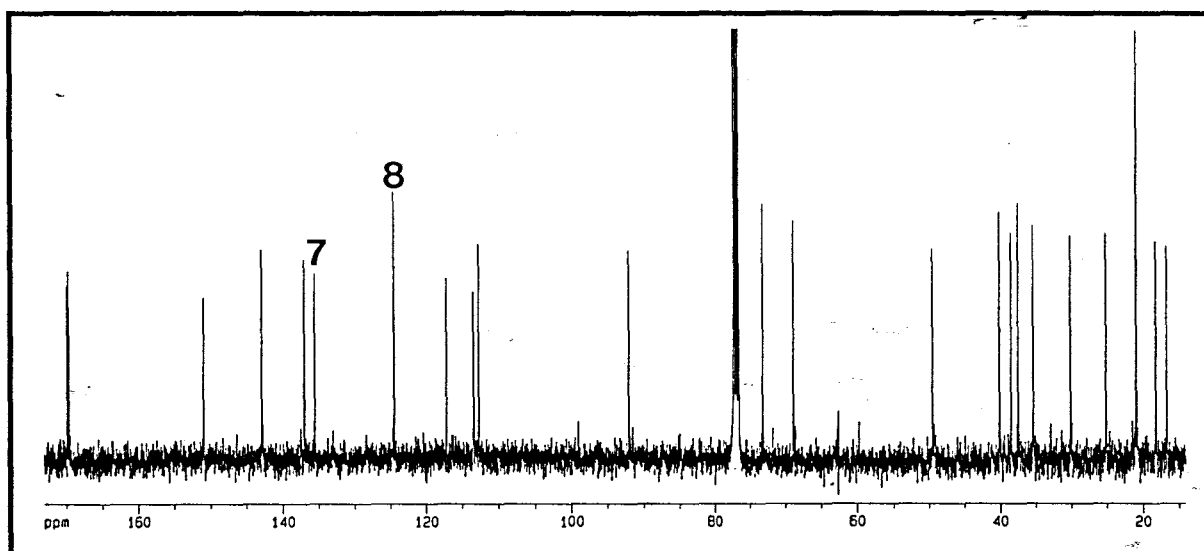
FIGURE 5.9 ^{13}C NMR (100 MHz, CDCl$_3$) spectrum of compound [16].
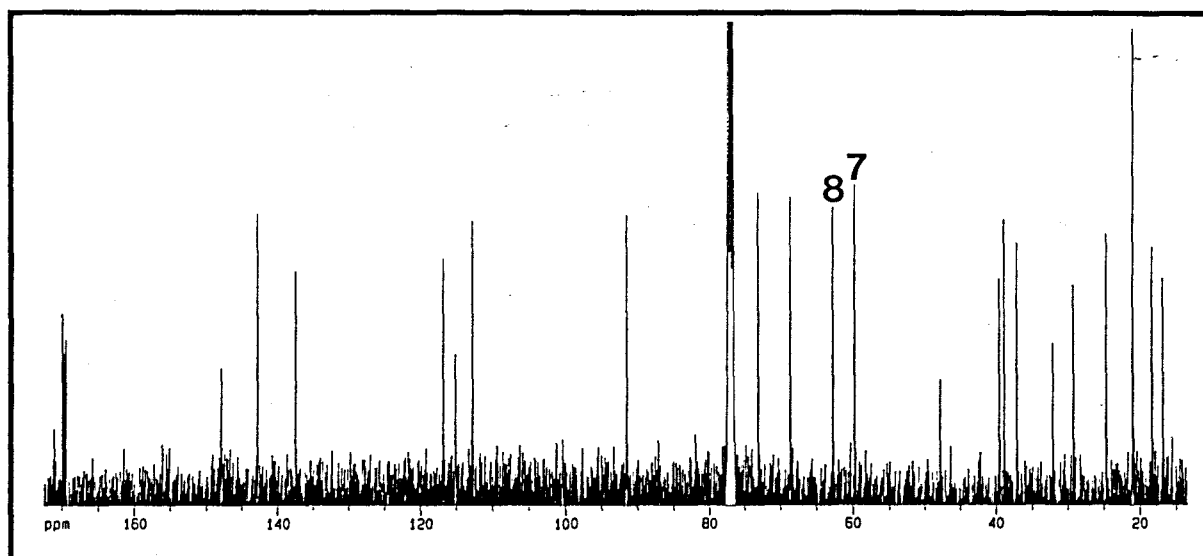
FIGURE 5.10 ^{13}C NMR (100 MHz, CDCl$_3$) spectrum of compound [123].

[124]   **[105]**
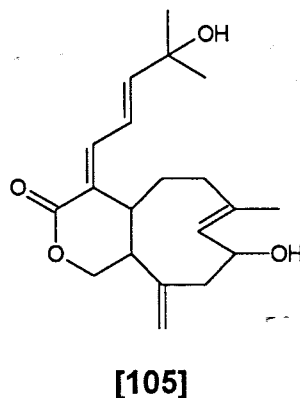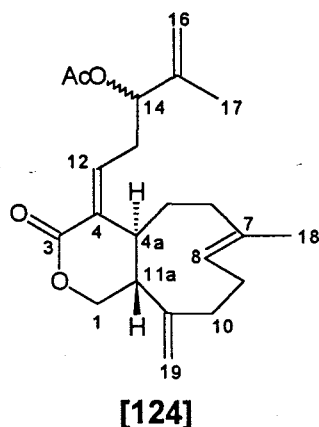
A molecular formula of $C_{22}H_{30}O_4$ (*m/z* 358.2158, Δmmu -15) was established from HREIMS data for compound **[124]**. The presence of two ester carbonyl functionalities and four double bonds in the ^{13}C NMR spectrum of **[124]** accounted for six of the eight degrees of unsaturation in the molecule. The remaining two degrees of unsaturation therefore required **[124]** to be bicyclic. Interestingly, certain similarities existed between the NMR spectra of **[124]** and **[16]**. The fact that the ^1H NMR spectrum of **[124]** had exocyclic methylene resonances at δ 4.95 and 4.84 (2H, s, H-19 and H-19'), a vinylic proton resonance at δ 5.41 (1H, br t, J = 7.8 Hz, H-8) and a vinylic methyl resonance at δ 1.70 (3H, s, H-18) suggested that **[124]** had the same 9-membered ring as found in **[16]**. Further common resonances included an olefinic methylene resonance at δ 5.00 and 4.95 (2H, s, H-16 and H-16'), a vinylic methyl resonance at δ 1.76 (3H, s, H-17) and an oxymethine resonance at δ 5.25 (1H, t, J = 6.4 Hz, H-14). These latter resonances implied that the same terminal portion existed in the six-carbon side chain of **[124]** as found in **[16]**. However, the absence of the H-1 and H-12 oxymethine signals and the absence of the H-3 enol ether proton resonance indicated that **[124]** did not have the 1-acetoxydihydropyran ring structure of **[16]**.

The HMQC NMR spectrum of **[124]** revealed the presence of two oxymethylene protons with multiplets centred at δ_H 4.06 (1H, dd, J = 5.8, 11.4 Hz, H-1) and 3.52 (1H, t, J = 12.0 Hz, H-1') coupled to a ^{13}C resonance at δ_C 71. These data were corroborated by a DEPT NMR experiment. A COSY NMR spectrum of **[124]** (FIGURE 5.12) showed that these oxymethylene protons were strongly coupled to the methine proton at the C-

122

11a ring junction (δ 2.12). The same oxymethylene protons showed a three bond correlation to the C-4a ring junction carbon atom at δ 44 and to an ester carbonyl carbon at δ 171 (C-3) (**FIGURE 5.11**) in the HMBC NMR spectrum of **[124]**.
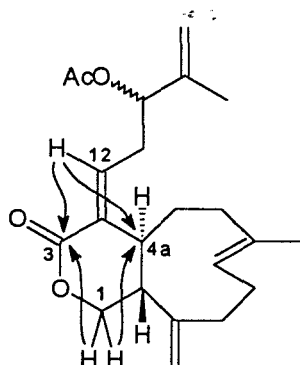
FIGURE 5.11 Selected three bond correlations from the HMBC NMR spectrum of **[124]**.

The COSY NMR spectrum of **[124]** revealed contiguous coupling from the oxymethine proton at δ 5.25 (H-14) to the methylene protons at δ 2.60 and 2.50 (2H, m, H-13 and H-13') and, finally, to an olefinic proton at δ 6.33 (1H, t, J = 7.3 Hz, H-12) (**FIGURE 5.12**). The H-12 olefinic proton, in turn, exhibited three bond long range correlations to the C-3 ester carbonyl and the C-4a ring junction carbon atom (**FIGURE 5.11**) in the HMBC spectrum of **[124]**. The above implied that a C-4/C-12 double bond was the site of attachment of the 6-carbon side chain. The ^{1}H and ^{13}C NMR data (**TABLE 5.6**) for the bicyclic ring system of **[124]** compared favourably with the corresponding data for the bicyclic ring system of xeniolide A **[105]** previously isolated from the soft coral *Xenia macrospiculata*[147] and the structure of **[124]**, xeniolide C, was thus established.
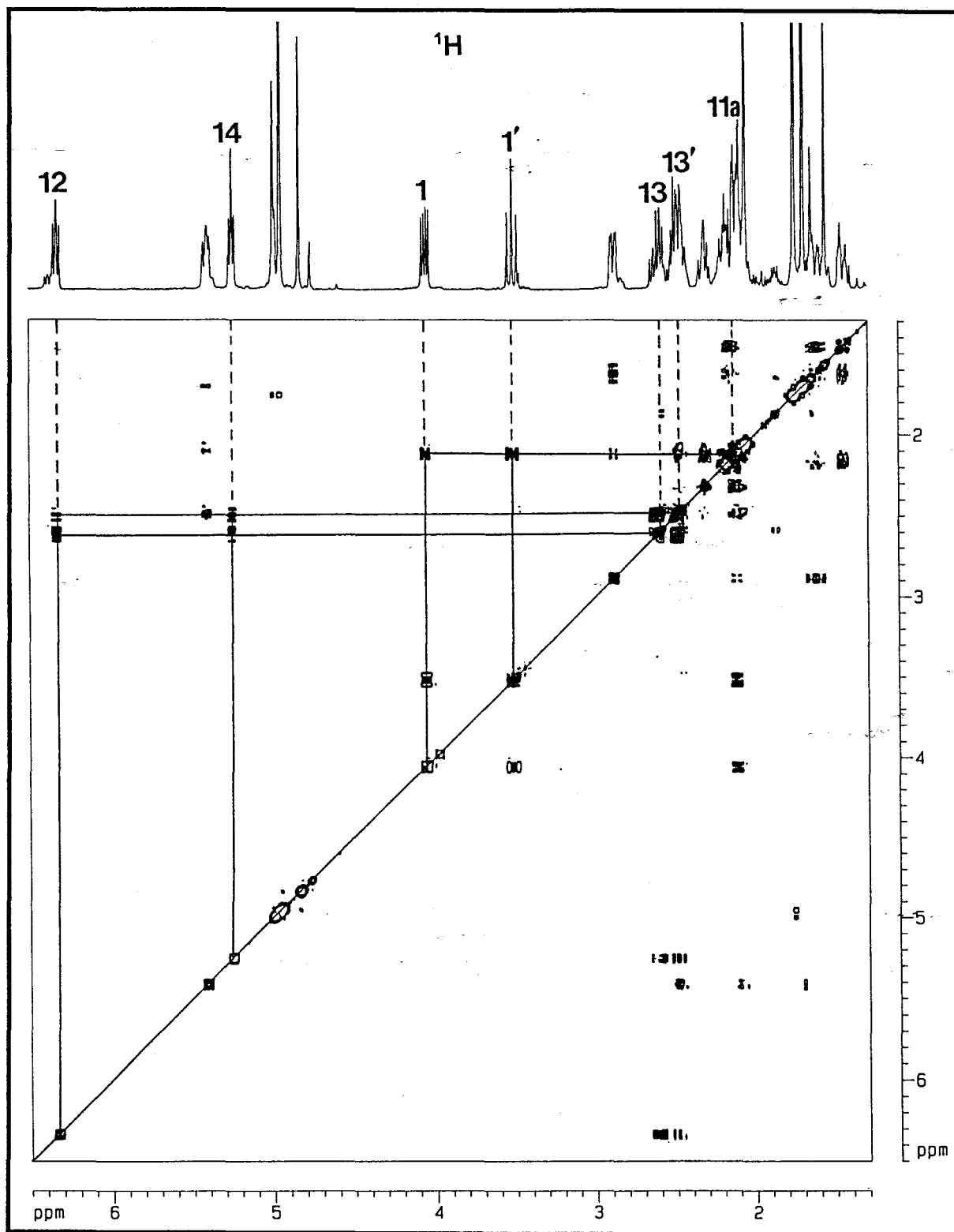
FIGURE 5.12 COSY NMR (400 MHz, CDCl$_3$) spectrum of compound **[124]**.

124

TABLE 5.6 NMR spectral data for compound **[124]**.

	^{13}C	^1H	COSY to	HMBC to
1	70.9 t	4.06 dd (5.8, 11.4)	H-1', H-11a	C3, C4a, C11a
1'	70.9 t	3.52 t (12.0)	H-1, H-11a	C3, C4a, C11, C11a
3	170.6 s			
4	136.8 s			
4a	43.8 d	2.88 dd (3.6, 10.8)	H-5, H-11a	C3, C4, C5, C6
5, 5'	37.6 t	1.62 m, 1.45 m	H-4a, H-6	C7
6, 6'	40.3 t	2.21 m, 2.17 m	H-5	
7	135.3 s			
8	124.6 d	5.41 br t (7.8)	H-9, H-9', H-18	C6, C18
9, 9'	24.8 t	2.47 m, 2.12 m	H-8, H-10	
10, 10'	34.6 t	2.32 m, 2.12 m	H-9, H-9'	C11, C11a
11	152.3 s			
11a	49.4 d	2.12 m	H-1, H-1', H-4a	
12	133.2 d	6.33 t (7.3)	H-13, H-13'	C3, C4a
13, 13'	31.0 t	2.60 m, 2.50 m	H-12, H-14	C4, C12, C14, C15
14	75.5 d	5.25 t (6.4)	H-13, H-13'	C12, C13, C15, C16
15	142.0 s			
16, 16'	113.5 t	5.00 s, 4.95 s	H-17	C14, C15, C17
17	18.5 q	1.76 s	H-16	C14, C15, C16
18	18.5 q	1.70 s	H-8	C6, C7, C8
19, 19'	113.1 t	4.95 s, 4.84 s		C10, C11a
COCH_3	21.1 q	2.06 s		$COCH_3$
$COCH_3$	169.9 s			

Spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C in $CDCl_3$ with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.

5.3.4 Stereochemistry of the xenicane diterpenes [110], [16], [123] and [124].
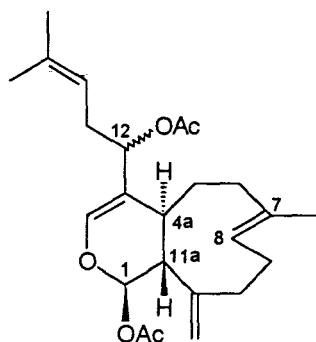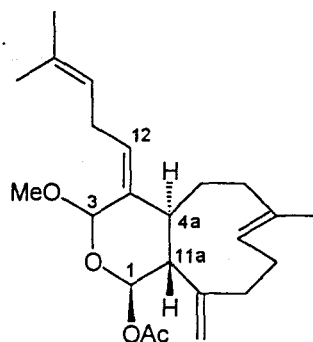
[110]   [112]

An E-configuration was assigned to the Δ^7 double bond in compound [110] based on the δ 16.7 chemical shift for the Me-18 group in the ^{1}H NMR spectrum of [110] (rather than δ 22-25 for the Z-configuration)[147,149]. As with the xenicanes in the previous chapter, the slightly broadened H-11a singlet in the ^{1}H NMR spectrum of [110] indicated a very small coupling to H-4a (a dihedral angle of approximately 90°) and this is consistent with the *trans*-fused bicyclic ring system found in other naturally occurring xenicane diterpenes. An α-configuration was assigned to the proton at C-1 based on the $J_{1,11a}$ = 2.2 Hz coupling and observed NOEs (see **TABLE 4.5**, section 4.3.4) which are in accordance with H-1α configured 1-acetoxyxenicane compounds found in the literature[63,145,146,148-150,160,163,164]. Comparison of the spectral data of compound [110] with that of 9-deacetoxy-14,15-deepoxyxeniculin isolated from the Red Sea soft coral *Xenia obscuronata*[149] suggested that they were in fact the same compound. However, this comparison was not definitive as no optical rotation was reported for the latter compound and the C-12 stereochemistry in both compounds remains unassigned. Previous attempts to define the stereochemistry of an acetoxy group at C-12 in xenicane compounds by chemical transformations and spectroscopy have failed[157,160]. Unsuccessful attempts were made to convert [110] to acalycigorgin D [112] ($[\alpha]_D^{21}$ -51°) by refluxing in MeOH for several hours. Compound [112], in which the stereochemistry has been defined, was assumed to be an artifact arising *via* an S_N2' *syn* reaction of [110] with MeOH during the extraction of the gorgonian *Alcalycigorgia* species[164].

[16]   **[123]**
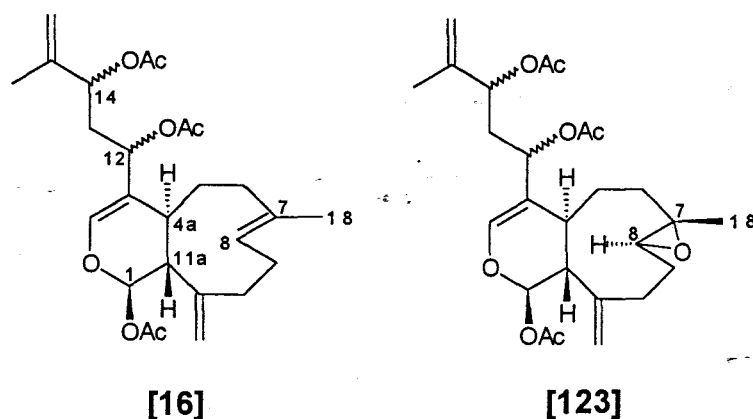
An *E*-configuration for the Δ^7 double bond in **[16]**, and a *trans* bicyclic ring system and α-proton at C-1 in **[16]** and **[123]**, were assigned using the same methods and reasoning as that applied to **[110]**. The assignment of the configuration for the 7(8)-epoxide in **[123]** revealed a disparity in the literature concerning the interpretation of NOEDS experiments. The observed enhancement of the α-proton at C-4a (19%) with the irradiation of H-8 and enhancement of the β-proton at C-11a (5%) with the irradiation of the Me-18 protons in NOEDS experiments on **[123]** indicated an α-orientated epoxide according to Shibata and co-workers[164]. Curiously, however, there was an apparent complete lack of enhancement of H-8 with the irradiation of the Me-18 protons and *vice versa*, and this, according to Higuchi and co-workers[157], indicated a 7(8)-*E*-epoxy configuration. In an α-orientated epoxide the H-8 and Me-18 protons would be close in space and would be expected to enhance upon their respective irradiation. The fact that there is an absence of any correlation between the H-8 and Me-18 protons in NOEDS experiments on **[123]**, therefore, must take precedence and supports a 7(8)-*E*-epoxy assignment for **[123]** rather than an α-orientated epoxide.

An attempt was made to assign the relative stereochemistries of the acetoxy groups at C-12 and C-14 in compound **[16]**. The proposed strategy (**SCHEME 5.3**) involved the removal of the acetate groups followed by elution through an Amberlyst 15 column in acetone to form the corresponding acetonide **[138]**[186]. This method relies on the fact that acetonides derived from *syn* 1,3-diols exist in a well defined chair conformation with the alkyl substituents in equatorial positions while acetonides derived

from *anti* 1,3-diols exist in a twist conformation in order to avoid the 1,3-diaxial interaction that would occur between the alkyl substituents in a chair conformation[186]. The ^{13}C NMR chemical shifts of the acetonide methyl groups allow the conformation and hence the relative stereochemistry of the diol to be distinguished. The reason for this is that the ^{13}C NMR spectra of *syn* 1,3-diol acetonides display an axial methyl resonance at about δ 19 and an equatorial methyl resonance at about δ 30, whereas the spectra of *anti* 1,3-diol acetonides show two methyl resonances at about δ 25[186].

Pilot scale reactions of **[16]** with K_2CO_3 in MeOH followed by NMR analysis suggested that, although the acetate groups had been removed, a mixture of products had resulted in which aldehyde and methoxy resonances were evident. Work up of the reaction followed by normal phase HPLC did not yield the desired saponified product **[137]**.
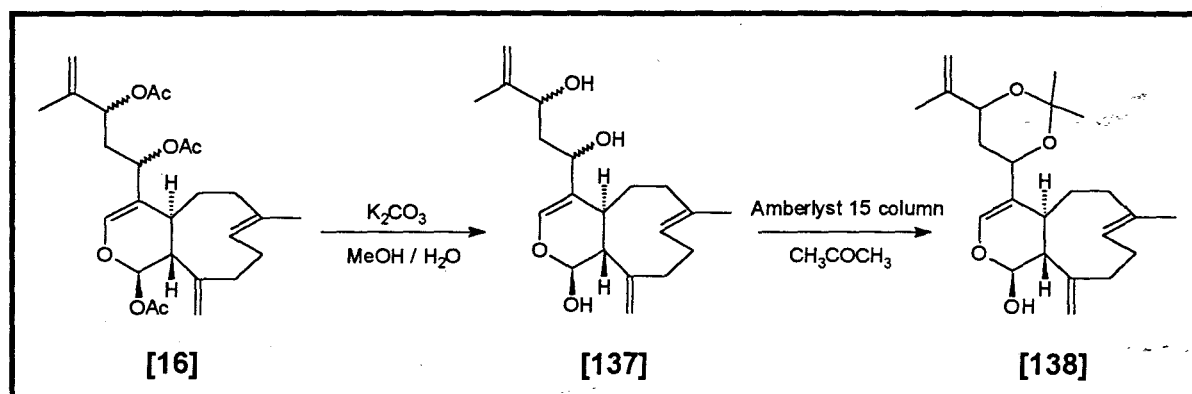
SCHEME 5.3 Reaction sequence proposed for the preparation of an acetonide derivative of compound **[16]**.

An interesting rearrangement compound **[139]** was, however, isolated in a pure state from the chromatography of the saponification reaction products. HREIMS data established the molecular formula of **[139]** as $C_{20}H_{26}O_2$ (*m/z* 298.1939, Δmmu +6). The presence of a single aldehyde carbonyl resonance and ten olefinic resonances in the ^{13}C NMR spectrum of **[139]** accounted for six of the eight degrees of unsaturation implied by the molecular formula and this required **[139]** to be bicyclic. Comparison of

the ^{1}H and ^{13}C NMR chemical shifts of compound [139] with those of zahavin A [16] indicated that the nine-membered ring and its associated substituents in [16] was still present in compound [139]. The absence of resonances characteristic of an acetylated hemi-acetal functionality in [139] implied that the second ring in [139] did not have the same 1-acetoxydihydropyran ring structure as in zahavin A. The comparison was also extended to the side chain. An olefinic methylene resonance at δ_H 5.19 and 5.00 (2H, s, H-18 and H-18') and δ_C 112, an olefinic methyl resonance at δ_H 1.63 (3H, s, H-19) and δ_C 19, and an oxymethine resonance at δ_H 4.61 (1H, s, H-16) and δ_C 77 indicated that the terminal portion of the six-membered side chain of [16] was still intact in [139]. Interestingly, an aldehyde functionality was present at δ_C 193 in [139] which was not present in the natural product [16].

[139]   [140]
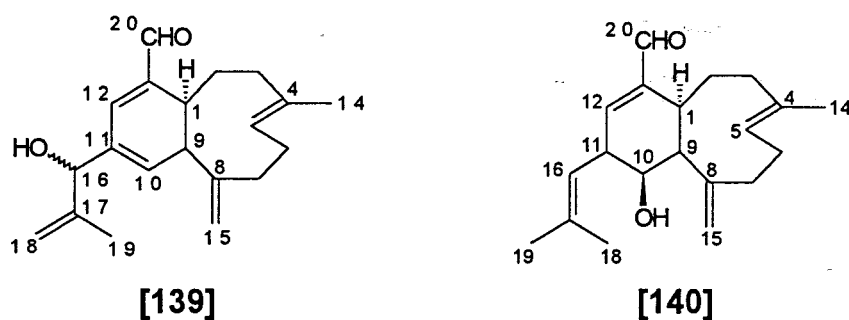
The COSY NMR spectrum of [139] (FIGURE 5.13) showed contiguous coupling from the methylene protons at δ 1.51 (2H, m, H-2) to the ring junction proton at δ 2.90 (1H, m, H-1) and to the other ring junction proton at δ 2.36 (1H, m, H-9). The latter ring junction proton was coupled to a vinylic proton at δ 5.91 (1H, d, J = 5.3 Hz, H-10) which showed long range coupling to a further vinylic proton at δ 6.65 (1H, s, H-12) and then to the H-1 ring junction proton. These COSY couplings suggested the presence of an unsaturated six-membered ring system. The existence, in the HMBC spectrum, of three bond correlations from the H-12 olefinic proton to the C-1 ring junction carbon atom, the aldehyde carbonyl and the C-10 vinylic carbon, together with three bond correlations from the H-10 olefinic proton to the C-1 ring junction carbon atom, the C-16 carbinol carbon and to the C-12 vinylic carbon atom, confirmed the six-membered ring structure and placed the aldehyde functionality at C-13 (δ 141) and the side chain at C-11 (δ 130). This completed the structure determination of the rearrangement product [139].

129
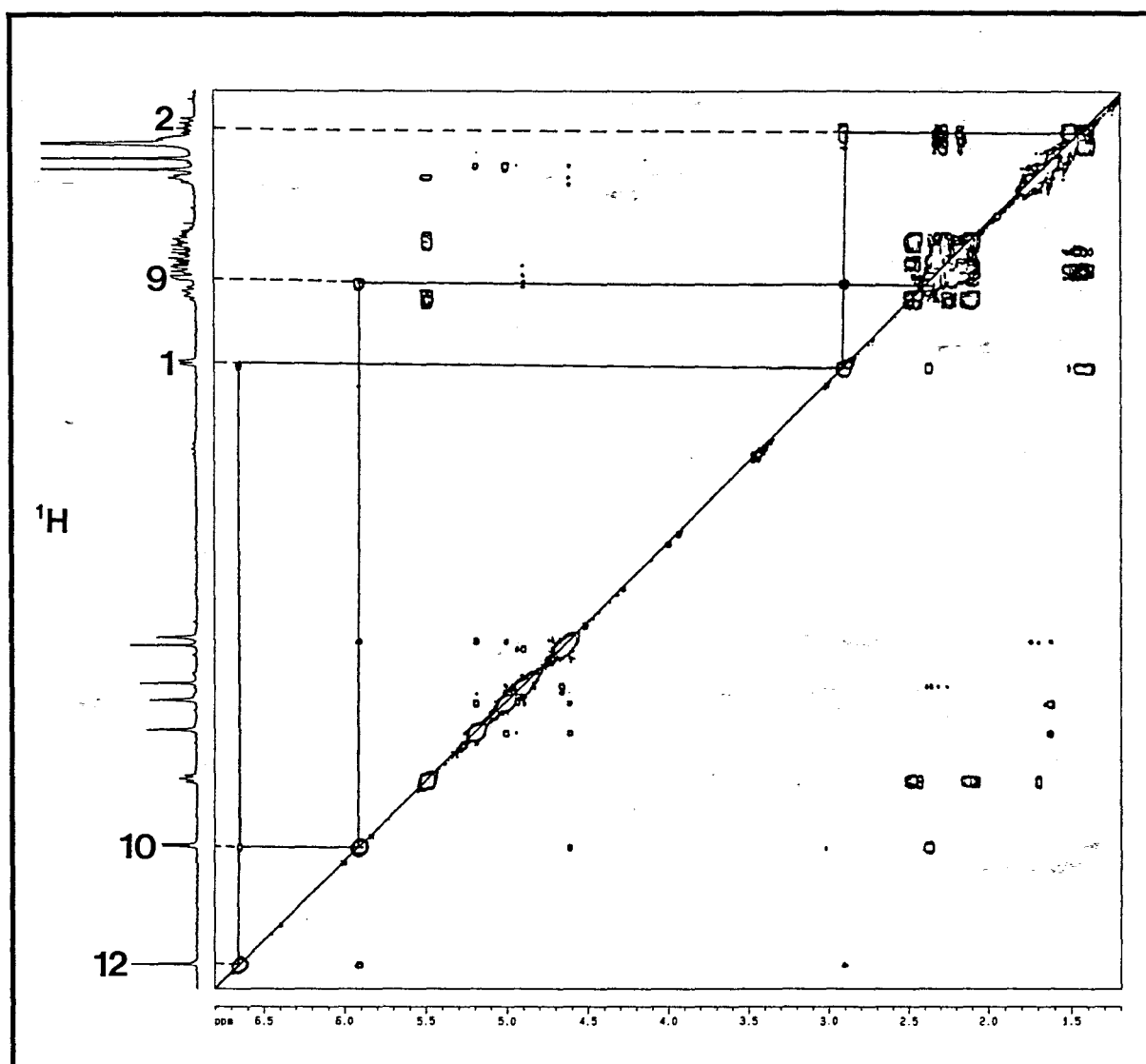
FIGURE 5.13 COSY NMR (400 MHz, CDCl₃) spectrum of compound [139].

A literature survey revealed the recently reported isolation, by Kashman and co-workers[187], of xeniafaraunol A [140], along with the known xeniolide A and B xenicane diterpenes from the Red Sea soft coral *Xenia faraunensis*. Xeniafaraunol A was described as being a novel compound with an unprecedented diterpene skeleton. Coincidently, the rearrangement product [139] contains the same bicyclo[7.4.0]tridecane carbon skeleton as xeniafaraunol A [140]. A comparison of the ¹H and ¹³C NMR data of [139] and [140] is contained in TABLE 5.7. The fact that xeniolide diterpenes were isolated from the same soft coral that yielded [140] suggests

a biosynthetic link between xenicane diterpene compounds and compounds with this new skeleton. The following mechanism (**SCHEME 5.4**) is proposed for the formation of the rearrangement product **[139]** from zahavin A **[16]**.
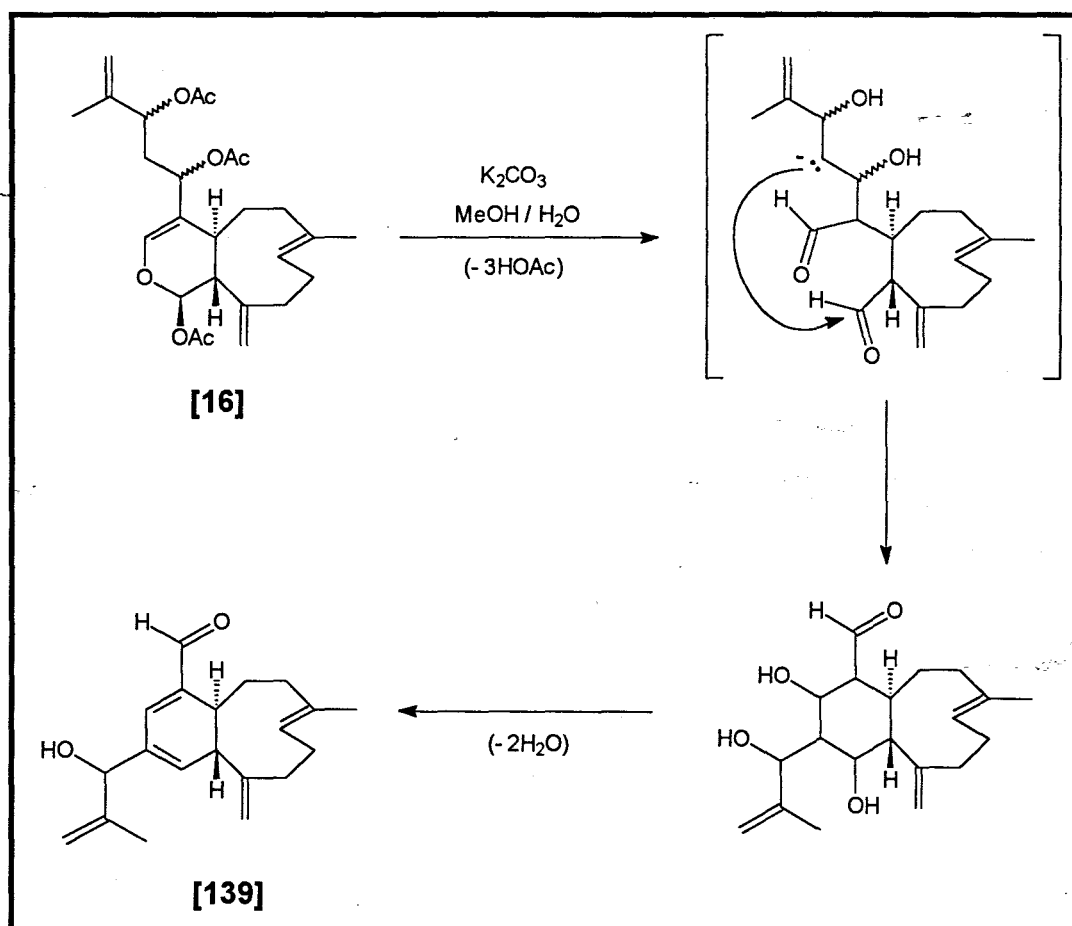
SCHEME 5.4 Mechanism proposed for the formation of the rearrangement product **[139]** which resulted from the saponification of zahavin A **[16]**.

TABLE 5.7 ^1H and ^{13}C NMR data for compounds **[139]** and **[140]**.

	[139]		[140]	
	^{13}C	^1H	^{13}C	^1H
1	37.7 d	2.90 m	36.6 d	2.80 m
2, 2'	34.4 t	1.51 m	36.3 t	1.65, 1.20 m
3, 3'	39.8 t	2.30, 2.17 m	40.6 t	2.72, 2.10 dt
4	136.1 s		136.0 s	
5	125.0 d	5.49 t (8.3)	125.1 d	5.40 dd
6, 6'	25.3 t	2.45, 2.17 m	28.5 t	2.30, 1.88 m
7, 7'	35.8 t	2.30 m	32.2 t	2.06, 1.62 m
8	156.3 s		146.9 s	
9	49.5 d	2.36 m	61.0 d	1.87 t (9)
10	134.3 d	5.91 d (5.3)	73.3 d	3.03 t (9)
11	130.2 s		44.0 d	3.06 m
12	139.8 d	6.65 s	150.1 d	5.90 s
13	140.9 s		146.0 s	
14	16.9 q	1.69 s	18.4 q	1.53 s
15, 15'	114.3 t	4.90, 4.66 s	118.0 t	4.80 s
16	77.2 d	4.61 s	125.6 d	4.90 d (7.5)
17	145.1 s		135.6 s	
18, 18'	111.6 t	5.19, 5.00 s	18.5 q	1.60 s
19	18.7 q	1.63 s	26.0 q	1.67 s
20	193.6 d	9.43 s	192.0 d	9.26 s

Spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C in $CDCl_3$ for **[139]** and at 500 MHz for ^1H and 125 MHz for ^{13}C in C_6D_6 for **[140]**. Chemical shifts (δ) are quoted in ppm. Coupling constants in Hz are in parentheses.
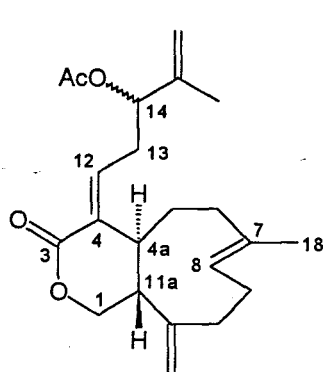
[124]
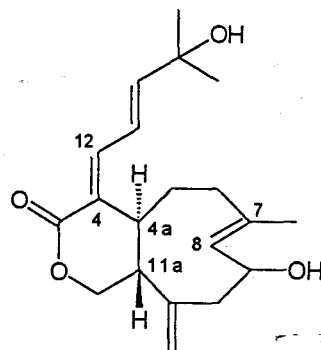
[105]
[126] $(Z) - \Delta^{4(12)}$

Overlapping resonances in the ^{1}H NMR spectrum of **[124]** did not permit unambiguous assignment of the relative stereochemistry at the site of ring fusion in xeniolide C **[124]**. However, based on the comparible $J_{1,11a}$ coupling between **[124]** and xeniolide A **[105]**[147] and isoxeniolide A **[126]**[148] it was presumed that these compounds have the same ring junction configuration. As for the configuration of the double bonds, an E-configuration was assigned to the Δ^7 double bond based on the ^{13}C NMR chemical shift for the Me-18 functionality at δ 18.5 (in a Z-configuration Me-18 resonates at δ 22-25)[147,149]. The $\Delta^{4(12)}$ double bond was tentatively assigned an E-configuration based on NOEDS experiments. The enhancement of the H_2-13 proton signals upon irradiation of H-4a and *vice versa* suggested the spatial closeness expected for an E-configuration. The stereochemistry of the acetoxy group at C-14 in **[124]** remains unassigned.

133

5.4 BIOLOGICAL ACTIVITY

Although the polyoxygenated sterol **[135]** isolated from the black coral *Antipathes subpinnata* was reported to be lethal toward the brine shrimp *(Artemia salina)*[185], this activity could not be reproduced with the identical compound derived from the reduction of **[121]** with $LiAlH_4$. In addition, neither the natural product **[121]** nor **[122]**, both isolated from *Alcyonium* species A, showed any toxicity towards *A. salina* when screened in the Microwell Cytotoxicity Assay[188].

In chapter four (section 4.4) the anti-inflammatory activity of the tsitsixenicins A **[100]** - D **[103]** was discussed. This activity was in the form of superoxide inhibition in rabbit and human neutrophils. Because of this positive activity, the xenicane diterpenes **[110]**, **[16]** and **[123]**, isolated in this chapter, were also screened for anti-inflammatory activity. The assay used, similar to that described in the previous chapter, involved measuring the inhibition of release of superoxide from rabbit blood neutrophils upon stimulation with the chemotactic factor FMPL. It is evident from the results (**TABLE 5.8**) that compounds **[110]**, **[16]** and **[123]** all show significant inhibition of superoxide release which is indicative of anti-inflammatory actvity.

TABLE 5.8 Effect of the xenicane diterpenes **[110]**, **[16]** and **[123]** on superoxide production in rabbit neutrophils at a concentration of 20 µg mL^{-1}.

Sample No.	V_{MAX}	% DMSO (0.2%) control	% Stim / Inhibit
Control	1.11	112.1	12.1
SOD	0.00	0.0	-100.0
0.2% DMSO	0.99	100.0	0.0
[110]	0.800	5.1	-91.9
[16]	0.021	2.1	-97.9
[123]	0.097	9.5	-90.2

Incubated at RT for 10 min and 37 °C for 5 min prior to stimulation with 1 µM FMLP.

Compounds **[110]**, **[16]** and **[123]** were also screened for antithrombotic activity. This was achieved by determining the extent to which the xenicane compounds prevented platelet aggregation caused by platelet activating factor (PAF). PAF is a local mediator of thrombus formation which is involved, particularly in the arterial system, in the pathogenesis of atherosclerosis. Compounds that inhibit platelet aggregation and therefore prevent the formation of blood clots have direct implication in the treatment of thrombosis and associated cardiovascular diseases[189]. The effect of compounds **[110]**, **[16]** and **[123]** on PAF-induced aggregation of rabbit platelets was determined using two different concentrations of PAF (**FIGURE 5.14**).

Although the results from the above two assays[†] clearly indicate that compounds **[110]**, **[16]** and **[123]** have good anti-inflammatory and antithrombotic activity, they must be interpreted with caution, and further studies at reduced concentration as well as cytotoxicity testing are necessary to assess more completely the anti-inflammatory and antithrombotic properties of these molecules.

---

† Assays courtesy of InflaZyme Pharmaceuticals Limited, Vancouver, Canada.

% Inhibition of Response to 0.1 nM PAF

% Inhibition of Response to 0.25 nM PAF

Compound [110]   Compound [16]   Compound [123]

	Conc. of compound	[110]	[16]	[123]
% Inhibition of Response to 0.1 nM PAF				
(1)	40 μg mL^{-1}	97.9	79.6	98.0
(2)	4 μg mL^{-1}	100.0	100.0	100.0
(3)	0.4 μg mL^{-1}	18.4	10.3	14.3
% Inhibition of Response to 0.25 nM PAF				
(4)	40 μg mL^{-1}	93.5	89.1	100.0
(5)	4 μg mL^{-1}	35.5	45.2	85.9
(6)	0.4 μg mL^{-1}	-	-	-
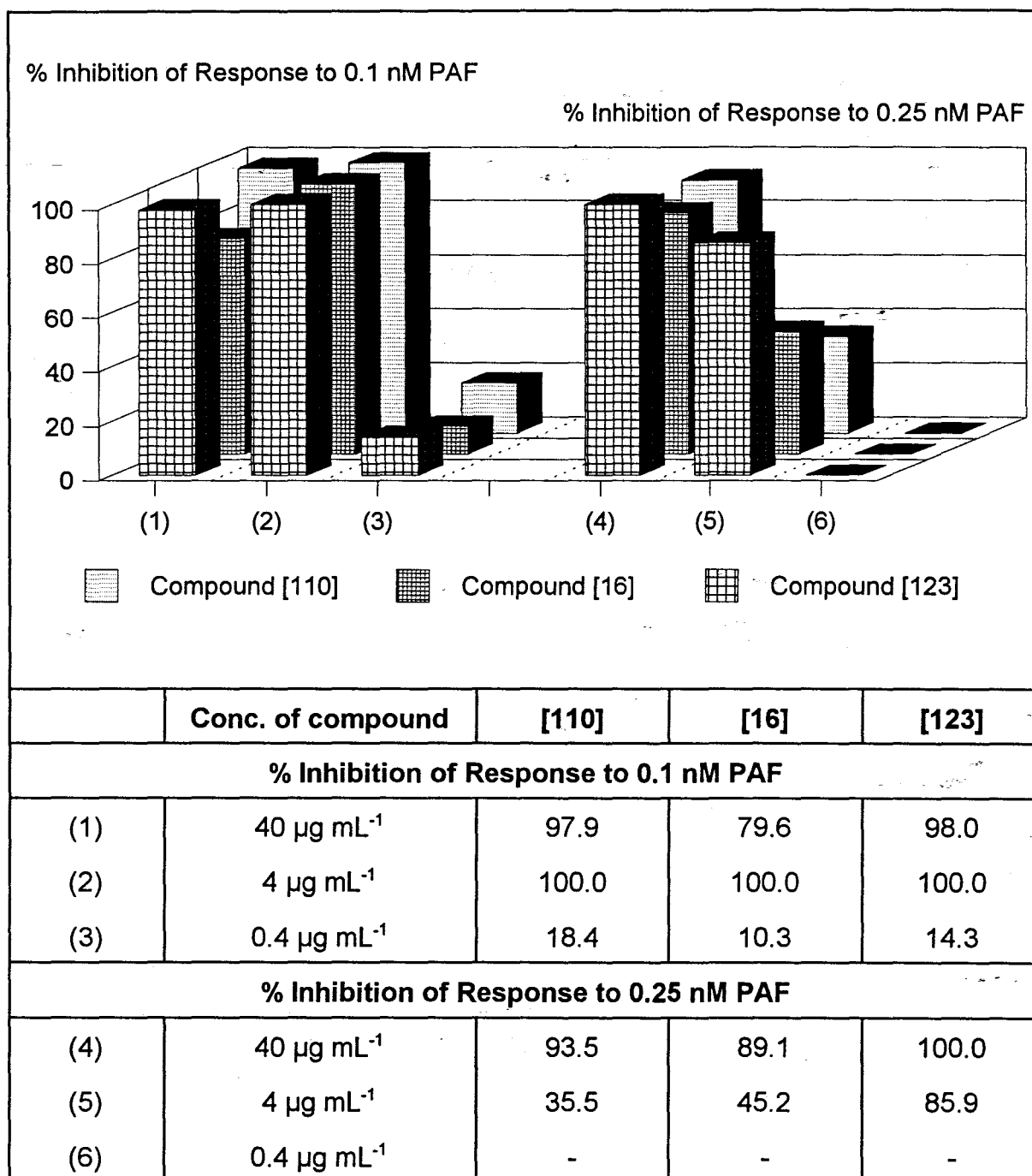
FIGURE 5.14 Effect of the xenicane diterpenes [110], [16] and [123] on PAF-induced aggregation of rabbit platelets.

CHAPTER SIX

SESQUITERPENE HYDROQUINONES FROM THE SOUTH AFRICAN SOFT CORAL *ALCYONIUM FAURI*

6.1   INTRODUCTION

The anti-inflammatory activity of the valdivones, isolated from the soft coral Alcyonium valdiviae[60], and the xenicane diterpenes, isolated from the soft corals Capnella thyrsoidea[84] and Alcyonium species B (see chapter five), has shown that South African soft corals are a good source of potential anti-inflammatory agents. A continuation of the search for new pharmaceuticals from South African Alcyonacea led to the chemical examination of the endemic southern African soft coral Alcyonium fauri. This is one of the most common and conspicuous soft corals occurring in the rocky intertidal and shallow sublittoral zones along the South African coast line[59]. The colour of the A. fauri colonies is extremely variable. They are most often vivid purple but can be pink, white, golden yellow, orange, grey or brown[59]. The chemical investigation of the EtOAc extract of bright pink specimens of A. fauri resulted in the isolation of three new sequiterpene hydroquinones, rietone [141], 8'-acetoxyrietone [142] and 8'-desoxyrietone [143][190]. Rietone exhibited moderate activity in the NCI's in vitro anti-HIV bioassays.
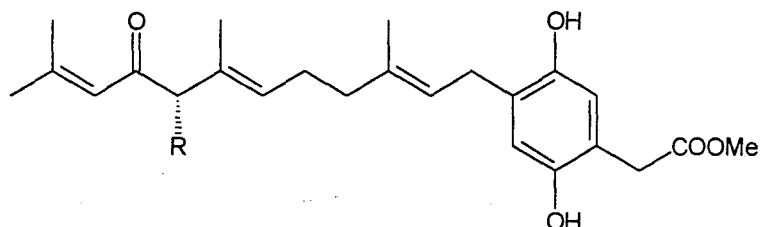
[141] R = OH
[142] R = OAc
[143] R = H

Marine brown algae and sponges have yielded a variety of metabolites based on a mixed sesquiterpene and quinone or hydroquinone biosynthesis. Examples include structures in which the sesquiterpene unit exists in an acyclic, monocyclic, bicyclic or tricyclic form and also in which there are varied degrees of oxygenation and substitution around the aromatic ring.

[144] $\Delta^{8\,(1\,3)}$
[145] Δ^7

[146]

[147]

[148]
[151] 8-epimer

[149]

[150]
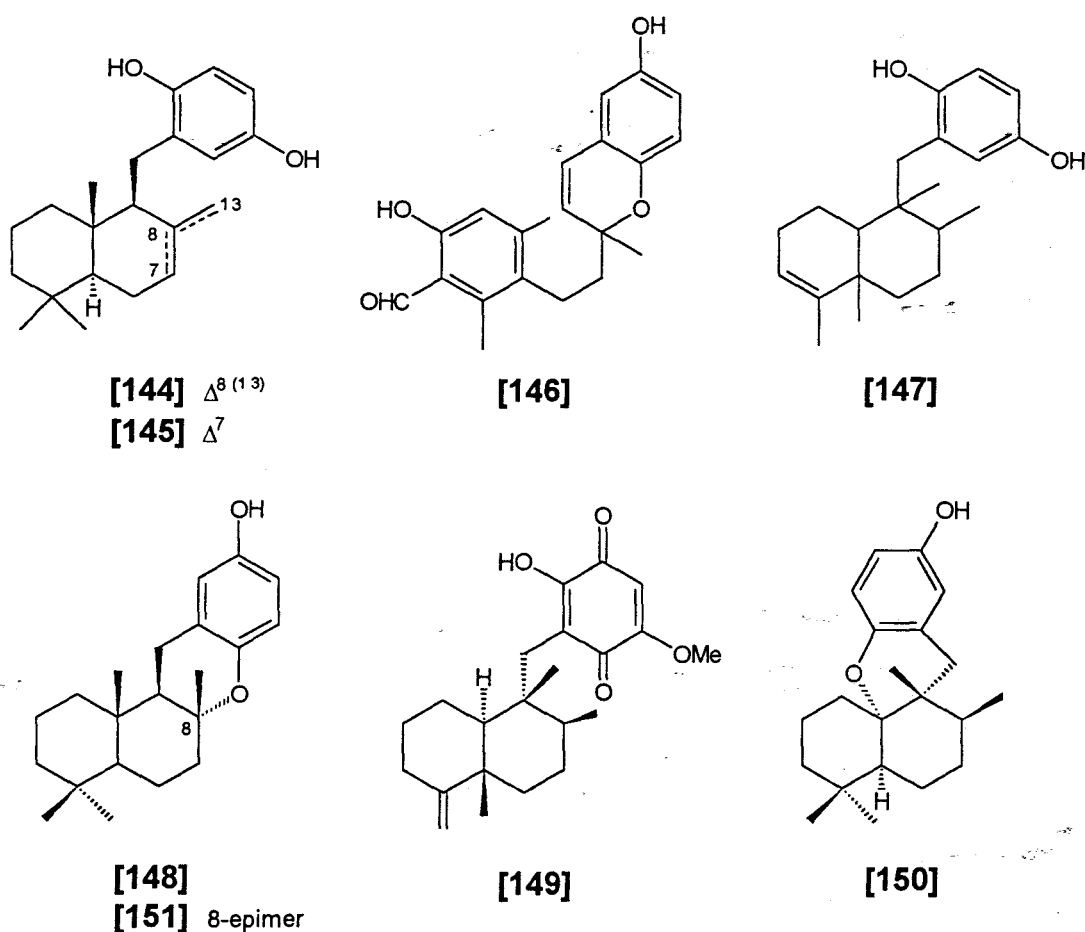
During the 1970's a number of sesquiterpene hydroquinones were isolated. Coupling of a hydroquinone functionality to an acyclic farnesyl moiety followed by cyclisation of the latter to give a rearranged drimane skeleton constitutes a plausible biosynthetic route to these sequiterpene hydroquinones. Examples include zonarol **[144]** and isozonarol **[145]** from the brown seaweed *Dictyopteris zonarioides*[191], the paniceins A, B$_1$, B$_2$ **[146]**, B$_3$ and C from the marine sponge *Halichondria panicea*[192], avarol **[147]** and its corresponding quinone, avarone, from the sponge *Disidea avara*[193], chromazonarol **[148]** and isochromazonarol from the brown seaweed *Dictyopteris undulata*[194], ilimaquinone **[149]** from a marine sponge tentatively identified as *Hippiospongia metachromia*[195], and aureol **[150]** and 8-epichromazonarol **[151]** from the marine sponge *Smenospongia aurea*[196].

There was considerable renewed interest in these sesquiterpene hydroquinone compounds in the late 1980's with the discovery that avarol [147] and the quinone, avarone, inhibited replication of the human immunodeficiency virus (HIV) believed to be responsible for acquired immune deficiency syndrome (AIDS)[197]. Further studies of the inhibitory effects of avarol, avarone, ilimaquinone [149] and derivatives of these compounds on HIV type1 reverse transcriptase have identified their active sites of inhibition [198,199] and this could lead to the rational design of further potential anti-HIV drugs.
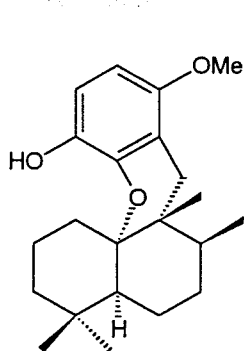
[152]
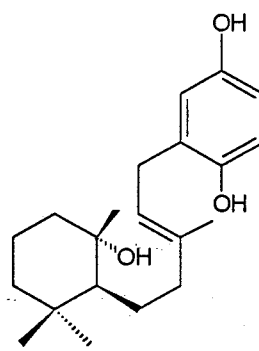
[153]
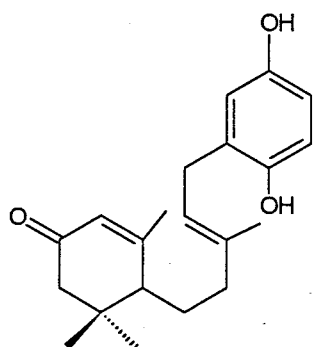
[154]
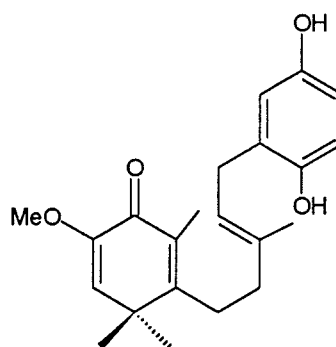
[155]

Recently, the number of sesquiterpene hydroquinones isolated from marine sponges and algae has grown steadily. An investigation of the marine sponge *Strongylophora hartmani* yielded strongylin A [152][200]. This compound was active in *in vitro* assays against the P-388 tumour cell line and the influenza strain PR-8. A new sesquiterpene linked to a hydroquinone, fulvanin 2 [153], together with four known paniceins have been isolated from the Mediterranean sponge *Reniera fulva*[201]. A further eight new compounds, six of them of the panicein class (as represented by [146]), and

140

two new cyclohexenones, renierin A [154] and B [155], together with seven related known compounds were isolated from the sponge Reniera mucosa[202]. In this report it was proposed that the cyclohexenones [154] and [155] might be biosynthetic intermediates between the hydroquinone-farnesyl precursors and the paniceins.

[156]   [157]   [158]
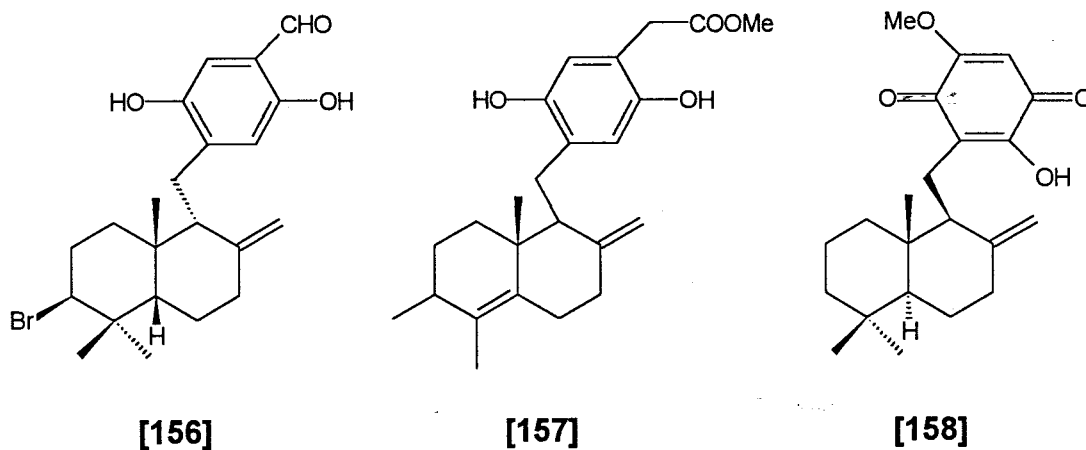
Three new sesquiterpene hydroquinones peyssonol A [156] and B [157] and hyatellaquinone [158] have been isolated from the alga Peyssonnelia species and the sponge Hyatella intestinalis, respectively[203]. Certain of these compounds, not disclosed in the report, were found, in a preliminary test, to inhibit HIV reverse transcriptase.
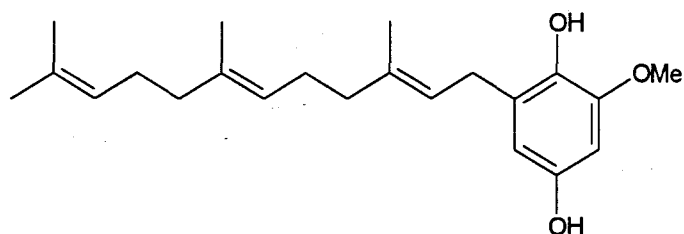
[159]

Capon and Bonny identified a new acyclic hydroquinone sesquiterpene [159], together with the corresponding quinone, from the southern Australian marine sponge Thorecta choanoides[204]. It was reported that the 2,3,5-oxygenation pattern around the aromatic ring in [159] was rare. Although hydroquinone sesquiterpene compounds have been reported to inhibit both bacterial growth and HIV replication, neither [159] nor its quinone analogue demonstrated such activity.

De Rosa and co-workers[205] screened twenty three hydroquinone and quinone derivatives for antimicrobial activity and brine shrimp and fish lethalities to establish relevant structure-activity relationships. From these data it was concluded that the optimum length of the side chain in the group of terpenoid - hydroquinones/quinones assayed ranged from five to fifteen carbon atoms.

[160] Me$_{12}$ = α
[161] Me$_{12}$ = β

[162]
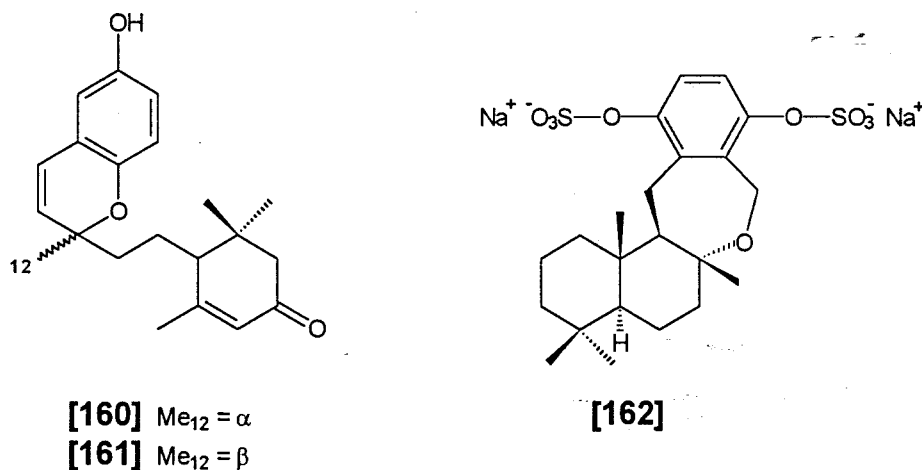
Crews et al. isolated cyclorenierins A [160] and B [161], which are closely related to panicein A$_2$ and renierin A, as an inseparable mixture from the marine sponge Haliclona species[206]. Recently a new disulphated sesquiterpene hydroquinone [162] has been isolated from a deep water collection of the marine sponge Siphonodictyon coralliphagum[207]. This compound inhibited the binding of leukotriene B$_4$ to intact human neutrophils and as a consequence may have possible applications for the treatment of inflammatory and allergic diseases.

From this introduction it is clearly seen that metabolites arising from mixed sesquiterpene and hydroquinone or quinone biogenesis are common in sponges and marine algae, particularly with a cyclisation of the sesquiterpene side chain. On the other hand, the isolation and structure determination of the sesquiterpene hydroquinones, described in section 6.2 and 6.3 of this chapter, is the first reported occurrence of these metabolites in a marine soft coral. In addition to containing the sesquiterpene side chain in the less common acyclic form, rietone and its analogues exhibit a novel oxygenation pattern in this side chain.

6.2 COLLECTION, EXTRACTION AND ISOLATION
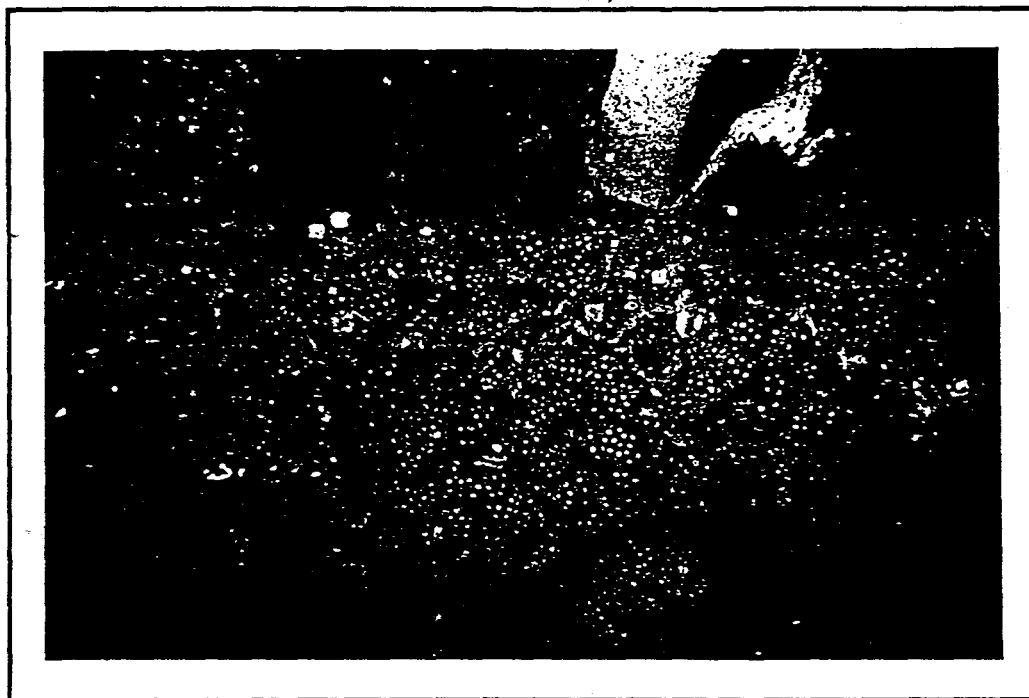
FIGURE 6.1 The bright pink soft coral, *Alcyonium fauri*, collected from Riet Point near Port Alfred.

A sample of the bright pink colour variant of the soft coral *Alcyonium fauri* was collected in September 1994 using SCUBA at a depth of -15 m at Riet Point. This reef is located near Port Alfred on the warm temperate south east coast of South Africa. The EtOAc extract of the freeze-dried soft coral (dry weight 380 g) was concentrated to give a dark red oil (5.8 g). TLC and ^{1}H NMR spectroscopy suggested that this extract consisted predominantly of one compound. Fractionation of the extract was performed according to **SCHEME 6.1** and resulted in the isolation of the new sesquiterpene hydroquinone rietone [141] in high yield along with the related minor compounds 8'-acetoxyrietone [142] and 8'-desoxyrietone [143].
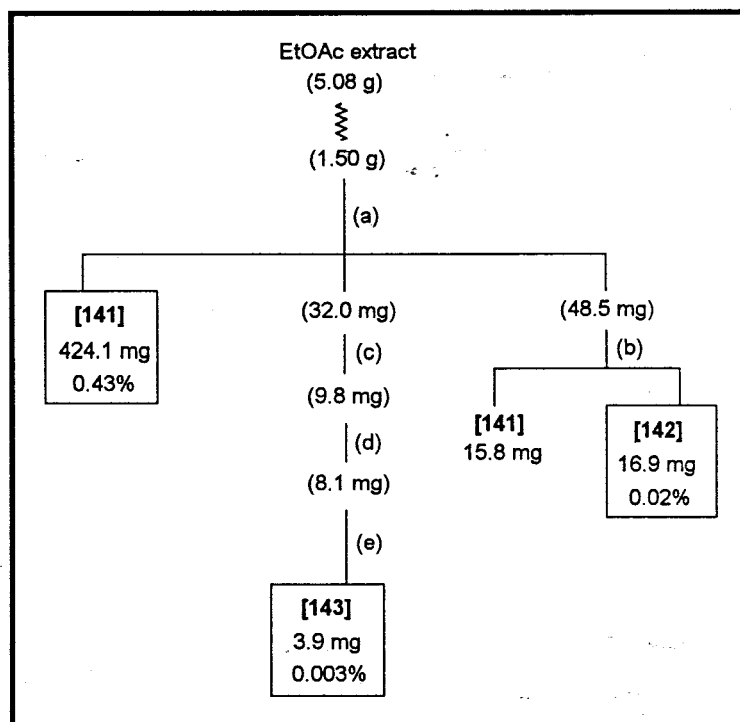
SCHEME 6.1 Chromatographic procedure for the isolation of compounds **[141]** - **[143]**. (a) flash chromatography (silica, 230-400 mesh, 150 g, elution with 33% EtOAc/hexane). (b) HPLC (silica, 10 μ, Whatman, 35% EtOAc/hexane, 4 mL min^{-1}). (c) HPLC (silica, 10 μ, Whatman, 35% EtOAc/hexane, 4 mL min^{-1}). (d) HPLC (silica, 10 μ, Whatman, 33% EtOAc/hexane, 4 mL min^{-1}). (e) HPLC (C18, 10 μ, Phenomenex, 40% H$_2$O/MeCN, 4 mL min^{-1}).

6.3 STRUCTURE DETERMINATION AND STEREOCHEMISTRY

6.3.1 Structure determination of the sesquiterpene hydroquinones [141] - [143].
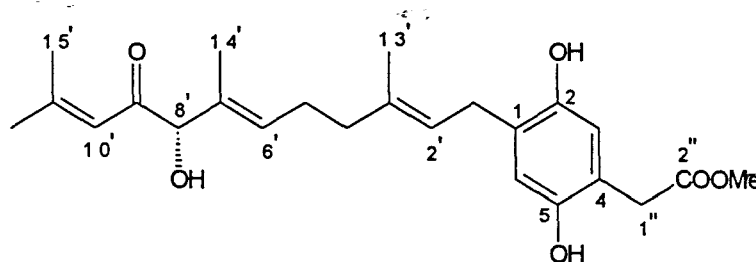
[141]

HREIMS established the molecular formula of rietone [141] as $C_{24}H_{32}O_6$ (m/z 416.2188, Δmmu -11). Rietone displayed UV (297, 235 and 209 nm) and IR (1510, 1620 and 1680 cm^{-1}) absorptions consistent with a substituted aromatic ring and an $\alpha\beta$-unsaturated carbonyl moiety. Other IR absorptions at 3340 and 1730 cm^{-1} indicated additional hydroxyl and ester functionalities respectively. The twelve well resolved aromatic and vinylic resonances (δ 116-161) together with the ester and $\alpha\beta$-unsaturated carbonyl signals (δ 173 and 198 respectively) in the ^{13}C NMR spectrum of rietone [141] (FIGURE 6.2) accounted for eight of the nine degrees of unsaturation implied by the molecular formula and required [141] to be monocyclic.
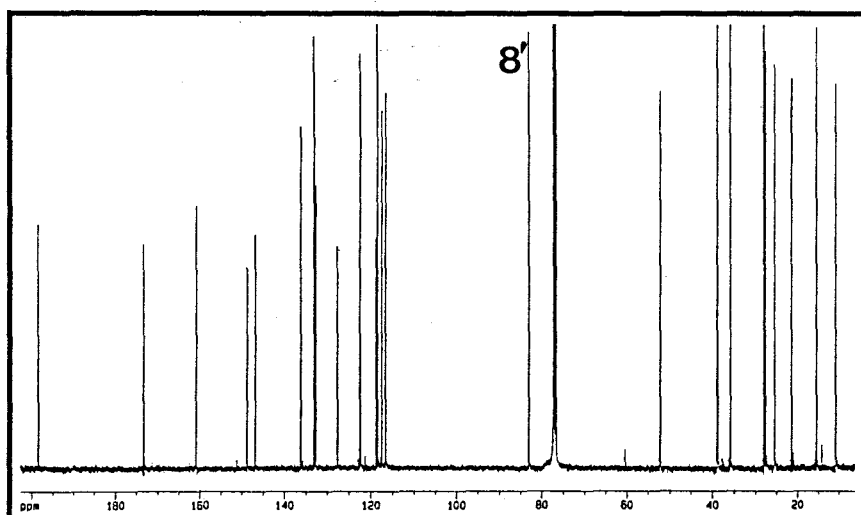
FIGURE 6.2 ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of rietone [141].

The HMQC NMR spectrum of rietone (**FIGURE 6.4**) exhibited correlations in accordance with four olefinic methyl groups (δ_H 1.43, 1.65, 1.91 and 2.21), one methoxy group (δ_H 3.68), two benzylic methylene groups (δ_H 3.25 and 3.55), three vinylic protons (δ_H 5.28, 5.59 and 6.12), one oxymethine proton (δ_H 4.52) and two aromatic protons (δ_H 6.55 and 6.58). The ^{1}H and ^{13}C NMR chemical shifts for the aromatic ring component of [141] are consistent with the values reported for the 1,2,4,5-tetrasubstituted *para*hydroquinone ring system, including the methyl ethanoate side chain, of peyssonol B [157], isolated by Kashman and co-workers from the Red Sea marine alga *Peyssonnelia* species[203].
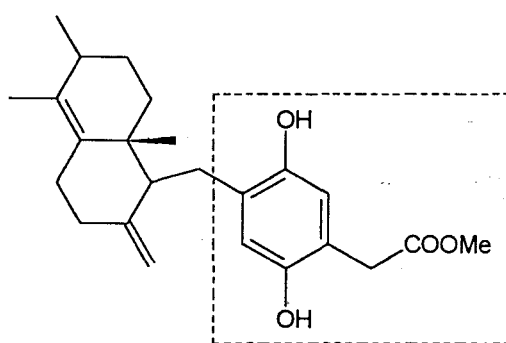
[157]

The position of the methyl ethanoate side chain relative to the phenolic hydroxyl groups and the unsaturated acyclic moiety in the aromatic substructure of rietone was unequivocally established from the two and three bond correlations (**FIGURE 6.3**) observed in the HMBC NMR spectrum of [141].
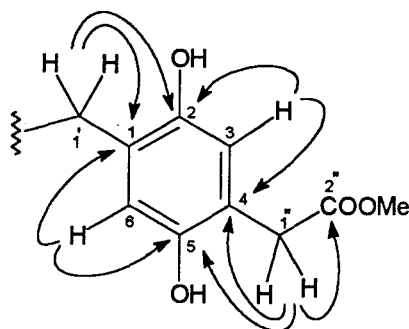
FIGURE 6.3 Selected two and three bond correlations observed in the HMBC NMR spectrum of [141].

The above data established the hydroquinone moiety as the monocyclic portion of **[141]**. This left fifteen carbon and two oxygen atoms unaccounted for suggesting the presence of an acyclic oxygenated farnesyl side chain. The COSY NMR spectrum of rietone (**FIGURE 6.5**) revealed two prominent coupling sequences. The first sequence involved coupling from the benzylic methylene protons at δ 3.25 (2H, dd, J = 6.7, 7.5 Hz, H-1') to the vinylic proton at δ 5.28 (1H, t, J = 7.1 Hz, H-2') and, by long range coupling, to the olefinic methyl protons at δ 1.65 (3H, s, H-13'). The second sequence indicated a contiguous coupling from the methylene protons at δ 2.20 (2H, m, H-4'), to the adjacent methylene protons at δ 2.39 and 2.17 (2H, m, H-5'), then to the vinylic proton at δ 5.59 (1H, dd, J = 7.9, 9.1 Hz, H-6') and finally, by long range coupling, to the olefinic methyl protons at δ 1.43 (3H, s, H-14'). These coupled systems, along with long range ^{1}H-^{13}C correlations, supported an oxygenated farnesyl side chain in which the oxygen functionalities were confined to the terminal prenyl unit of this side chain.

A sharp exchangeable doublet at δ 4.69 (1H, d, J = 4.1 Hz, OH-8'), with no correlating carbon atom in the HMQC NMR spectrum of **[141]**, but strongly coupled to an oxymethine doublet at δ 4.52 (1H, d, J = 4.1 Hz, H-8') in the COSY NMR spectrum (**FIGURE 6.5**) established the existence of a secondary alcohol. Prominent two bond correlations between the oxymethine proton and the carbon atoms at δ 132 (C-7') and δ 198 (C-9') in the HMBC NMR spectrum placed the secondary alcohol group at C-8', between a terminal αβ-unsaturated carbonyl functionality and the Δ^6 double bond. This completed the assignment of rietone **[141]**. The NMR spectral data for rietone are presented in **TABLE 6.1** and the stereochemical assignment of the 8'-OH functionality is discussed in section 6.3.2.
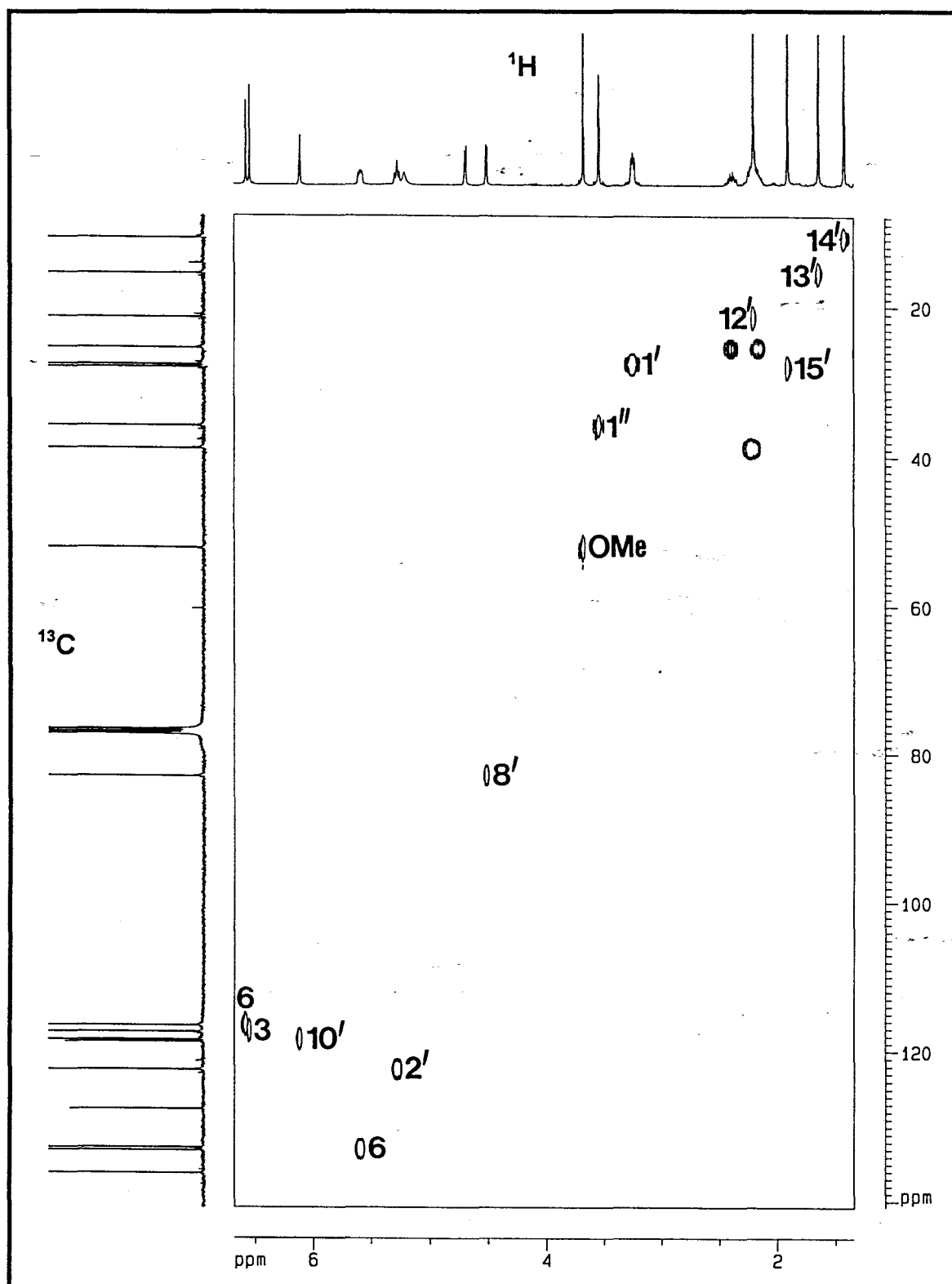
FIGURE 6.4 HMQC NMR (400 MHz, $CDCl_3$) spectrum of **[141]**.
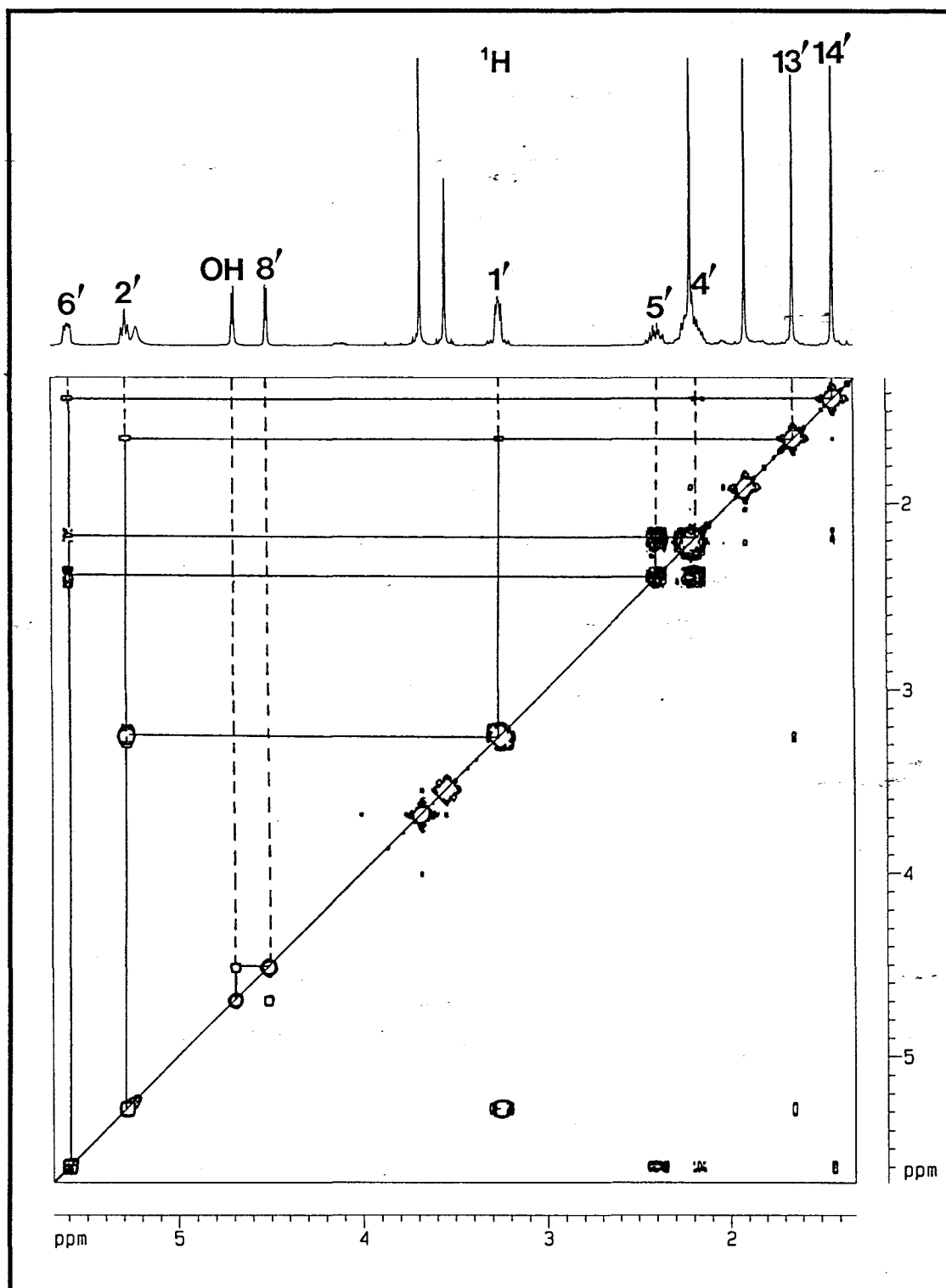
FIGURE 6.5 COSY NMR (400 MHz, CDCl$_3$) spectrum of **[141]**.

TABLE 6.1   NMR spectral data for rietone **[141]**.

	^{13}C	^1H	COSY to ^1H	HMBC to ^{13}C
1	127.6 s			
2	146.9 s			
3	117.4 d	6.55 s	*1"	1, 5, 1"
4	118.6 s			
5	148.6 s			
6	116.4 d	6.58 s	*1'	2, 4, 1'
1'	27.7 t	3.25 dd (6.7, 7.5)	*6, 2', *13'	1, 2, 6, 2', 3'
2'	122.5 d	5.28 t (7.1)	1', *13'	1', 4', 13'
3'	136.1 s			
4'	38.8 t	2.20 m	5'	
5'	25.5 t	2.39 m, 2.41 m	4', 6'	4', 6', 7'
6'	133.1 d	5.59 dd (7.9, 9.1)	5', *14'	
7'	132.6 s			
8'	83.0 d	4.52 d (4.1)	8'-OH	6', 7', 9', 14'
9'	198.4 s			
10'	118.5 d	6.12 s	*12', *15'	9', 12', 15'
11'	160.8 s			
12'	21.4 q	2.21 s	*10'	10', 11', 15'
13'	15.5 q	1.65 s	*1', *2'	2', 3', 4'
14'	10.8 q	1.43 s	*6'	6', 7', 8'
15'	28.0 q	1.91 s	*10'	10', 11', 12'
1"	35.8 t	3.55 s	*3	3, 4, 5, 2"
2"	173.4 s			
2-OH		5.22 s		
5-OH		7.51 s		4, 5, 6
8'-OH		4.69 d (4.1)	8'	8', 9'
OMe	52.2 q	3.68 s		2"

* long-range COSY coupling.

Spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C in $CDCl_3$ with chemical shifts (δ) in ppm. Coupling constants in Hz are in parentheses.
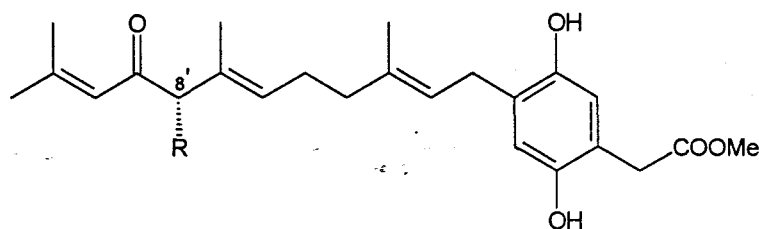
[141] R = OH
[142] R = OAc
[143] R = H

The molecular formula of **[142]** was determined from HREIMS data as $C_{26}H_{34}O_7$ (m/z 458.2294, Δmmu -9) and differed from that of rietone **[141]** by the presence of a single acetate unit in **[142]**. This was corroborated by the acetate methyl singlet at δ 2.11 and the extra ester carbonyl resonance at δ 171 in the ^1H and ^{13}C NMR spectra of **[142]**, respectively. The position of the acetate group at C-8' was confirmed firstly, by the downfield chemical shift of H-8' (δ 5.40) in **[142]** relative to **[141]** (δ 4.52), and secondly, by a correlation between H-8' and the acetate carbonyl in the HMBC NMR spectrum of **[142]**. The stereochemical assignment of the 8'-acetoxy functionality in **[142]** is discussed in section 6.3.2.

The molecular ion at m/z 400.2265 (Δmmu +15) in the HREIMS spectrum of **[143]** is compatible with the molecular formula $C_{24}H_{32}O_5$. This molecular formula corresponds to the loss of a single oxygen atom from rietone **[141]**. The replacement of the C-8' oxymethine carbon resonance (**FIGURE 6.2**) with a deshielded methylene signal at δ 55 in the ^{13}C NMR spectrum of **[143]** (**FIGURE 6.6**), coupled with the disappearance of optical activity, supported the loss of the chiral oxygen functional group in this compound. The ^1H and ^{13}C NMR data for 8'-acetoxyrietone **[142]** and 8'-desoxyrietone **[143]** are presented in **TABLE 6.2**.
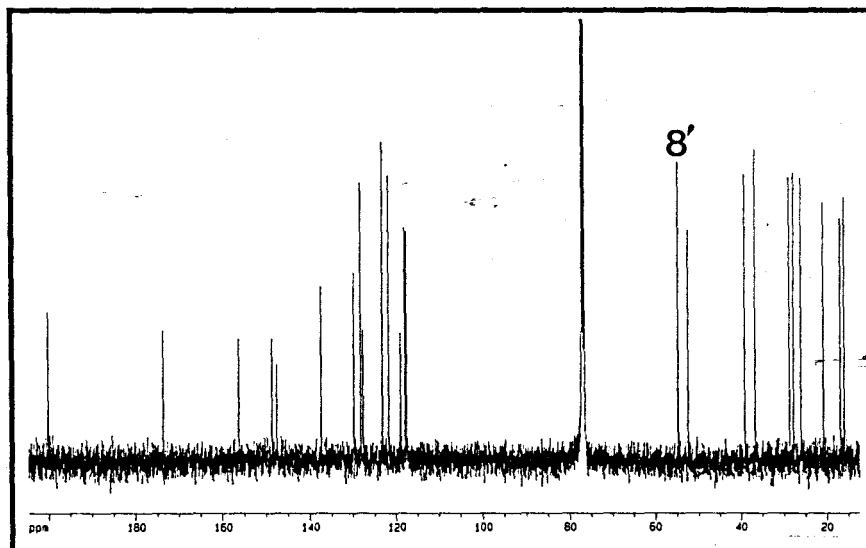
FIGURE 6.6 ^{13}C NMR (100 MHz, CDCl$_3$) spectrum of 8'-desoxyrietone [143].

6.3.2 Stereochemistry of rietone [141] and 8'-acetoxyrietone [142].

Mosher's method for the determination of the absolute stereochemistry in secondary alcohols has found wide application in marine natural products[208-212]. This method requires the formation of diastereomers of the compound containing the chiral secondary alcohol, followed by ^1H NMR analysis of the diastereomers. In order to simplify the interpretation of the NMR data in the application of Mosher's method to rietone it was desirable to generate the mono-ester of rietone. This was achieved by first converting rietone to the corresponding quinone. Mild oxidation of rietone [141] with silver oxide[213] followed by normal phase HPLC of the oxidation products gave unchanged [141] and the quinone [163] as a red oil in low yield (20%).

[163]

152
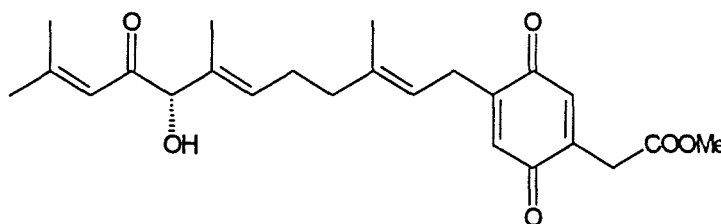
TABLE 6.2 ^{1}H and ^{13}C NMR data for compounds **[142]** and **[143]**.

	[142] ^{13}C	[142] ^{1}H	[143] ^{13}C	[143] ^{1}H
1	127.7 s		127.8 s	
2	147.5 s		147.6 s	
3	117.6 d	6.57 s	117.7 s	6.57 s
4	119.0 s		119.0 s	
5	148.6 s		148.7 s	
6	117.9 d	6.65 s	118.1 d	6.68 s
1'	28.5 t	3.27 d (7.2)	28.8 t	3.27 d (7.2)
2'	122.4 d	5.28 t (7.3)	121.8 d	5.28 t (7.2)
3'	136.6 s		137.5 s	
4'	38.6 t	2.14 m	39.1 t	2.12 m
5'	25.8 t	2.22 m	26.0 t	2.18 m
6'	133.3 d	5.62 t (6.5)	128.4 d	5.18 t (6.7)
7'	129.3 s		129.9 s	
8'	84.1 d	5.40 s	54.8 t	3.04 s
9'	194.5 s		200.2 s	
10'	119.9 d	6.09 s	123.3 d	6.10 s
11'	158.4 s		156.3 s	
12'	21.2 q	2.15 s	20.8 q	2.13 s
13'	15.9 q	1.69 s	16.0 q	1.69 s
14'	13.1 q	1.62 s	16.9 q	1.56 s
15'	28.0 q	1.88 s	27.8 q	1.87 s
1"	36.5 t	3.58 s	36.7 t	3.58 s
2"	173.7 s		173.8 s	
2-OH		4.92 s		4.78 s
5-OH		7.13 s		7.19 s
8'-OH				
OMe	52.4 q	3.70 s	52.5 q	3.71 s
OAc	20.8 q	2.11 s		
	170.6 s			

Spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$ with chemical shifts (δ) quoted in ppm. Coupling constants in Hz are in parentheses.

The MTPA esters of the quinone **[163]** were prepared using the modified Mosher's method[208,210]. In this method the derivatising agent 2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) was used to convert the quinone to its corresponding (S)- and (R)-MTPA esters. Interpretation of stereochemistry in Mosher's method relies on the fact that the most stable conformation of the ester in solution is when the C_α-proton and the ester carbonyl and trifluoromethyl groups of the MTPA ester lie in the same plane and *cis* to one another (**FIGURE 6.7**)[208].
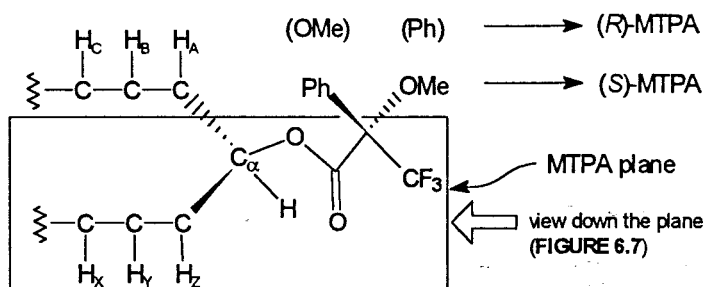
FIGURE 6.7 Representation of the MTPA plane of an MTPA ester with $H_{A,B,C}$ on the right hand side and $H_{x,y,z}$ on the left side of the plane respectively.

In this conformation the ^1H NMR signals $H_{A,B,C}$ of the (R)-MTPA ester will appear upfield relative to those of the (S)-MTPA ester due to the diamagnetic effect of the benzene ring[208]. The reverse is true for $H_{x,y,z}$. Therefore, when calculating $\Delta\delta_H$ ($\delta_{H(S)}$ - $\delta_{H(R)}$) the protons on the right side of the MTPA plane should have positive values and those on the left should have negative values (**FIGURE 6.8**). By calculating the $\Delta\delta_H$ values it is thus possible to determine the stereochemistry of the chiral secondary alcohol.
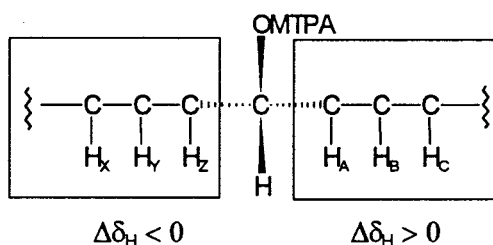
FIGURE 6.8 A view down the plane of the MTPA ester (see **FIGURE 6.7**).

Application of the MTPA determination rule[208] to the $\Delta\delta_H$ values of the proton resonances in the diastereomeric MTPA-quinone esters (**TABLE 6.3**) established negative values on the right hand side of the MTPA plane and positive values on the left hand side as represented in **FIGURE 6.9**. This demonstrated an 8'(S)-configuration for the chiral centre in the quinone **[163]** and therefore also for the hydroquinone rietone **[141]**.

TABLE 6.3 Calculation of the $\Delta\delta_H$ values from the MTPA esters of **[163]**.

¹H	δ (S) -	δ (R) =	Δδ
12'	1.8636	1.8406	+0.023
15'	2.1727	2.1531	+0.020
10'	6.1027	6.0560	+0.047
14'	1.4926	1.5579	-0.065
6'	5.6416	5.7120	-0.070
5'	2.0204	2.0697	-0.049
13'	1.5469	1.5687	-0.022
2'	5.1383	5.1632	-0.025
1'	3.0887	3.1005	-0.012

Chemical shifts (δ) quoted in ppm.
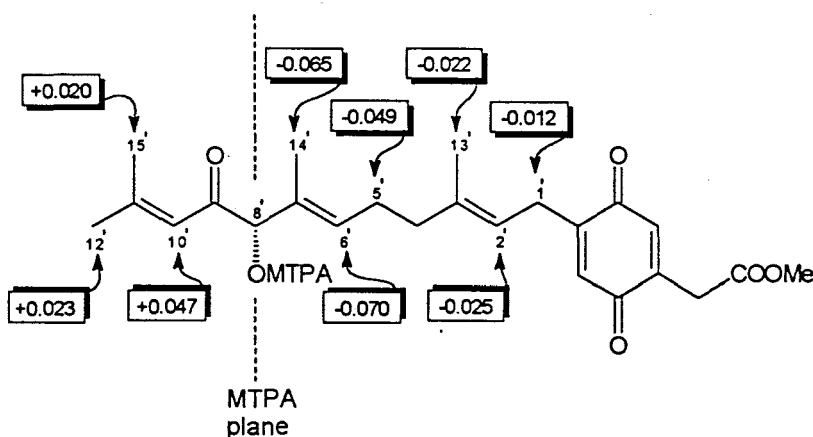
FIGURE 6.9 Determination of the absolute configuration at C-8' in the MTPA ester of **[163]** ($\Delta\delta_H$ values represented in boxes in ppm).

The shielded chemical shifts of the C-13' and C-14' olefinic methyl groups at δ 15 and δ 11, respectively, in the ^{13}C NMR spectrum of rietone [141] defined an E-geometry around the two exocyclic, asymmetrically substituted double bonds (a Z-configuration requires δ_C 22-25)[214].
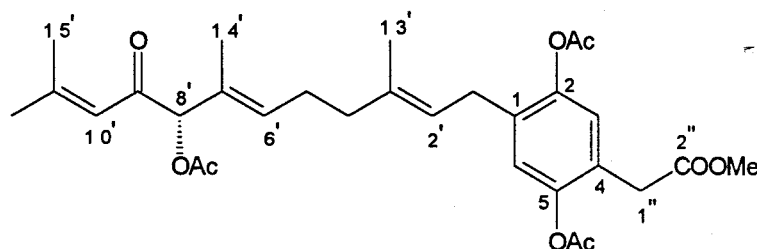
[164]

- Acetylation of 8'-acetoxyrietone [142] with Ac_2O in pyridine gave the triacetate [164] which was identical ($[\alpha]_D^{21}$, IR, ^1H NMR) to the acetylation product of rietone [141] obtained under the same conditions. These data, therefore, established an 8'(S) stereochemistry at C-8' in [142] (based on the 8'(S) stereochemistry determined for rietone [141] using Mosher's method) and an E-geometry at the $\Delta^{2'}$ and $\Delta^{6'}$ olefins.

6.4 BIOLOGICAL ACTIVITY

The anti-HIV reverse transcriptase activity reported for several sesquiterpene hydroquinone compounds[197-199,203] prompted an investigation of the anti-HIV activity of rietone [141]. With almost two million people worldwide having already progressed to full-blown AIDS and with approximately thirteen million people infected with HIV, the magnitude of the AIDS problem continues to grow[215]. Tremendous resources have focussed on the study of the etiological agent, the retrovirus HIV. The target that has been most actively pursued for the chemotherapeutic treatment of HIV is the enzyme reverse transcriptase[198,215].

Rietone was screened in the NCI's Anti-HIV Drug Testing System[†] which, rather than specifically targeting reverse transcriptase, is designed to detect agents acting at any stage of the virus reproductive cycle[216,217]. The assay procedure involves the killing of T4 lymphocytes by HIV. Compounds that interact with the virions, cells or virus gene-products to interfere with the HIV activity will protect the lymphocyte cells from cytolysis. The assay uses a tetrazolium reagent that is metabolically reduced by viable cells to yield a soluble, coloured formazan product measurable by conventional colourimetric techniques. In other words, any drug-induced suppression of the HIV cytopathic effects is detected by the generation of the soluble formazan in the surviving cells[216].

Rietone was tested in several assay protocols. A representative example of the results of one of these tests is contained in **FIGURE 6.10**. The solid line depicts the percentage of surviving HIV-infected cells treated with rietone (at the indicated concentration) relative to uninfected, untreated controls, *i.e.* the line expresses the *in vitro* anti-HIV activity of rietone. The dashed line depicts the percentage of surviving uninfected cells treated with rietone relative to the same uninfected, untreated controls, *i.e.* the line expresses the *in vitro* growth inhibitory properties of rietone.

From **FIGURE 6.10** it is evident that rietone offers a maximum protection of 67.5 per cent against HIV in the assay at a concentration of 6.33×10^{-6} M. However, at higher concentrations rietone causes cytolysis. The 50% effective concentration (EC_{50}) against HIV cytopathic effects was at a concentration of 1.23×10^{-6} M, the 50% inhibitory concentration (IC_{50}) for cell growth was at a concentration of 9.32×10^{-6} M and, therefore, the Therapeutic Index of rietone (TI = IC_{50} / EC_{50}) was calculated to be 7.55. From these results it was concluded that rietone exhibited moderate anti-HIV activity, however, its cytotoxicity mitigated against the further development of rietone as an anti-HIV agent.

† Assays courtesy of Dr D. Newman, National Cancer Institute.

National Cancer Institute
Developmental Therapeutics Program
In Vitro Testing Results

NSC: 675010-P / -1 /	Plate: 7876	Lab: 9W	SSPL: 0FPI	Assay: Primary Screen
Test Date: December 28, 1994	Solubility Ind.: 1			COMI: Rietone 1
Report Date: January 31, 1995	Cell Line: CEM-SS			Solvent: DMSO 100%

In Vitro Anti-HIV Drug Screening Results
Primary Screen

Log_{10} of Sample Concentration (Molar)
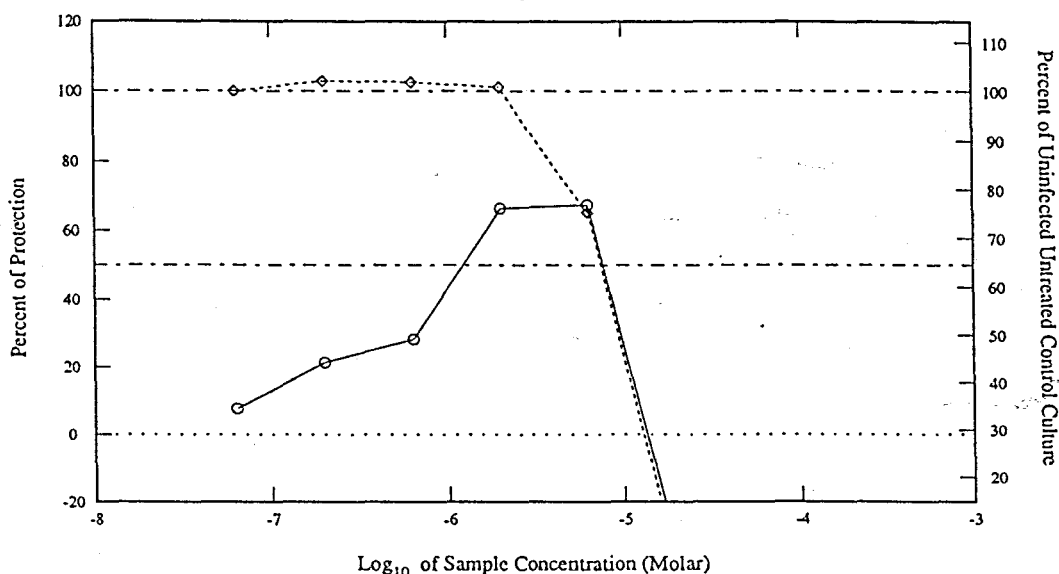
100%, 50% Reference Lines   Viral Cytopathic Effect   Infected Treated Culture   Uninfected Treated Culture

Summary		Dose (Molar)	Percent of Protection	Percent of Control	
Index	Concentration			Infected	Uninfected
IC50 (Molar)	9.32×10^{-6}	6.35×10^{-8}	7.77	34.52	100.07
EC50 (Molar)	1.23×10^{-6}	2.00×10^{-7}	21.34	44.15	102.08
TI50 (IC/EC)	7.55×10^{0}	6.34×10^{-7}	28.35	49.13	101.87
Conclusion		2.00×10^{-6}	66.45	76.18	100.83
CONFIRMED MODERATE		6.33×10^{-6}	67.46	76.90	75.28
		2.00×10^{-5}	-34.99	4.16	0.21
		6.32×10^{-5}	-47.92	-5.02	-2.84
		2.00×10^{-4}	-36.80	2.87	-2.77

FIGURE 6.10 Selected test results from the anti-HIV screening of rietone by the NCI.

CHAPTER SEVEN

EXPERIMENTAL

7.1   EXPERIMENTAL : GENERAL

All solvents were redistilled before use. When necessary solvents were dried according to standard procedures. Normal phase TLC was performed on DC-Alufolien Kieselgel 60F$_{254}$ plates and reverse phase TLC was performed on DC-Fertigplatten RP-18F$_{254}$s and RP-8F$_{254}$s plates. The plates were viewed under 254 nm wavelength UV light and were developed by spraying with 10% H$_2$SO$_4$ in MeOH followed by heating for several minutes. Flash column chromatography was performed using Kieselgel 60 (230 - 400 mesh) silica.

Analytical grade solvents were used for HPLC. The HPLC system used in the fractionation of the organic extracts included a Spectra-Physics IsoChrom LC pump equipped with a Rheodyne injector, a Waters R401 differential refractometer and a Rikadenki chart recorder. In normal phase separations a Whatman Magnum 9 Partisil column was used and in reverse phase separations a Phenomenex Selectosil C18 column was used. Chapter three involved the addtional use of a Spectra-Physics SpectraSERIES P100 pump, a SpectraSERIES UV100 detector and Phenomenex Selectosil C8 and CN columns.

Melting points were determined on a Gallenkamp melting point apparatus. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR spectra were recorded on a Perkin-Elmer 180 Grating infrared spectrophotometer with the exception of spectra in chapters four and six which were recorded on a Perkin-Elmer series 7 FTIR spectrophotometer. UV spectra in chapter three were obtained in MeOH on a GBC UV/VIS 916 spectrophotometer and UV spectra in chapters one and six were obtained in MeOH on a Shimadzu UV/VIS UV160A spectrophotometer.

The ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR experiments, in addition to the 2-D and NOEDS experiments, were recorded on a Bruker AMX400 spectrometer. ^{1}H and ^{13}C NMR chemical shifts are reported in δ units (ppm) and are referenced to residual protonated solvent.

Low-resolution mass spectra (EIMS) were recorded on a Hewlett-Packard 5988A spectrometer. All high-resolution spectra (HREIMS) were obtained by Dr P. Boshoff of the Mass Spectrometry Unit at the Cape Technikon, Cape Town, with the exception of spectra in chapter six which were obtained by Professor Ferreira at the University of Orange Freestate, Bloemfontein. In chapter three the HRFABMS data were obtained by Dr M. Hemling of SmithKline Beecham Pharmaceuticals, US.

7.2 EXPERIMENTAL : CHAPTER ONE

COLLECTION AND IDENTIFICATION — The grey-brown sponge *Ircinia* species (dry weight 252 g) was collected using SCUBA (-20 m) in the Tsitsikamma Marine Reserve in March 1992. The sponge was identified by M. K. Harper of Scripps Institute of Oceanography, California. A total of 593 *Siphonaria concinna* marine molluscs were collected by hand from Old Woman's River mouth near Port Alfred in December 1992. The animals were identified by Professor A. Hodgson of the Zoology Department at Rhodes University, Grahamstown. Voucher specimens of the sponge (No. 92-011) and the molluscs (No. 92-005) are housed at Rhodes University.

EXTRACTION AND ISOLATION — The frozen *Ircinia* sponge was freeze-dried and extracted with hexane, EtOAc and MeOH respectively. The crude EtOAc extract exhibited moderate antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* bacteria. The EtOAc extract was fractionated by flash chromatography on silica gel using a hexane/EtOAc solvent gradient. The fraction which showed the strongest antimicrobial activity was further purified by normal phase HPLC (50% EtOAc/hexane) to yield variabilin [35] (47.2 mg, 0.26%). The *Siphonaria concinna* marine molluscs were steeped in acetone for seven weeks after which the combined aqueous/acetone extract was partitioned between EtOAc and H_2O. The EtOAc layer was concentrated and fractionated by flash chromatography using a hexane/EtOAc solvent gradient. The fraction eluted with pure EtOAc crystallised on standing. This crystalline fraction showed strong antimicrobial activity against *Staphylococcus aureus*. Recrystallisation with EtOAc gave pectinatone [36] (88.6 mg).

Variabilin [35] — stable, yellow oil; $[\alpha]_D^{21}$ -29° (c 0.83, $CHCl_3$); IR (film) 3140, 2925, 1725, 1630, 1305, 1060, 750 cm^{-1}; UV (MeOH)λ_{max} 204.8 (ϵ 20 100), 250.8 (ϵ 15 700), 308.0 (ϵ 8 700); ^{1}H NMR ($CDCl_3$) δ 7.32 (1H, s, H-1), 7.19 (1H, s, H-4), 6.26 (1H, s, H-2), 5.31 (1H, d, J = 10.1 Hz, H-20), 5.15 (1H, t, J = 7.0 Hz, H-7), 5.07 (1H, t, J = 6.8 Hz, H-12), 2.77 (1H, m, H-18), 2.22 (2H, q, J = 7.5 Hz, H-6), 2.04 (2H, m, H-11), 1.97 (2H, t, J = 7.7 Hz, H-10), 1.93 (2H, t, J = 7.6 Hz, H-15), 1.81 (3H, s, Me-25), 1.57 (3H, s, Me-

9), 1.54 (3H, s, Me-14), 1.34 (2H, m, H-16), 1.28 (2H, m, H-17), 1.05 (3H, d, J = 6.7 Hz, Me-19); ^{13}C NMR (CDCl$_3$) δ 172.0 (s), 162.0 (s), 142.7 (s), 142.5 (d), 138.8 (d), 135.8 (s), 134.8 (s), 125.0 (s), 124.4 (d), 123.7 (d), 116.2 (d), 111.1 (d), 99.5 (s), 39.7 (t), 39.5 (t), 36.6 (t), 30.9 (d), 28.4 (t), 26.6 (t), 25.7 (t), 25.0 (t), 20.6 (q), 16.0 (q), 15.8 (q), 6.1 (q); EIMS (70 eV) m/z (int. %) No M$^+$, 175 (2), 149 (6), 135 (8), 109 (9), 95 (20), 83 (35), 81 (100), 69 (56), 55 (46), 44 (57); HREIMS m/z 398.2440 (calcd for C$_{25}$H$_{34}$O$_4$, 398.2457).

Pectinatone [36] — white crystals; $[\alpha]_D^{21}$ +59° (c 1.15, CHCl$_3$), IR (KBr) 3090, 2940, 2910, 2850, 1655, 1620, 1540, 1440, 1370, 1210, 1110, 1070, 1020, 960, 740 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 5.36 (1H, dq, J = 9.9 Hz), 2.63 (1H, m), 2.01 (6H, s), 1.89 (3H, d, J = 1.3 Hz), 1.46 (2H, m), 1.31 (4H, m), 1.24 (4H, m), 1.51 (2H, m), 1.05 (2H, m), 0.97 (3H, d, J = 6.6 Hz), 0.93 (2H, m), 0.85 (3H, t, J = 7.1 Hz), 0.83 (3H, d, J = 6.5 Hz), 0.79 (3H, d, J = 6.6 Hz); ^{13}C NMR (CDCl$_3$) δ 165.9 (s), 165.4 (s), 159.3 (s), 142.9 (d), 126.2 (s), 106.4 (s), 98.7 (s), 45.8 (t), 44.7 (t), 39.3 (t), 30.5 (d), 29.7 (d), 28.3 (d), 21.2 (q), 20.2 (q), 20.1 (q), 20.0 (t), 14.8 (q), 14.4 (q), 11.5 (q), 8.6 (q); EIMS (70 eV) m/z (int. %) 334 (M$^+$, 5), 278 (2), 221 (4), 181 (72), 168 (100), 125 (6), 83 (49), 69 (22), 43 (37); HREIMS m/z 334.2503 (calcd for C$_{21}$H$_{34}$O$_3$, 334.2508).

COLLECTION AND IDENTIFICATION — The colonial ascidian *Pseudodistoma* species was collected using SCUBA (-20 to -30 m) in the Tsitsikamma Marine Reserve in May 1993. A voucher specimen (No. 93-002) resides at Rhodes University. The animal was identified by Dr P. Coetzee of the Zoology Department, University of Port Elizabeth, South Africa.

EXTRACTION AND ISOLATION — The freshly collected ascidian was immediately frozen and stored for approximately one month. Upon removal it was freeze-dried and extracted with hexane, EtOAc and MeOH respectively. The crude EtOAc extract proved to be most active in the antimicrobial disc assay. Fractionation of this extract was conducted according to SCHEME 2.1 (section 2.2) to yield four new amino alcohols as their acetyl derivatives **[47]** (33.0 mg), **[48]** (10.7 mg), **[49]** (17.3 mg) and **[50]** (21.1 mg).

Preparation of Acetyl Derivatives — a mixture of amino alcohols (267 mg) was treated with Ac_2O (5 mL) in pyridine and left overnight at RT. After evaporation of the solvents the mixture was separated by HPLC (SCHEME 2.1).

2S^*-Acetamido-3S^*-acetoxy-5E,13-tetradecadiene [47] — stable, colourless oil; $[\alpha]_D^{21}$ -20° (c 0.83, $CHCl_3$); IR (film) 3290, 2924, 2850, 1738, 1647, 1545, 1368, 1229, 1018, 950 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.79 (1H, dq, H-13), 5.50 (1H, br s, NH), 5.48 (1H, dt, $J_{5,6}$ = 15.3 Hz, $J_{6,7}$ = 6.9 Hz, H-6), 5.30 (1H, dt, $J_{4,5}$ = 6.8 Hz, $J_{5,6}$ = 15.3 Hz, H-5), 4.94 (2H, dd, H-14), 4.82 (1H, m, H-3), 4.20 (1H, m, H-2), 2.22 (2H, m, H-4), 2.06 (3H, s, OAc), 2.01 (2H, m, H-12), 1.98 (3H, s, OAc), 1.95 (2H, m, H-7), 1.36 - 1.25 (8H, m, H_2-8 to H_2-11), 1.09 (3H, d, J = 6.8 Hz, H-1); ^{13}C NMR ($CDCl_3$) see TABLE 2.1(section 2.3) ; EIMS (70 eV) m/z (int. %) 309 (M^+, 2), 249 (32), 206 (19), 149 (13), 98 (99), 86 (100), 83 (70); HREIMS m/z 309.2292 (calcd for $C_{18}H_{31}NO_3$, 309.2304).

2S*-Acetamido-3S*-acetoxy-5E-tetradecene [48] — stable, colourless oil; $[\alpha]_D^{21}$ +14° (c 0.68, CHCl$_3$); IR (film) 3290, 2920, 2850, 1738,1645,1539,1366,1229,1016, 949 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 5.50 (1H, br s, NH), 5.47 (1H, dt, $J_{5,6}$ = 15.1 Hz, $J_{6,7}$ = 6.7 Hz, H-6), 5.30 (1H, dt, $J_{4,5}$ = 7.5 Hz, $J_{5,6}$ = 15.0 Hz, H-5), 4.82 (1H, m, H-3), 4.21 (1H, m, H-2), 2.22 (2H, m, H-4), 2.06 (3H, s, OAc), 2.01 (2H, m, H-7), 1.98 (3H, s, OAc), 1.30 - 1.23 (12H, m, H$_2$-8 to H$_2$-13), 1.09 (3H, d, J = 6.8 Hz, H-1), 0.87 (3H, t, J = 6.7 Hz, H-14); ^{13}C NMR (CDCl$_3$) see TABLE 2.1 (section 2.3) ; EIMS (70 eV) m/z (int. %) 311 (M$^+$, 2), 251 (20), 192 (20), 149 (71), 99 (65), 98 (61), 86 (100), 43 (55); HREIMS m/z 311.2478 (calcd for C$_{18}$H$_{33}$NO$_3$, 311.2460).

1-Acetamido-2-acetoxy-4E,12-tridecadiene [49] — stable, colourless oil; $[\alpha]_D^{21}$ +14° (c 0.97, CHCl$_3$); IR (film) 3290, 2921, 2848, 1738, 1652, 1548, 1425, 1366, 1229, 1039, 948 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 5.79 (1H, dq, H-12), 5.70 (1H, br s, NH), 5.48 (1H, dt, $J_{4,5}$ = 15.3 Hz, $J_{5,6}$ = 6.9 Hz, H-5), 5.31 (1H, dt, $J_{3,4}$ = 7.1 Hz, $J_{4,5}$ = 15.3 Hz, H-4), 4.95 (2H, m, H-13), 4.90 (1H, m, H-2), 3.40 (2H, m, H-1), 2.25 (2H, t, J = 6.8 Hz, H-3), 2.05 (3H, s, OAc), 2.02 (2H, m, H-11), 1.98 (2H, m, H-6), 1.96 (3H, s, OAc), 1.38 - 1.26 (8H, m, H$_2$-7 to H$_2$-10); ^{13}C NMR (CDCl$_3$) see TABLE 2.2 (section 2.3); EIMS (70 eV) m/z (int. %) 295 (M$^+$, 2), 279 (10), 235 (74), 176 (19), 149 (100), 111 (41), 102 (46), 96 (46), 82 (58), 80 (67), 43 (62); HREIMS m/z 295.2156 (calcd for C$_{17}$H$_{29}$NO$_3$, 295.2147).

1-Acetamido-2-acetoxy-4E-tridecene [50] — stable, colourless oil; $[\alpha]_D^{21}$ +10° (c 0.75, CHCl$_3$); IR (film) 3290, 2925, 2851, 1740, 1655, 1550, 1425, 1368, 1231, 1041, 948 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 5.69 (1H, br s, NH), 5.50 (1H, dt, $J_{4,5}$ = 15.2 Hz, $J_{5,6}$ = 8.2 Hz, H-5), 5.31 (1H, dt, $J_{3,4}$ = 7.1 Hz, $J_{4,5}$ = 15.2 Hz, H-4), 4.91 (1H, m, H-2), 3.41 (2H, m, H-1), 2.26 (2H, t, J = 6.7 Hz, H-3), 2.05 (3H, s, OAc), 1.98 (2H, m, H-6), 1.96 (3H, s, OAc), 1.31 - 1.25 (12H, m, H$_2$-7 to H$_2$-12), 0.87 (3H, t, J = 6.6 Hz, H-13); ^{13}C NMR (CDCl$_3$) see TABLE 2.2 (section 2.3); EIMS (70 eV) m/z (int. %) 297 (M$^+$, 1), 237 (100), 178 (28), 149 (23), 124 (18), 82 (34), 80 (45), 43 (25); HREIMS m/z 297.2305 (calcd for C$_{17}$H$_{31}$NO$_3$, 297.2304).

Preparation of Oxazolidinones — a mixture of the amino alcohols (140 mg) was reacted with 1,1-carbonyl-di-imidazole (330 mg) in refluxing C_6H_6 for 6 h. The solution was washed with H_2O, dried over anhyd. Na_2SO_4, concentrated under reduced pressure and chromatographed by normal phase HPLC (50% EtOAc/hexane) and reverse phase HPLC (20% H_2O/MeOH) to afford pure **[57]** (4.1 mg) and **[58]** (2.5 mg).

Oxazolidinone [57] — oil; $[\alpha]_D^{21}$ -32° (c 0.41, $CHCl_3$); IR (film) 3280, 2917, 2848, 1749, 1385, 1232, 1015, 949 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.79 (1H, dq, H-13), 5.58 (1H, dt, $J_{5,6}$ = 15.3 Hz, $J_{6,7}$ = 6.7 Hz, H-6), 5.36 (1H, dt, $J_{4,5}$ = 7.0 Hz, $J_{5,6}$ = 15.2 Hz, H-5), 5.15 (1H, br s, NH), 4.94 (2H, dd, H-14), 4.11 (1H, q, J = 6.2 Hz, H-2), 3.60 (1H, m, H-3, transformed into a doublet with J = 6.1 Hz by spin decoupling at δ 1.25), 2.40 (2H, m, H-4), 2.01 (4H, m, H_2-7 and H_2-12), 1.29 (8H, m, H_2-8 to H_2-11), 1.25 (3H, d, J = 6.2 Hz, Me-1); ^{13}C NMR ($CDCl_3$) δ 158.7 (s, CO), 139.1 (d, C-13), 135.7 (d, C-6), 122.5 (d, C-5), 114.2 (t, C-14), 83.4 (d, C-3), 52.5 (d, C-2), 37.0 (t, C-4), 33.7 (t, C-12), 32.5 (t, C-7), 29.2 / 29.0 / 28.9 / 28.8 (t, C-8 to C-11), 20.8 (q, Me-1); EIMS (70 eV) m/z (int. %) 251 (M^+, 1), 190 (6), 149 (18), 112 (31), 100 (52), 99 (33), 88 (100), 56 (50); HREIMS m/z 251.1890 (calcd for $C_{15}H_{15}NO_2$, 251.1885).

Oxazolidinone [58] — oil; $[\alpha]_D^{21}$ -26° (c 0.25, $CHCl_3$); IR (film) 3280, 2917, 2848, 1749, 1385, 1232, 1015, 949 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.58 (1H, dt, $J_{5,6}$ = 15.2 Hz, $J_{6,7}$ = 6.6 Hz, H-6), 5.35 (1H, dt, $J_{4,5}$ = 7.0 Hz, $J_{5,6}$ = 15.3 Hz, H-5), 4.99 (1H, br s, NH), 4.11 (1H, q, J = 6.1 Hz, H-2), 3.60 (1H, m, transformed into a doublet with J = 6.1 Hz by spin decoupling at δ 1.24), 2.40 (2H, m, H-14), 2.04 (2H, m, H-7), 1.40 (12H, m, H_2-8 to H_2-13), 1.24 (3H, d, J = 6.2 Hz, Me-1), 0.97 (3H, t, J = 7.5 Hz, Me-14); ^{13}C NMR ($CDCl_3$) δ 158.6 (s, CO), 135.4 (d, C-6), 122.9 (d, C-5), 83.4 (d, C-3), 52.5 (d, C-2), 37.0 (t, C-4), 32.2 (t, C-7), 29.2 / 29.2 / 29.2 / 26.7 / 25.5 / 20.5 (t, C-8 to C-13), 20.8 (q, Me-1), 14.3 (q, Me-14); EIMS (70 eV) m/z (int. %) 253 (M^+, 1), 149 (57), 99 (89), 88 (100), 81 (74), 57 (96), 55 (79), 43 (63).

Preparation of Hydrogenated Derivatives — a solution of **[47]** (9.8 mg) in EtOH (6 mL) containing Adams catalyst (10 mg) was stirred under an atmosphere of hydrogen

for 3.5 h. The catalyst was removed by filtration and the solvent evaporated to give the tetrahydroacetate **[59]** (12.2 mg). By the same procedure **[59]** (8.2 mg) was obtained from **[48]** (6.5 mg).

Hydrogenated Derivative [59] — oil; $[\alpha]_D^{21}$ -8° (c 1.06, CHCl$_3$); IR (film) 3290, 2920, 2850, 1732, 1654, 1550, 1458, 1366, 1233 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 5.73 (1H, br s, NH), 4.89 (1H, m, H-12), 3.40 (2H, m, H-1), 2.06 (3H, s, OAc), 1.96 (3H, s OAc), 1.53 (2H, m, H-3), 1.24 (18H, m, H$_2$-4 to H$_2$-12), 0.87 (3H, t, J = 6.7 Hz, H-13); ^{13}C NMR (CDCl$_3$) δ 171.3 (s, OAc), 170.1 (s, OAc), 73.6 (d, C-2), 43.2 (t, C-1), 31.9 / 29.6 / 29.6 / 29.5 / 29.4 / 29.3 / 29.3 / 29.3 / 25.2 / 22.7 (t, C-3 to C-12), 23.2 (q, OAc), 21.2 (q, OAc), 14.1 (q, C-13); EIMS (70 eV) m/z (int. %) No M$^+$, 149 (5), 115 (38), 73 (100), 72 (17), 60 (19), 43 (26).

COLLECTION AND IDENTIFICATION — The black nodular Latrunculid sponge was collected using SCUBA (-20 m) in the Tsitsikamma Marine Reserve in May 1993 and May 1995. A voucher specimen (No. 94-006) is housed at Rhodes University. The animal is presently being identified by Dr M. Kelly-Borges of The Natural History Museum, London.

EXTRACTION AND ISOLATION — The freshly collected sponge was stored frozen. Upon removal it was freeze-dried and extracted with a 1:1 MeOH/CHCl$_3$. Fractionation of this extract was conducted according to SCHEME 3.2 and SCHEME 3.3 (section 3.2) to yield four new alkaloids, tsitsikammamine A **[90]** (7.9 mg), B **[91]** (8.9 mg), C **[92]** (63.2 mg) and D **[93]** (5.7 mg).

Tsitsikammamine A [90] — characterised as its TFA salt; brown solid; intense pigmentation precluded measurement of $[\alpha]_D^{21}$; IR (film) 3150, 1670, 1620, 1570, 1540, 1430, 1370, 1200, 1130, 1050, 780 cm^{-1}; UV (MeOH) λ_{max} 242.9 (ϵ 11 700), 317.1 (ϵ 6 500), 377.3 (ϵ 2 500); ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) see TABLE 3.1 (section 3.3); ^{1}H NMR (CD$_3$OD) δ 7.38 (2H, d, J = 8.3 Hz, H-18 and H-20), 6.99 (1H, s, H-13), 6.95 (1H, s, H-2), 6.92 (2H, d, J = 8.4 Hz, H-17 and H-21), 3.90 (2H, t, J = 7.8 Hz, H-5), 3.03 (2H, t, J = 7.8 Hz, H-4); ^{13}C NMR (CD$_3$OD) δ 167.8 (s, C-10), 159.9 (s, C-19), 159.6 (s, C-7), 136.7 (s, C-11), 130.4 (d, C-18), 130.4 (d, C-20), 130.2 (s, C-9), 129.5 (s, C-16), 125.9 (d, C-13), 124.5 (s, C-14), 123.9 (d, C-2), 122.0 (s, C-8), 120.7 (s, C-3), 117.8 (d, C-17), 117.8 (d, C-21), 115.0 (s, C-15), 46.1 (t, C-5), 19.2 (t, C-4); HRFABMS m/z 304.1087 (M+H)$^+$ (calcd for C$_{18}$H$_{14}$N$_3$O$_2$, 304.1086).

Tsitsikammamine B [91] — characterised as its TFA salt; brown solid; intense pigmentation precluded measurement of $[\alpha]_D^{21}$; UV (MeOH) λ_{max} 242.9 (ϵ 7 900), 317.1 (ϵ 3 900), 374.7 (ϵ 1 900); ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) see TABLE 3.2 (section 3.3); HRFABMS m/z 318.1252 (M+H)$^+$ (calcd for C$_{19}$H$_{16}$N$_3$O$_2$, 318.1243).

Tsitsikammamine C [92] — characterised as its TFA salt; brown solid; intense pigmentation precluded measurement of $[\alpha]_D^{21}$; IR (film) 1690, 1670, 1590, 1550, 1540, 1430, 1410, 1320, 1200, 1170, 1130 cm^{-1}; UV (MeOH) λ_{max} 204.5 (ε 13 600), 254.4 (ε 20 200), 382.4 (ε 7 600); ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) see TABLE 3.3 (section 3.3); ^{1}H NMR (CD$_3$OD) δ 7.70 (2H, s, H-1 and H-5), 3.81 (2H, t, J = 7.4 Hz, H-17), 3.72 (2H, t, J = 5.4 Hz, H-8), 2.78 (2H, t, J = 7.4 Hz, H-16), 2.10 (2H, t, J = 5.5 Hz, H-7); ^{13}C NMR (CD$_3$OD) δ 172.8 (s, C-3), 165.4 (s, C-11), 155.2 (s, C-19), 153.6 (s, C-10), 152.6 (d, C-1), 152.6 (d, C-5), 126.1 (s, C-21), 125.4 (s, C-12), 124.4 (s, C-2), 124.4 (s, C-4), 121.3 (s, C-15), 112.9 (s, C-14), 93.3 (s, C-20), 46.1 (s, C-6), 45.1 (t, C-17), 39.7 (t, C-8), 35.2 (t, C-7), 19.1 (t, C-16); HRFABMS m/z 539.8544 (M+H)$^+$ (calcd for C$_{18}$H$_{13}$N$_3$O$_2$^{79}Br$_3$, 539.8558).

Tsitsikammamine D [93] — characterised as its TFA salt; brown solid; intense pigmentation precluded measurement of $[\alpha]_D^{21}$; UV (MeOH) λ_{max} 249.3 (ε 11 900), 381.8 (ε 5 400); ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) see TABLE 3.4 (section 3.3); HRFABMS m/z 541.8710 (M+H)$^+$ (calcd for C$_{18}$H$_{15}$N$_3$O$_2$^{79}Br$_3$, 541.8714)..

169

COLLECTION AND IDENTIFICATION — Yellow and grey colour variants of the soft coral *Capnella thyrsoidea* were collected using SCUBA (-30 m) in the Tsitsikamma Marine Reserve in May 1993 and May 1994. Voucher specimens of the yellow (No. 94-003) and grey (No 94-004) soft corals reside at Rhodes University. The animals were identified by Dr G. Williams of the California Academy of Sciences.

EXTRACTION AND ISOLATION — Portions of the EtOAc extracts of the freeze-dried yellow and grey colour variants were fractionated according to SCHEME 4.1 and SCHEME 4.2 (section 4.2) respectively. The yellow colour variant yielded 5α-pregna-1,20-dien-3-one [97] (80.2 mg) together with the diterpene, tsitsixenicin A [100] (23.3 mg), while the grey colour variant gave 16β-hydroxy-5α-pregna-1,20-dien-3-one 16-acetate [98] (7.5 mg), 3α,16β-dihydroxy-5α-pregna-1,20-diene 3,16-diacetate [99] (4.8 mg) and tsitsixenicins A [100] (67.8 mg), B [101] (5.6 mg), C [102] (5.9 mg) and D [103] (8.3 mg).

5α-Pregna-1,20-dien-3-one [97] — white needles; mp 125-126 °C; $[\alpha]_D^{21}$ +45° (c 1.11, CHCl$_3$); IR (CHCl$_3$) 2943, 2873, 1674, 1448, 1274, 1213, 918 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 7.13 (1H, d, $J_{1,2}$ = 10.2 Hz, H-1), 5.83 (1H, d, $J_{1,2}$ = 10.0 Hz, H-2), 5.74 (1H, td, J = 16.5, 10.8, 8.8 Hz, H-20), 4.95 (2H, m, H-21), 2.35 (1H, dd, J = 17.7, 14.1 Hz, H-4), 2.20 (1H, dd, J = 17.7, 3.6 Hz, H-4), 1.95 (1H, m, H-17), 1.66 (1H, m, H-16), 1.55 (1H, m, H-6), 1.45 (1H, m, H-8), 1.42 (2H, m, H-11), 1.42 (1H, m, H-15), 1.18 (1H, m, H-16), 1.08 (1H, m, H-14), 1.07 (1H, m, H-12), 1.00 (3H, s, H-19), 0.99 (1H, m, H-7), 0.97 (1H, m, H-9), 0.61 (3H, s, H-18); ^{13}C NMR (CDCl$_3$) see TABLE 4.1 (section 4.3); EIMS (70 eV) m/z (int. %) 298 (M$^+$, 11), 283 (29), 134 (36), 122 (100), 121 (41), 95 (42), 79 (52), 67 (35); HREIMS m/z 298.2313 (calcd for C$_{21}$H$_{30}$O, 298.2296).

16β-Hydroxy-5α-pregna-1,20-dien-3-one 16-acetate [98] — colourless oil; $[\alpha]_D^{21}$ +91° (c 0.58, CHCl$_3$); IR (CHCl$_3$) 2939, 1729, 1669, 1609, 1256 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 7.12 (1H, d, $J_{1,2}$ = 10.2 Hz, H-1), 5.85 (1H, d, $J_{1,2}$ = 10.2 Hz, H-2), 5.77 (1H, td, J = 17.1,

10.5, 8.9 Hz, H-20), 5.11 (1H, m, H-16), 5.08 (2H, m, H-21), 2.36 (1H, dd, J = 17.6, 14.1 Hz, H-4), 2.21 (2H, dd, J = 17.7, 4.0 Hz, H-4), 2.12 (1H, dd, $J_{17,20}$ = 7.7 Hz, $J_{16,17}$ = 4.1 Hz, H-17), 2.02 (3H, s, OAc), 1.95 (1H, m, H-5), 1.85 (1H, m, H-11), 1.77 (1H, m, H-15), 1.74 (1H, m, H-12), 1.67 (1H, m, H-7), 1.56 (1H, m, H-11), 1.47 (1H, m, H-8), 1.45 (1H, m, H-6), 1.42 (1H, m, H-15), 1.41 (1H, m, H-6), 1.38 (1H, m, H-14), 1.20 (1H, m, H-12), 1.02 (1H, m, H-9), 1.01 (3H, s, H-19), 0.98 (1H, m, H-7), 0.69 (3H, s, H-18); ^{13}C NMR (CDCl$_3$) see TABLE 4.1 (section 4.3); EIMS (70 eV) m/z (int. %) No M$^+$, 296 (41), 122 (73), 121 (45), 107, (45), 94 (73), 79 (76), 43 (100); HREIMS m/z 356.2338 (calcd for C$_{23}$H$_{32}$O$_3$, 356.2351).

3α,16β-Dihydroxy-5α-pregna-1,20-diene 3,16-diacetate [99] — colourless oil; $[\alpha]_D^{21}$ +133° (c 0.53, CHCl$_3$); IR (CHCl$_3$) 3029, 2934, 1726, 1482, 1378, 1257,1039 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 6.18 (1H, d, 10.0 Hz, H-1), 5.76 (1H, td, J = 17.1, 10.4, 8.9 Hz, H-20), 5.60 (1H, ddd, $J_{1,2}$ = 10.0 Hz, $J_{2,3}$ = 5.5 Hz, $J_{2,4}$ = 1.2 Hz, H-2), 5.15 (1H, m, H-3), 5.08 (1H, m, H-16), 5.04 (2H, m, H-21), 2.13 (1H, t, J = 7.7 Hz, H-17), 2.04 (3H, s, OAc), 2.02 (3H, s, OAc), 1.85 (1H, m, H-11), 1.81 (1H, m, H-4), 1.76 (1H, m, H-15), 1.71 (1H, m, H-12), 1.68 (1H, m, H-7), 1.58 (1H, m, H-11), 1.58 (1H, m, H-5), 1.53 (1H, m, H-4), 1.40 (1H, m, H-8), 1.37 (1H, m, H-15), 1.37 (1H, m, H-14), 1.37 (2H, m, H-6), 1.14 (1H, m, H-12), 1.02 (1H, m, H-7), 0.99 (1H, m, H-9), 0.81 (3H, s, H-19), 0.66 (3H, s, H-18); ^{13}C NMR (CDCl$_3$) see TABLE 4.1 (section 4.3); EIMS (70 eV) m/z (int. %) No M$^+$, 120 (43), 105 (76), 95 (36), 94 (54), 91 (72), 43 (100); HREIMS m/z 400.2609 (calcd for C$_{25}$H$_{36}$O$_4$, 400.2613).

Saponification of 16β-hydroxy-5α-pregna-1,20-dien-3-one 16-acetate [98] — a solution of [98] (3.4 mg) in EtOH (1 mL) was refluxed at 80 °C for 1 h with ethanolic KOH (0.24 mmol). H$_2$O was added and the EtOH removed under reduced pressure. The aqueous solution was acidified, extracted with ether, dried over anhyd. Na$_2$SO$_4$, filtered and concentrated to yield [107] (3.0 mg).

16β-Hydroxy-5α-pregna-1,20-dien-3-one [107] — yellow oil; $[\alpha]_D^{17}$ +12° (c 0.24, CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 7.13 (1H, d, J = 10.2 Hz, H-1), 5.85 (1H, d, J = 10.7 Hz, H-

2), 5.81 (1H, m, H-20), 5.14 (2H, m, H-21), 4.22 (1H, m, H-16), 2.36 (1H, dd, J = 17.6, 14.1 Hz, H-4), 2.22 (1H, dd, J = 18.3, 4.2 Hz, H-4), 1.92 (1H, m, H-5), 1.00 (3H, s, H-19), 0.68 (3H, s, H-18); ^{13}C NMR (CDCl$_3$) δ 200.1 (s, C-3), 158.2 (d, C-1), 136.8 (d, C-20), 127.5 (d, C-2), 117.2 (t, C-21), 76.5 (d, C-16), 66.2 (d, C-17), 53.2 (d, C-14), 50.3 (d, C-9), 44.9 (s, C-13), 44.4 (d, C-5), 41.0 (t, C-4), 39.9 (s, C-10), 37.3 (s, C-12), 35.6 (d, C-8), 35.3 (t, C-11), 31.3 (t, C-7), 27.6 (t, C-6), 20.4 (t, C-15), 14.4 (q, C-18), 13.1 (q, C-19).

Reduction of 3α,16β-dihydroxy-5α-pregna-1,20-diene 3,16-diacetate [99] — LiAlH$_4$ (8 mg) was added to a stirred solution of [99] (3.5 mg) in dry ether (2 mL). The solution was cooled in ice and 5% NaOH (2 mL) was added. The basic solution was extracted with ether, dried over anhyd. Na$_2$SO$_4$, filtered and concentrated to give [108] (3.1 mg).

5α-pregna-1,20-dien-3α,16β-diol [108] — white solid; $[\alpha]_D^{17}$-63° (c 0.20, CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 6.06 (1H, d, J = 10.0 Hz, H-1), 5.81 (1H, m, H-20), 5.65 (1H, m, H-2), 5.12 (2H, m, H-21), 4.22 (1H, td, J = 8.8, 2.1 Hz, H-16), 4.08 (1H, td, J = 4.5 Hz, H-3); ^{13}C NMR (CDCl$_3$) δ 140.3 (d, C-1), 136.9 (d, C-20), 126.2 (d, C-2), 116.9 (t, C-21), 76.6 (d, C-16), 66.3 (d, C-17), 64.4 (d, C-3), 53.4 (d, C-14), 51.3 (d, C-9), 45.0 (s, C-13), 39.0 (s, C-5), 38.3 (s, C-10), 35.7 (t, C-12), 35.4 (d, C-8), 34.8 (t, C-11), 31.9 (t, C-4), 29.7 (t, C-7), 27.9 (t, C-6), 20.3 (t, C-15), 14.4 (q, C-18), 13.9 (q, C-19).

Tsitsixenicin A [100] — yellow oil; $[\alpha]_D^{17}$-64° (c 0.9, CHCl$_3$); IR (CHCl$_3$) 3090, 3038, 1960, 1821, 1735, 1482, 1230, 1039 cm^{-1}; ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$) see TABLE 4.2 (section 4.3); EIMS (70 eV) m/z (int. %) No M$^+$, 231 (11), 83 (38), 69 (19), 55 (17), 43 (100); HREIMS m/z 402.2398 (calcd for C$_{24}$H$_{34}$O$_5$, 402.2406).

Tsitsixenicin B [101] — colourless oil; $[\alpha]_D^{21}$ -38° (c 0.49, CHCl$_3$); IR (CHCl$_3$) 2960, 2934, 2864, 1726, 1239, 1074 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 6.51 (1H, d, J = 1.3 Hz, H-3), 5.87 (1h, d, J = 3.3 Hz, H-1), 5.24 (1H, t, J = 7.6 Hz, H-12), 5.06 (1H, br s, H-19), 4.95 (1H, m, H-14), 4.93 (1H, br s, H-19'), 2.98 (1H, dd, J = 3.2, 10.8 Hz, H-8), 2.43 (1H, m, H-11a), 2.41 (1H, m, H-5), 2.41 (1H, m, H-13), 2.38 (1H, m, H-4a), 2.23 (1H, m, H-5'),

2.23 (1H, m, H-13'), 2.22 (1H, m, H-9), 2.19 (1H, m, H-6), 2.08 (3H, s, OAc), 2.00 (3H, s, OAc), 1.67 (3H, s, H-17), 1.61 (3H, s, H-16), 1.43 (1H, m, H-9'), 1.35 (2H, m, H-10), 1.31 (3H, s, H-18), 1.19 (1H, m, H-6'); ^{13}C NMR (CDCl$_3$) see TABLE 4.3 (section 4.3); EIMS (70 eV) m/z (int. %) No M$^+$, 167 (31), 149 (100), 83 (25), 71 (22), 43 (75); HREIMS m/z 418.2378 (calcd for C$_{24}$H$_{34}$O$_6$, 418.2355).

Tsitsixenicin C [102] — colourless oil; $[\alpha]_D^{21}$ -139° (c 0.60, CHCl$_3$); IR (CHCl$_3$) 3620, 3029, 2934, 1735, 1613, 1221, 1013 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 7.25 (1H, d, J = 2.0 Hz, H-3), 6.82 (1H, d, J$_{13,14}$ = 15.8 Hz, H-14), 6.51 (1H, d, J$_{13,14}$ = 15.8 Hz, H-13), 6.14 (1H, d, J = 2.3 Hz, H-1), 5.59 (1H, m, H-9), 5.40 (1H, d, J$_{8,9}$ = 11.9 Hz, H-8), 5.14 (2H, d, J = 7.0 Hz, H-19), 3.44 (1H, m, H-4a), 3.17 (1H, m, H-10), 2.89 (1H, m, H-6), 2.63 (1H, m, H-10'), 2.63 (1H, m, H-11a), 2.06 (3H, s, OAc), 2.04 (3H, s, OAc), 1.73 (1H, m, H-5), 1.61 (3H, s, H-18), 1.57 (1H, m, H-5'), 1.46 (1H, m, H-6'), 1.40 (3H, s, H-16), 1.39 (3H, s, H-17); ^{13}C NMR (CDCl$_3$) see TABLE 4.3 (section 4.3); EIMS (70 eV) m/z (int. %) No M$^+$, 91 (13), 85 (24), 83, (32), 69 (15), 55 (14), 43 (100); HREIMS m/z 432.1961 (calcd for C$_{24}$H$_{32}$O$_7$, 432.1948).

Tsitsixenicin D [103] — colourless oil; $[\alpha]_D^{21}$ -126° (c 0.81, CHCl$_3$); IR (CHCl$_3$) 3029, 2934, 1735, 1613, 1230, 1013 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 7.26 (1H, d, J = 2.0 Hz, H-3), 6.12 (1H, d, J = 2.3 Hz, H-1), 5.57 (1H, m, H-9), 5.41 (1H, d, J$_{8,9}$ = 11.8 Hz, H-8), 5.30 (1H, t, J = 7.0 Hz, H-14), 5.12 (2H, d, J = 8.3 Hz, H-19), 3.32 (1H, m, H-4a), 3.25 (2H, t, J = 5.6 Hz, H-13), 3.16 (1H, m, H-10), 2.84 (1H, br t, J = 12.2 Hz, H-6), 2.65 (1H, m, H-10'), 2.60 (1H, d, J = 12.3 Hz, H-11a), 2.04 (3H, s, OAc), 2.04 (3H, s, OAc), 1.74 (3H, d, J = 1.0 Hz, H-16), 1.69 (1H, m, H-5), 1.64 (3H, s, H-17), 1.59 (3H, s, H-18), 1.55 (1H, m, H-5'), 1.43 (1H, m, H-6'); ^{13}C NMR (CDCl$_3$) see TABLE 4.3 (section 4.3); EIMS (70 eV) m/z (int. %) No M$^+$, 245 (19), 135 (14), 85 (22), 83 (27), 69 (22), 55, (14), 43 (100); HREIMS m/z 416.2174 (calcd for C$_{24}$H$_{32}$O$_6$, 416.2199).

Methylation of tsitsixenicin A [100] — a solution of tsitsixenicin A (33 mg) in MeOH (4 mL) was refluxed for 90 min, concentrated, and purified by normal phase HPLC (7% EtOAc/hexane) to give compound **[111]** (17 mg).

Methylation product [111] — colourless oil; $[\alpha]_D^{21}$ -95° (c 0.15, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 5.78 (1H, d, J = 2.3 Hz, H-1), 5.41 (1H, dd, J = 11.2, 3.6 Hz, H-8), 5.39 (1H, t, J = 7.3 Hz, H-12), 5.15 (1H, t, J = 7.0 Hz, H-14), 5.06 (1H, s, H-3), 3.37 (3H, s, OMe), 1.96 (3H, s, OAc); ^{13}C NMR ($CDCl_3$) δ 170.0 (s), 135.2 (s), 132.7 (s), 125.4 (d), 121.5 (d), 113.9 (t), 103.4 (d), 90.2 (d), 54.8 (q), 40.4 (t), 27.0 (t), 25.6 (t), 25.6 (q), 21.3 (q), 17.4 (q).

COLLECTION AND IDENTIFICATION — The orange soft coral *Alcyonium* species A was collected using SCUBA (-24 m) in the Tsitsikamma Marine Reserve in May 1994. The yellow-white soft coral *Alcyonium* species B was collected using SCUBA (-20 m) from Aliwal Shoal in June 1994. Voucher specimens for species A (No. 94-001) and for species B (No. SAF94-013) are housed at Rhodes University.

EXTRACTION AND ISOLATION — A portion of the EtOAc extract of the freeze-dried species A soft coral was fractionated according to SCHEME 5.1 (section 5.2). The fractionation yielded the new polyhydroxysterols, cholest-5-ene-3β,7β,19-triol 19-acetate [121] (11.9 mg) and cholest-5,24-diene-3β,7β,19-triol 19-acetate [122] (21.5 mg). Two separate portions of the EtOAc extract of the freeze-dried species B soft coral were fractionated according to SCHEME 5.2 (section 5.2). The fractionation yielded the xenicane compounds 9-deacetoxy-14,15-deepoxyxeniculin [110] (184.6 mg), zahavin A [16] (15.9 mg), 7-epoxyzahavin A [123] (8.9 mg) and xeniolide C [124] (11.7 mg).

Cholest-5-ene-3β,7β,19-triol 19-acetate [121] — colourless oil; $[\alpha]_D^{21}$ -24° (c 0.47, $CHCl_3$); IR (film) 3380, 2940, 2880, 1740, 1230, 1040 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.52 (1H, br s, H-6), 4.48 (1H, d, J_{gem} = 11.9 Hz, H-19), 3.98 (1H, d, J_{gem} = 11.9 Hz, H-19'), 3.80 (1H, d, $J_{7,8}$ = 7.8 Hz, H-7), 3.56 (1H, m, H-3), 2.39 (1H, m, H-4eq), 2.27 (1H, m, H-4ax), 2.05 (3H, s, OAc), 1.65 (1H, m, H-8), 0.85 (3H, d, $J_{25,26}$ = 6.6 Hz, H-26), 0.91 (3H, d, $J_{21,20}$ = 6.5 Hz, H-21), 0.85 (3H, d, $J_{25,27}$ = 6.6 Hz, H-27), 0.70 (3H, s, H-18); ^{13}C NMR ($CDCl_3$) see TABLE 5.2 (section 5.3) m/z (int. %) No M^+, 442 (24), 400 (22), 382 (19), 174 (36), 161 (66), 143 (49), 105 (50), 91 (52), 43 (100); HREIMS m/z 460.3541 (calcd for $C_{29}H_{48}O_4$, 460.3552).

Cholest-5,24-diene-3β,7β,19-triol 19-acetate [122] — colourless oil; $[\alpha]_D^{21}$ -25° (c 0.58, $CHCl_3$); IR (film) 3380, 2940, 2880, 1740, 1230, 1040 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.52 (1H, br s, H-6), 5.08 (1H, t, $J_{23,24}$ = 7.1 Hz, H-24), 4.48 (1H, d, J_{gem} = 11.9 Hz, H-19), 3.98 (1H, d, J_{gem} = 11.9 Hz, H-19'), 3.80 (1H, d, $J_{7,8}$ = 7.8 Hz, H-7), 3.57 (1H, m, H-

3), 2.39 (1H, m, H-4eq), 2.27 (1H, m, H-4ax), 2.06 (3H, s, OAc), 2.02 (2H, m, H-23), 1.67 (3H, s, H-27), 1.59 (3H, s, H-26), 0.93 (3H, d, $J_{21,20}$ = 6.5 Hz, H-21), 0.70 (3H, s, H-18); ^{13}C NMR (CDCl$_3$) see TABLE 5.2 (section 5.3);EIMS (70 eV) m/z (int. %) No M$^+$, 440 (13), 349 (13), 197 (16), 161 (38), 143 (47), 141 (52), 91 (42), 69 (100), 43 (45); HREIMS m/z 458.3391 (calcd for $C_{29}H_{46}O_4$, 458.3396).

Acetylation of cholest-5-ene-3β,7β,19-triol 19-acetate [121] — Ac$_2$O (1 mL) was added to a solution of **[121]** (7.1 mg) in pyridine (1 mL) and the reaction was left overnight. EtOH (1.5 mL) was added to quench the excess Ac$_2$O and the solution was concentrated *in vacuo*. The product was dissolved in EtOAc, filtered through a celite column and concentrated under reduced pressure to yield **[134]** (8.5 mg).

Cholest-5-ene-3β,7β,19-triol 3β,7β,19-triacetate [134] — colourless oil; $[\alpha]_D^{21}$ +18° (c 0.70, CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 5.47 (1H, d, $J_{6,7}$ = 2.1 Hz, H-6), 4.99 (1H, d, $J_{7,8}$ = 8.4 Hz, H-7), 4.61 (1H, m, H-3), 4.59 (1H, d, J_{gem} = 11.9 Hz, H-19), 3.94 (1H, d, J_{gem} = 11.8 Hz, H-19'), 2.38 (2H, br d, $J_{3,4}$ = 7.9 Hz, H-4), 2.08 (3H, s, OAc), 2.01 (3H, s, OAc), 2.00 (3H, s, OAc), 0.90 (3H, d, $J_{21,20}$ = 6.5 Hz, H-21), 0.85 (3H, d, $J_{26,25}$ = 6.6 Hz, H-26), 0.85 (3H, d, $J_{27,25}$ = 6.6 Hz, H-27), 0.70 (3H, s, H-18); ^{13}C NMR (CDCl$_3$) δ 171.0 (s, OAc), 170.6 (s, OAc), 170.3 (s, OAc), 139.0 (s, C-5), 126.4 (d, C-6), 74.5 (d, C-7), 72.7 (d, C-3), 64.0 (t, C-19), 56.2 (d, C-14), 55.4 (d, C-17), 48.2 (d, C-9), 43.0 (s, C-13), 39.8 (t, C-12), 39.6 (s, C-10), 39.5 (t, C-24), 37.6 (t, C-4), 37.6 (d, C-8), 36.1 (t, C-22), 35.7 (d, C-20), 33.2 (t, C-1), 28.3 (t, C-16), 28.0 (d, C-25), 27.8 (t, C-2), 24.9 (t, C-15), 23.8 (t, C-23), 22.8 (q, C-26), 22.5 (q, C-27), 21.6 (t, C-11), 21.6 (q, OAc), 21.3 (q, OAc), 21.0 (q, OAc), 18.7 (q, C-21), 11.8 (q, C-18); EIMS (70 eV) m/z (int. %) No M$^+$, 424 (33), 382 (32), 157 (43), 156 (48), 145 (38), 143 (100), 141((70), 43 (94).

Reduction of cholest-5-ene-3β,7β,19-triol 19-acetate [121] — the fractionation procedure in SCHEME 5.1 (section 5.2) was repeated in order to isolate further **[121]** for this reduction. LiAlH$_4$ was added to a stirred solution of **[121]** (19.2 mg) in dry ether (6 mL). After 20 min 5% m/v KOH (8 mL) was added to terminate the reaction. The basic solution was extracted with ether which was then dried over anhyd. Na$_2$SO$_4$,

filtered and concentrated under reduced pressure to yield **[135]** (10.4 mg).

Cholest-5-ene-3β,7β,19-triol [135] — white solid; mp 160 - 162 °C; $[\alpha]_D^{21}$ +35° (c 0.17, CHCl₃); ^{1}H NMR (CDCl₃) δ 5.65 (1H, br s, H-6), 3.83 (1H, d, J_{gem} = 11.6 Hz, H-19), 3.62 (1H, d, J_{gem} = 11.6 Hz, H-19'), 0.91 (3H, d, $J_{21,20}$ = 6.5 Hz, H-21), 0.86 (3H, d, $J_{26,25}$ = 6.6 Hz, H-26), 0.86 (3H, d, $J_{27,25}$ = 6.6 Hz, H-27), 0.74 (3H, s, H-18); ^{13}C NMR (CDCl₃) see TABLE 5.2 (section 5.3).

9-Deacetoxy-14,15-deepoxyxeniculin [110] — colourless oil; $[\alpha]_D^{21}$ = +126.9° (c 0.86, CHCl₃); IR (film) 2970, 2930, 2860, 1735, 1670, 1445, 1370, 1230, 1155, 1015, 1015 cm⁻¹; ^{1}H NMR (CDCl₃) and ^{13}C NMR (CDCl₃) see TABLE 5.4 (section 5.3); EIMS (70 eV) m/z (int. %) No M⁺, 359 (2), 349 (21), 342 (4), 333 (13), 291 (14), 231 (25), 149 (25), 91 (37), 69 (38), 43 (100); HREIMS m/z 402.2419 (calcd for C₂₄H₃₄O₅, 402.2406).

Zahavin A [16] — colourless oil; $[\alpha]_D^{21}$ +21.5° (c 0.77, CHCl₃); IR (film) 2960, 2920, 2850, 1735, 1365, 1230, 1155, 1010 cm⁻¹; ^{1}H NMR (CDCl₃) and ^{13}C NMR (CDCl₃) see TABLE 5.5 (section 5.3); EIMS (70 eV) m/z (int. %) No M⁺, 417 (1), 297 (4), 243 (9), 149 (30), 105 (23), 91 (20), 83 (23), 69 (16), 43 (100); HREIMS m/z 460.2448 (calcd for C₂₆H₃₆O₇, 460.2459).

7-Epoxyzahavin A [123] — colourless oil; $[\alpha]_D^{21}$ +121.7° (c 0.34, CHCl₃); IR (film) 2970, 2940, 2870, 1740, 1440, 1372, 1235, 1162, 1020, 915 cm⁻¹; ^{1}H NMR (CDCl₃) and ^{13}C NMR (CDCl₃) see TABLE 5.5 (section 5.3); EIMS (70 eV) m/z (int. %) No M⁺, 417 (1), 356 (5), 297 (6), 243 (15), 149 (23), 145 (23), 105 (23), 91 (27), 81 (25), 69 (17), 43 (100); HREIMS m/z 476.2419 (calcd for C₂₆H₃₆O₈, 476.2408).

Xeniolide C [124] — colourless oil; $[\alpha]_D^{21}$ +96.4° (c 1.17, CHCl₃); IR (film) 2970, 2925, 2870, 1735, 1645, 1438, 1365, 1225, 1025 cm⁻¹; ^{1}H NMR (CDCl₃) and ^{13}C NMR (CDCl₃) see TABLE 5.6 (section 5.3); EIMS (70 eV) m/z (int. %) No M⁺, 298 (9), 246 (60), 203 (20), 145 (37), 105 (48), 91 (82), 79 (57), 43 (100); HRIEMS m/z 358.2158 (calcd for C₂₂H₃₀O₄, 358.2143).

Attempted saponification of zahavin A [16] — dry K_2CO_3 (56.0 mg) was dissolved in H_2O (0.5 mL) and added to a stirred solution of **[16]** (18.0 mg) in MeOH (6 mL). The reaction was monitored by reverse phase TLC. After 16 h, H_2O (4.5 mL) was added and the MeOH was removed under reduced pressure. The aqueous layer was extracted with EtOAc which was then dried over anhyd. Na_2SO_4 and concentrated *in vacuo*. TLC and ^1H NMR spectroscopy suggested the presence of numerous rearrangment products. These products could not separated and purified by normal phase HPLC, with the exception of the rearrangement compound **[139]** (1.3 mg) (80% hex/EtOAc).

Rearrangment compound [139] — colourless oil; $[\alpha]_D^{21}$ -76.4° (*c* 0.16, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 9.43 (1H, s, H-20), 6.65 (1H, s, H-12), 5.91 (1H, d, J = 5.3 Hz, H-10), 5.49 (1H, t, J = 8.3 Hz, H-5), 5.19 (1H, s, H-18), 5.00 (1H, s, H-18'), 4.90 (1H, s, H-15), 4.66 (1H, s, H-15'), 4.61 (1H, s, H-16), 2.90 (1H, m, H-1), 2.45 (1H, m, H-6), 2.36 (1H, m, H-9), 2.30 (1H, m, H-3), 2.30 (2H, m, H-7), 2.17 (1H, m, H-3'), 2.17 (1H, m, H-6'), 1.69 (3H, s, H-14), 1.63 (3H, s, H-19), 1.51 (2H, m, H-2); ^{13}C NMR ($CDCl_3$) δ 193.6 (d), 156.3 (s), 145.1 (s), 140.9 (s), 139.8 (d), 136.1 (s), 134.3 (d), 130.2 (s), 125.0 (d), 114.3 (t), 111.6 (t), 77.2 (d), 49.5 (d), 39.8 (t), 37.7 (d), 35.8 (t), 34.4 (t), 25.3 (t), 18.7 (q), 16.9 (q); HREIMS *m/z* 298.1939 (calcd for $C_{20}H_{36}O_2$, 298.1933).

COLLECTION AND IDENTIFICATION — A bright pink colour variant of the soft coral *Alcyonium fauri* was collected using SCUBA (-15 m) at Riet Point near Port Alfred in September 1994. A voucher specimen (No. PA94-002) resides at Rhodes University. The animals were identified by Dr G. Williams of the California Academy of Sciences.

EXTRACTION AND ISOLATION — A portion of the EtOAc extract of the freeze-dried soft coral was fractionated according to SCHEME 6.1 (section 6.2). The fractionation yielded the new sesquiterpene hydroquinone rietone [141] (424.1 mg) in high yield, together with the minor compounds 8'-acetoxyrietone [142] (16.9 mg) and 8'-desoxyrietone [143] (3.9 mg).

Rietone [141] — orange oil; $[\alpha]_D^{19}$ +95° (c 0.8, CHCl$_3$); IR (CHCl$_3$) 3340, 3030, 2920, 1730, 1680, 1620, 1510, 1432, 1230, 1020 cm^{-1}; UV (MeOH) λ_{max} 209.0 (ϵ 9 600), 235.0 (ϵ 7 400), 297.0 (ϵ 2 400); ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$) see TABLE 6.1 (section 6.3); EIMS (70 eV) m/z (int. %) 416 (M$^+$, 2), 333 (17), 195 (34), 163 (26), 149 (11), 83 (100), 55 (28); HREIMS m/z 416.2188 (calcd for C$_{24}$H$_{32}$O$_6$, 416.2199).

8'-Acetoxyrietone [142] — orange oil; $[\alpha]_D^{21}$ +133° (c 0.9, CHCl$_3$); IR (CHCl$_3$) 3350, 3030, 2930, 1730, 1620, 1440, 1230, 1020 cm^{-1}; ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$) see TABLE 6.2 (section 6.3); EIMS (70 eV) m/z (int. %) 457 (M$^+$, 2), 233 (9), 189 (11), 149 (19), 135 (8), 85 (14), 83 (100), 55 (18), 43 (18); HREIMS m/z 458.2294 (calcd for C$_{26}$H$_{34}$O$_7$, 458.2303).

8'-Desoxyrietone [143] — orange oil; IR (CHCl$_3$) 3340, 3030, 2920, 1730, 1680, 1620, 1440, 1350, 1200, 1000 cm^{-1}; ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$) see TABLE 6.2 (section 6.3); EIMS (70 eV) m/z (int. %) 400 (M$^+$, 5), 212 (3), 151 (4), 123 (5), 84 (6), 83 (100), 55 (17); HREIMS m/z 400.2265 (calcd for C$_{24}$H$_{32}$O$_5$, 400.2250).

Oxidation of reitone [141] — Ag$_2$O (500 mg) and Mg$_2$SO$_4$ (500 mg) were added to a stirred solution of reitone (167 mg) in ether (20 mL). The solution was refluxed for 30 min, filtered through celite, and concentrated *in vacuo* to yield a dark red oil. TLC indicated the presence of starting material which was removed by normal phase HPLC (35% EtOAc/hexane) to give the sesquiterpene quinone [163] (21.3 mg) in low yield.

Sesquiterpene quinone [163] — red oil; $[\alpha]_D^{21}$ +231° (c 0.9, CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 6.68 (1H, s, ^{A}H-6), 6.54 (1H, s, ^{A}H-3), 6.12 (1H, s, H-10'), 5.59 (1H, t, J = 5.8 Hz, H-6'), 5.18 (1H, t, J = 7.2 Hz, H-2'), 4.42 (1H, d, J = 4.3 Hz, ^{B}H-8'), 4.13 (1H, d, J = 4.4 Hz, ^{B}OH-8'), 3.70 (3H, s, OMe), 3.43 (2H, d, J = 1.1 Hz, H-1''), 3.13 (2H, d, J = 7.2 Hz, H-1'), 2.23 (2H, m, H-5'), 2.20 (3H, s, H-12'), 2.14 (2H, m, H-4'), 1.91 (3H, s, H-15'), 1.64 (3H, s, H-13'), 1.42 (3H, s, H-14'); ^{13}C NMR (CDCl$_3$) δ 199.1 (s), 187.3 (s), 186.8 (s), 169.7 (s), 159.8 (s), 148.6 (s), 141.5 (s), 139.6 (s), 135.1 (d), 133.4 (d), 132.2 (d), 131.8 (d), 118.8 (d), 118.2 (d), 83.3 (d), 52.4 (q), 39.0 (t), 34.4 (t), 28.0 (q), 27.2 (t), 26.5 (t), 21.3 (q), 16.1 (q), 10.9 (q) - (protons with identical superscripts may be interchanged).

Acetylation of rietone [141] — Ac$_2$O (1 mL) was added to a solution of rietone (27.8 mg) in pyridine (1 mL) and the reaction was left overnight. EtOH (1.5 mL) was added to quench the excess Ac$_2$O and the solution was concentrated *in vacuo* to yield 2,5,8'-triacetoxyrietone [164] (30.8 mg).

Acetylation of 8'-acetoxyrietone [142] — the acetylation of [142] (4.8 mg) was conducted under the same conditions as were used for rietone and the resulting 2,5,8'-triacetoxyrietone product (5.8 mg) was identical to [164] with respect to $[\alpha]_D^{21}$, IR and NMR data.

2,5,8'-Triacetoxyrietone [164] — orange oil; $[\alpha]_D^{21}$ +142° (c 1.5, CHCl$_3$); IR (CHCl$_3$) 3030, 1740, 1620, 1440, 1370, 1230, 1170, 1020, 920 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 7.00 (1H, s, H-3), 6.93 (1H, s, H-6), 6.08 (1H, s, H-10'), 5.67 (1H, t, J = 6.5 Hz, H-6'), 5.36 (1H, s, H-8'), 5.22 (1H, t, J = 7.2 Hz, H-2'), 3.65 (3H, s, OMe), 3.49 (2H, s, H-1''), 3.20 (2H, d, J = 7.2 Hz, H-1'), 2.27 (6H, s, OAc-2 and OAc -5), 2.18 (2H, m, H-5'), 2.14 (3H,

s, H-12'), 2.13 (3H, s, OAc-8'), 2.10 (2H, m, H-4'), 1.86 (3H, s, H-15'), 1.66 (3H, s, H-13'), 1.58 (3H, s, H-14'); ^{13}C NMR (CDCl$_3$) δ 193.7 (s, C-9'), 170.7 (s, C-2''), 170.1 (s, OAc-8'), 169 (s, OAc-2 and OAc -5), 158.7 (s, C-11'), 146.6 (s, C-5), 146.3 (s, C-2), 136.8 (s, C-3'), 133.5 (d, C-6'), 133.7 (s, C-1), 129.2 (s, C-7'), 125.1 (s, C-4), 124.7 (d, C-3), 123.6 (d, C-6), 121.3 (d, C-2'), 119.6 (d, C-10'), 84.7 (d, C-8'), 52.1 (q, OMe), 38.7 (t, C-4'), 35.9 (t, C-1''), 28.3 (q, C-12'), 27.9 (t, C-1'), 26.5 (t, C-5'), 20.8 (q, C-15'), 20.8 (q, OAc-8', OAc-5 and OAc-2), 16.2 (q, C-13'), 12.2 (q, C-14'); EIMS (70 eV) m/z (int. %) 542 (M$^+$, 1), 417 (14), 275 (4), 196 (8), 195 (7), 149 (9), 84 (9), 83 (100), 55 (14), 43 (63); HREIMS m/z 542.2517 (calcd for C$_{30}$H$_{38}$O$_9$, 542.2513).

REFERENCES

182

1.   De Vries, D.J.; Beart, P.M. *TiPS* **1995**, *16*, 275.

2.   Ireland, C.M.; Roll, D.M.; Molinski, T.F.; McKee, T.C.; Zabriskie, T.M.; Swersey, J.C. In *Biomedical Importance of Marine Organisms*; Fautin, D. Ed.; California Academy of Sciences: San Francisco, **1988**, Vol. 13, pp 41-47.

3.   Scheuer, P.J. In *Chemistry of Marine Natural Products*; Academic Press: New York, 1973.

4.   Faulkner, D.J. *Tetrahedron* **1977**, *33*, 1421.

5.   Faulkner, D.J. *Nat. Prod. Reports* **1984**, *1*, 251.

6.   Faulkner, D.J. *Nat. Prod. Reports* **1984**, *1*, 551.

7.   Faulkner, D.J. *Nat. Prod. Reports* **1986**, *3*, 1.

8.   Faulkner, D.J. *Nat. Prod. Reports* **1987**, *4*, 539.

9.   Faulkner, D.J. *Nat. Prod. Reports* **1988**, *5*, 613.

10.   Faulkner, D.J. *Nat. Prod. Reports* **1990**, *7*, 269.

11.   Faulkner, D.J. *Nat. Prod. Reports* **1991**, *8*, 97.

12.   Faulkner, D.J. *Nat. Prod. Reports* **1992**, *9*, 323.

13.   Faulkner, D.J. *Nat. Prod. Reports* **1994**, *11*, 355.

14.   Faulkner, D.J. *Nat. Prod. Reports* **1995**, *12*, 223.

15.   *Marine Natural Products, Chemical and Biological Perspectives*; Scheuer, P.J. Ed.; Academic Press: New York, 1978; Vol. 1.

16.   *Marine Natural Products, Chemical and Biological Perspectives*; Scheuer, P.J. Ed.; Academic Press: New York, 1978; Vol. 2.

17.   *Marine Natural Products, Chemical and Biological Perspectives*; Scheuer, P.J. Ed.; Academic Press: New York, 1980; Vol. 3.

18.   *Marine Natural Products, Chemical and Biological Perspectives*; Scheuer, P.J. Ed.; Academic Press: New York, 1981; Vol. 4.

19.   *Marine Natural Products, Chemical and Biological Perspectives*; Scheuer, P.J. Ed.; Academic Press: New York, 1983; Vol. 5.

20. *Bioorganic Marine Chemistry*; Scheuer, P.J. Ed.; Springer-Verlag: Heidelberg, 1987; Vol. 1.

21. *Bioorganic Marine Chemistry*; Scheuer, P.J. Ed.; Springer-Verlag: Heidelberg, 1988; Vol. 2.

22. *Bioorganic Marine Chemistry*; Scheuer, P.J. Ed.; Springer-Verlag: Heidelberg, 1989; Vol. 3.

23. *Bioorganic Marine Chemistry*; Scheuer, P.J. Ed.; Springer-Verlag: Heidelberg, 1991; Vol. 4.

24. Faulkner, D.J. *Chemistry in Britain* **1995**, *31*, 680.

25. Pawlik, J.R. *Chem. Rev.* **1993**, *93*, 1911.

26. Williams, D.H.; Stone, M.J.; Hauck, P.R.; Rahman, S.K. *J. Nat. Prod.* **1989**, *52*, 1189.

27. Rouhi, A.M. *C&EN* **1995**, 42.

28. Puentes, J.L.F.; Rinehart, K. *Pharmaceutical Manufacturing International* **1995**, 17.

29. Cragg, G.M.; Schepartz, S.A.; Suffness, M.; Grever, M.R. *J. Nat. Prod.* **1993**, *56*, 1657.

30. Anderson, I. *New Scientist* **1995**, *2005*, 5.

31. Pettit, G.R.; Herald, C.L.; Cichacz, Z.A.; Gao, F.; Schmidt, J.M.; Boyd, M.R.; Christie, N.D.; Boettner, F.E. *J. Chem. Soc., Chem. Commun.* **1993**, 1805.

32. Schaufelberger, D.E.; Koleck, M.P.; Beutler, J.A.; Vatakis, A.M.; Alvarado, A.B.; Andrews, P.; Marzo, L.V.; Muschik, G.M.; Roach, J.; Ross, J.T.; Lebherz, W.B.; Reeves, M.P.; Eberwein, R.M.; Rodgers, L.L.; Testerman, R.P.; Snader, K.M.; Forenza, S. *J. Nat. Prod.* **1991**, *54*, 1265.

33. Putterman, D.M. *Nature* **1994**, *371*, 553.

34. Baker, J.T.; Borris, R.P.; Carté, B.; Cordell, G.A.; Soejarto, D.D.; Cragg, G.M.; Gupta, M.P.; Iwu, M.M.; Madulid, D.R.; Tyler, V.E. *J. Nat. Prod.* **1995**, *58*, 1325.

35. Rapson, W.S.; Schwartz, H.M. *J. Soc. Chem. Ind.* **1944**, *63*, 18.

36. Rapson, W.S.; Schwartz, H.M.; Van Rensburg, N.J. *J. Soc. Chem. Ind.* **1943**, *62*, 221.

37.   Silk, M.H.; Stephton, H.H.; Hahn, H.H. *Biochem. J.* **1954**, *57*, 574.

38.   Silk, M.H.; Hahn, H.H. *Biochem. J.* **1954**, *57*, 577.

39.   Silk, M.H.; Hahn, H.H. *Biochem. J.* **1954**, *57*, 582.

40.   Whiting, M.C. *Annual Reports of the Chemical Society* **1954**, 186.

41.   Clingman, A.L.; Nunn, J.R.; Stephen, A.M. *J. Chem. Soc.* **1957**, *34*, 197.

42.   Nunn, J.R.; Von Holdt, M.M. *J. Chem. Soc.* **1957**, *215*, 1094.

43.   Clingman, A.L.; Nunn, J.R. *J. Chem. Soc.* **1959**, *102*, 493.

44.   Parolis, H. PhD. Thesis, Rhodes University, Grahamstown, South Africa, 1967.

45.   Farrant, A.J. PhD. Thesis, Rhodes University, Grahamstown, South Africa, 1971.

46.   Russell, I. PhD. Thesis, Rhodes University, Grahamstown, South Africa, 1971.

47.   Allsobrook, A.J.R. PhD. Thesis, Rhodes University, Grahamstown, South Africa, 1973.

48.   Riekert, H.C. In *Drugs from the Sea*; The Government Printer: Pretoria, 1972; pp 1-20.

49.   Elsworth, J.F.; Cragg, G.M.L. In *UCT: Natural Products Research Group Report 2*; UCT: Cape Town, 1978; pp 1-13.

50.   Ballantine, J.A.; Lavis, A.; Roberts, J.C.; Morris, R.J.; Elsworth, J.F.; Cragg, G.M.L. *Comp. Biochem. Physiol.* **1978**, *61 B*, 43.

51.   Elsworth, J.F.; Cragg, G.M.L. In *UCT: Natural Products Research Group Report 3*; UCT: Cape Town, 1979; pp 1-21.

52.   Elsworth, J.F. In *UCT: Natural Products Research Group Report 4*; UCT: Cape Town, 1980; pp 1-21.

53.   Pelling, S. Honours Project Thesis, University of Cape Town, Cape Town, South Africa, 1979.

54.   Elsworth, J.F. In *UCT: Natural Products Research Group Report 7*; UCT: Cape Town, 1983; pp 1-55.

55.   Elsworth, J.F.; Naude, W.D. *South African Journal of Science* **1986**, *82*, 47.

56.   Elsworth, J.F.; Thomson, R.H. *J. Nat. Prod.* **1989**, *52*, 893.

57. Pettit, G.R.; Cichacz, Z.A.; Herald, C.L.; Gao, F.; Boyd, M.R.; Schmidt, J.M.; Hamel, E.; Bai, R. *J. Chem. Soc., Chem. Commun.* **1994**, 1605.

58. Pettit, G.R.; Cichacz, Z.A.; Gao, F.; Herald, C.L.; Boyd, M.R.; Schmidt, J.M.; Hooper, J.N.A. *J. Org. Chem.* **1993**, *58*, 1302.

59. Williams, G.C. *Annals of the South African Museum* **1992**, *100*, 249.

60. Lin, Y.; Bewley, C.A.; Faulkner, D.J. *Tetrahedron* **1993**, *49*, 7977.

61. D'Ambrosio, M.; Guerriero, A.; Pietra, F. *Helv. Chim. Acta* **1987**, *70*, 2019.

62. D'Ambrosio, M.; Guerriero, A.; Pietra, F. *Helv. Chim. Acta* **1988**, *71*, 964.

63. Rudi, A.; Ketzinel, S.; Goldberg, I.; Stein, Z.; Kashman, Y.; Benayahu, Y.; Schleyer, M. *J. Nat. Prod.* **1995**, *58*, 1581.

64. Green, D.; Carmely, S.; Benayahu, Y.; Kashman, Y. *Tetrahedron Lett.* **1988**, *29*, 1605.

65. Rudi, A.; Talpir, R.; Kashman, Y.; Benayahu, Y.; Schleyer, M. *J. Nat. Prod.* **1993**, *56*, 2178.

66. De Guzman, F.S.; Schmitz, F.J. *J. Nat. Prod.* **1990**, *53*, 926.

67. Kushlan, D.M.; Faulkner, D.J. *J. Nat. Prod.* **1991**, *54*, 1451.

68. Rudi, A.; Goldberg, I.; Stein, Z.; Benayahu, Y.; Schleyer, M.; Kashman, Y. *Tetrahedron Lett.* **1993**, *34*, 3943.

69. Rudi, A.; Kashman, Y.; Benayahu, Y.; Schleyer, M. *J. Nat. Prod.* **1994**, *57*, 1416.

70. Carmely, S.; Kashman, Y. *J. Org. Chem.* **1983**, *48*, 3517.

71. Carmely, S.; Loya, Y.; Kashman, Y. *Tetrahedron Lett.* **1983**, *24*, 3673.

72. Carmely, S.; Kashman, Y. *J. Org. Chem.* **1986**, *51*, 784.

73. Cimino, G.; Crispino, A.; Madaio, A.; Trivellone, E.; Uriz, M. *J. Nat. Prod.* **1993**, *56*, 534.

74. Rudi, A.; Goldberg, I.; Stein, Z.; Kashman, Y.; Benayahu, Y.; Schleyer, M.; Gravalos, M.D.G. *J. Nat. Prod.* **1995**, *58*, 1702.

75. Rudi, A.; Stein, Z.; Green, S.; Goldberg, I.; Kashman, Y.; Benayahu, Y.; Schleyer, M. *Tetrahedron Lett.* **1994**, *35*, 2589.

76. Rudi, A.; Goldberg, I.; Stein, Z.; Frolow, F.; Benayahu, Y.; Schleyer, M.; Kashman, Y. *J. Org. Chem.* **1994**, *59*, 999.

77. Andersen, R.J.; Faulkner, D.J.; Cun-heng, H.; Van Duyne, G.D.; Clardy, J. *J. Am. Chem. Soc.* **1985**, *107*, 5492.

78. Lindquist, N.; Fenical, W.; Van Duyne, G.D.; Clardy, J. *J. Org. Chem.* **1988**, *53*, 4570.

79. Faulkner, D.J. In *Biomedical Importance of Marine Organisms*; Fautin, D. Ed.; California Academy of Sciences: San Francisco, **1988**, Vol. 13, pp 29-36.

80. Carté, B.; Faulkner, D.J. *J. Chem. Ecol.* **1986**, *12*, 795.

81. Pika, J.; Faulkner, D.J. *Tetrahedron* **1994**, *50*, 3065.

82. D'Ambrosio, M.; Guerriero, A.; Pietra, F. *Helv. Chim. Acta* **1987**, *70*, 612.

83. Coll, J.C. *Chem. Rev.* **1992**, *92*, 613.

84. Hooper, G.J.; Davies-Coleman, M.T. *Tetrahedron* **1995**, *51*, 9973.

85. Pika, J.; Faulkner, D.J. *Tetrahedron* **1995**, *51*, 8189.

86. Groweiss, A.; Shmueli, U.; Kashman, Y. *J. Org. Chem.* **1983**, *48*, 3512.

87. Branch, G.M.; Griffiths, C.L.; Branch, M.L.; Beckley, L.E. In *Two Oceans: A Guide to the Marine Life of Southern Africa*; David Philip: Cape Town, 1994; p 1.

88. Day, J.H.; Field, J.G.; Penrith, M.J. *Transactions of the Royal Society of South Africa* **1970**, *39*, 1.

89. Faulkner, D.J. *Tetrahedron Lett.* **1973**, 3821.

90. Martinez, A.; Duque, C.; Sato, N.; Tanaka, R.; Fujimoto, Y. *Nat. Prod. Lett.* **1995**, *6*, 1.

91. Perry, N.B.; Battershill, C.N.; Blunt, J.W.; Fenwick, G.D.; Munro, M.H.G.; Bergquist, P.R. *Biochemical Systematics and Ecology* **1987**, *15*, 373.

92. Barrow, C.J.; Blunt, J.W.; Munro, M.H.G.; Perry, N.B. *J. Nat. Prod.* **1988**, *51*, 275.

93. Ishibashi, M.; Kurosaki, M.; Mikami, Y.; Kobayashi, J. *Nat. Prod. Lett.* **1993**, *3*, 189.

94.	Capon, R.J.; Dargaville, T.R.; Davis, R. *Nat. Prod. Lett.* **1994**, *4*, 51.

95.	Biskupiak, J.E.; Ireland, C.M. *Tetrahedron Lett.* **1983**, *24*, 3055.

96.	Garson, M.J.; Small, C.J.; Skelton, B.W.; Thinapong, P.; White, A.H. *J. Chem. Soc., Perkin Trans. 1* **1990**, 805.

97.	Davidson, B.S. *Chem. Rev.* **1993**, *93*, 1771.

98.	Perry, N.B.; Blunt, J.W.; Munro, M.H.G. *Aust. J. Chem.* **1991**, *44*, 627.

99.	Jares-Erijman, E.A.; Bapat, C.P.; Lithgow-Bertelloni, A.; Rinehart, K.L.; Sakai, R. *J. Org. Chem.* **1993**, *58*, 5732.

100.	Searle, P.A.; Molinski, T.F. *J. Org. Chem.* **1993**, *58*, 7578.

101.	Gulavita, N.K.; Scheuer, P.J. *J. Org. Chem.* **1989**, *54*, 366.

102.	Jiménez, C.; Crews, P. *J. Nat. Prod.* **1990**, *53*, 978.

103.	Hooper, G.J.; Davies-Coleman, M.T.; Coetzee, P.S. *Nat. Prod. Lett.* **1995**, *6*, 31.

104.	Williams, D.H.; Fleming, I. In *Spectroscopic Methods in Organic Chemistry*, 4th ed.; McGraw-Hill: London, 1987; p 93.

105.	Harada, N.; Nakanishi, K. In *Circular Dichroic Spectroscopy: Exciton Coupling in Organic Stereochemistry*; University Science Books: Mill Valley, CA, 1983; pp 1-460.

106.	Kawai, M.; Nagai, U.; Katsumi, M. *Tetrahedron Lett.* **1975**, 3165.

107.	Martin, A.; Swarbrick, J.; Cammarata, A. In *Physical Pharmacy: Physical Chemical Principles in the Pharmaceutical Sciences*, 3rd ed.; Lea & Febiger: Philadelphia, 1983; p 453.

108.	Leiner, J.; Simek, A.; Capek, A. *Pharm. Ind.* **1970**, *32*, 940.

109.	Albericci, M.; Braekman, J.C.; Daloze, D.; Tursch, B. *Tetrahedron* **1982**, *38*, 1881.

110.	Capon, R.J.; MacLoed, J.K.; Willis, A.C. *J. Org. Chem.* **1987**, *52*, 339.

111.	Capon, R.J.; MacLoed, J.K. *Tetrahedron* **1985**, *41*, 3391.

112.	Kashman, Y.; Groweiss, A.; Lidor, R.; Blasberger, D.; Carmely, S. *Tetrahedron* **1985**, *41*, 1905.

113. Perry, N.B.; Blunt, J.W.; McCombs, J.D.; Munro, M.H.G. *J. Org. Chem.* **1986**, *51*, 5476.

114. Perry, N.B.; Blunt, J.W.; Munro, M.H.G. *Tetrahedron* **1988**, *44*, 1727.

115. Kobayashi, J.; Cheng, J.; Ishibashi, M.; Nakamura, H.; Ohizumi, Y.; Hirata, Y.; Sasaki, T.; Lu, H.; Clardy, J. *Tetrahedron Lett.* **1987**, *28*, 4939.

116. Perry, N.B.; Blunt, J.W.; Munro, M.H.G.; Higa, T.; Sakai, R. *J. Org. Chem.* **1988**, *53*, 4127.

117. Cheng, J.; Ohizumi, Y.; Wälchli, M.R.; Nakamura, H.; Hirata, Y.; Sasaki, T.; Kobayashi, J. *J. Org. Chem.* **1988**, *53*, 4621.

118. Blunt, J.W.; Munro, M.H.G.; Battershill, C.N.; Copp, B.R.; McCombs, J.D.; Perry, N.B.; Prinsep, M.; Thompson, A.M. *New J. Chem.* **1990**, *14*, 761.

119. Kobayashi, J.; Cheng, J.; Yamamura, S.; Ishibashi, M. *Tetrahedron Lett.* **1991**, *32*, 1227.

120. Sakemi, S.; Sun, H.H.; Jefford, C.W.; Bernardinelli, G. *Tetrahedron Lett.* **1989**, *30*, 2517.

121. Sun, H.H.; Sakemi, S.; Burres, N.; McCarthy, P. *J. Org. Chem.* **1990**, *55*, 4964.

122. Stierle, D.B.; Faulkner, D.J. *J. Nat. Prod.* **1991**, *54*, 1131.

123. Copp, B.R.; Ireland, C.M.; Barrows, L.R. *J. Org. Chem.* **1991**, *56*, 4596.

124. Lee, K.H.; Imakura, Y.; Haruna, M.; Beers, S.A.; Thurston, L.S.; Dai, H.J.; Chen, C.H.; Liu, S.Y.; Cheng, Y.C. *J. Nat. Prod.* **1989**, *52*, 606.

125. Radisky, D.C.; Radisky, E.S.; Barrows, L.R.; Copp, B.R.; Kramer, R.A.; Ireland, C.M. *J. Am. Chem. Soc.* **1993**, *115*, 1632.

126. Carney, J.R.; Scheuer, P.J.; Kelly-Borges, M. *Tetrahedron* **1993**, *49*, 8483.

127. Lill, R.E.; Major, D.A.; Blunt, J.W.; Munro, M.H.G.; Battershill, C.N.; McLean, M.G.; Baxter, R.L. *J. Nat. Prod.* **1995**, *58*, 306.

128. Yang, A.; Baker, B.J.; Grimwade, J.; Leonard, A.; McClintock, J.B. *J. Nat. Prod.* **1995**, *58*, 1596.

129. Kita, Y.; Tohma, H.; Inagaki, M.; Hatanaka, K.; Yakura, T. *J. Am. Chem. Soc.* **1992**, *114*, 2175.

130.   Izawa, T.; Nishiyama, S.; Yamamura, S. *Tetrahedron* **1994**, *50*, 13593.

131.   Copp, B.R.; Fulton, K.F.; Perry, N.B.; Blunt, J.W.; Munro, M.H.G. *J. Org. Chem.* **1994**, *59*, 8233.

132.   Gunatilaka, A.A.L.; Kingston, D.G.I.; Johnson, R.K. *Pure & Appl. Chem.* **1994**, *66*, 2219.

133.   Tursch, B. *Pure & Appl. Chem.* **1976**, *48*, 1.

134.   Kaisin, M.; Sheikh, Y.M.; Durham, L.J.; Djerassi, C.; Tursch, B.; Daloze, D.; Braekman, J.C.; Losman, D.; Karlsson, R. *Tetrahedron Lett.* **1974**, 2239.

135.   Sheikh, Y.M.; Singy, G.; Kaisin, M.; Eggert, H.; Djerassi, C.; Tursch, B.; Daloze, D.; Braekman, J.C. *Tetrahedron* **1976**, *32*, 1171.

136.   Sheikh, Y.M.; Djerassi, C.; Braekman, J.C.; Daloze, D.; Kaisin, M.; Tursch, B.; Karlsson, R. *Tetrahedron* **1977**, *33*, 2115.

137.   Kaisin, M.; Tursch, B.; Declercq, J.P.; Germain, G.; Van Meerssche, M. *Bull. Soc. Chim. Belg.* **1979**, *88*, 253.

138.   Ayanoglu, E.; Gebreyesus, T.; Beechan, C.M.; Djerassi, C. *Tetrahedron* **1979**, *35*, 1035.

139.   Kaisin, M.; Braekman, J.C.; Daloze, D.; Tursch, B. *Tetrahedron* **1985**, *41*, 1067.

140.   Blackman, A.J.; Heaton, A.; Skelton, B.W.; White, A.H. *Aust. J. Chem.* **1985**, *38*, 565.

141.   Higgs, M.D.; Faulkner, D.J. *Steroids* **1977**, *30*, 379.

142.   Ballantine, J.A.; Williams, K.; Burke, B.A. *Tetrahedron Lett.* **1977**, 1547.

143.   Kingston, J.F.; Gregory, B.; Fallis, A.G. *Tetrahedron Lett.* **1977**, 4261.

144.   Cimino, G.; Desiderio, B.; De Stefano, S.; Sodano, G. *Experientia* **1979**, *35*, 298.

145.   Vanderah, D.J.; Steudler, P.A.; Ciereszko, L.S.; Schmitz, F.J.; Ekstrand, J.D.; Van der Helm, D. *J. Am. Chem. Soc.* **1977**, *99*, 5780.

146.   Groweiss, A.; Kashman, Y. *Tetrahedron Lett.* **1978**, 2205.

147.   Kashman, Y.; Groweiss, A. *Tetrahedron Lett.* **1978**, 4833.

148. Braekman, J.C.; Daloze, D.; Tursch, B.; Declercq, J.P.; Germain, G.; Van Meerssche, M. *Bull. Soc. Chim. Belg.* **1979**, *88*, 71.

149. Kashman, Y.; Groweiss, A. *J. Org. Chem.* **1980**, *45*, 3814.

150. Bowden, B.F.; Coll, J.C.; Ditzel, E.; Mitchell, S.J.; Robinson, W.T. *Aust. J. Chem.* **1982**, *35*, 997.

151. Groweiss, A.; Kashman, Y. *Tetrahedron* **1983**, *39*, 3385.

152. Lelong, H.; Ahond, A.; Chiaroni, A.; Poupat, C.; Riche, C.; Potier, P.; Pusset, J.; Pusset, M.; Laboute, P.; Menou, J.L. *J. Nat. Prod.* **1987**, *50*, 203.

153. Almourabit, A.; Ahond, A.; Chiaroni, A.; Poupat, C.; Riche, C.; Potier, P.; Laboute, P.; Menou, J.L. *J. Nat. Prod.* **1988**, *51*, 282.

154. Almourabit, A.; Gillet, B.; Ahond, A.; Beloeil, J.C.; Poupat, C.; Potier, P. *J. Nat. Prod.* **1989**, *52*, 1080.

155. König, G.M.; Coll, J.C.; Bowden, B.F.; Gulbis, J.M.; MacKay, M.F.; La Barre, S.C.; Laurent, D. *J. Nat. Prod.* **1989**, *52*, 294.

156. Almourabit, A.; Ahond, A.; Poupat, C.; Potier, P. *J. Nat. Prod.* **1990**, *53*, 894.

157. Miyamoto, T.; Takenaka, Y.; Yamada, K.; Higuchi, R. *J. Nat. Prod.* **1995**, *58*, 924.

158. Iwagawa, T.; Amano, Y.; Hase, T.; Shiro, M. *Tetrahedron* **1995**, *51*, 11111.

159. Iwagawa, T.; Amano, Y.; Hase, T.; Shiro, M. *Chem. Lett.* **1995**, 695.

160. Coval, S.J.; Scheuer, P.J.; Matsumoto, G.K.; Clardy, J. *Tetrahedron* **1984**, *40*, 3823.

161. Tanaka, J.; Ogawa, N.; Liang, J.; Higa, T.; deNys, R.; Bowden, B.F.; Carroll, A.R.; Coll, J.C.; Bernardinelli, G.; Jefford, C.W. *Tetrahedron* **1994**, *50*, 9989.

162. Fusetani, N.; Asano, M.; Matsunaga, S.; Hashimoto, K. *Tetrahedron Lett.* **1987**, *28*, 5837.

163. Hokama, S.; Tanaka, J.; Higa, T.; Fusetani, N.; Asano, M.; Matsunaga, S.; Hashimoto, K. *Chem. Lett.* **1988**, 855.

164. Ochi, M.; Kataoka, K.; Tatsukawa, A.; Kotsuki, H.; Shibata, K. *Heterocycles* **1994**, *38*, 151.

165. Eggert, H.; VanAntwerp, C.L.; Bhacca, N.S.; Djerassi, C. *J. Org. Chem.* **1976**, *41*, 71.

166. Das, B.; Srinivas, K.V.N.S. *J. Nat. Prod.* **1992**, *55*, 1310.

167. Chihiro, M.; Nagamoto, H.; Takemura, I.; Kitano, K.; Komatsu, H.; Sekiguchi, K.; Tabusa, F.; Mori, T.; Tominaga, M.; Yabuuchi, Y. *J. Med. Chem.* **1995**, *38*, 353.

168. Kobayashi, M.; Kanda, F.; Damarla, S.R.; Rao, D.V.; Rao, C.B. *Chem. Pharm. Bull.* **1990**, *38*, 2400.

169. Zeng, L.; Li, X.; Su, J.; Fu, X.; Schmitz, F.J. *J. Nat. Prod.* **1995**, *58*, 296.

170. Jagodzinska, B.M.; Trimmer, J.S.; Fenical, W.; Djerassi, C. *J. Org. Chem.* **1985**, *50*, 1435.

171. Jagodzinska, B.M.; Trimmer, J.S.; Fenical, W.; Djerassi, C. *J. Org. Chem.* **1985**, *50*, 2988.

172. Su, J.; Yu, X.; Zeng, L.; Mak, T.C.W. *J. Nat. Prod.* **1989**, *52*, 934.

173. Tillekeratne, L.M.V.; Liyanage, G.K.; Ratnasooriya, W.D.; Ksebati, M.B.; Schmitz, F.J. *J. Nat. Prod.* **1989**, *52*, 1143.

174. Kobayashi, M. *J. Chem. Research(S)* **1994**, 44.

175. Kobayashi, M.; Rao, K.M.C.A.; Anjaneyulu, V. *J. Chem. Research(S)* **1994**, 140.

176. Anjaneyulu, A.S.R.; Sagar, K.S. *J. Chem. Research(S)* **1995**, 142.

177. Kashman, Y.; Carmely, S. *Tetrahedron Lett.* **1980**, *21*, 4939.

178. Kobayashi, M.; Krishna, M.M.; Haribabu, B.; Anjaneyulu, V. *Chem. Pharm. Bull.* **1993**, *41*, 87.

179. Findlay, J.A.; Patil, A.D. *Can. J. Chem.* **1985**, *63*, 2406.

180. Piccialli, V.; Sica, D. *J. Nat. Prod.* **1986**, *49*, 779.

181. Notaro, G.; Piccialli, V.; Sica, D.; Pronzato, R. *J. Nat. Prod.* **1992**, *55*, 773.

182. Fattorusso, E.; Lanzotti, V.; Magno, S.; Novellino, E. *J. Org. Chem.* **1985**, *50*, 2868.

183. Morand, P.; Van Tongerloo, A. *Steroids* **1973**, *21*, 65.

184. Bortolotto, M.; Braekman, J.C.; Daloze, D.; Losman, D.; Tursch, B. *Steroids* **1976**, *28*, 461.

185. Aiello, A.; Fattorusso, E.; Menna, M. *J. Nat. Prod.* **1992**, *55*, 321.

186. Rychnovsky, S.D.; Skalitzky, D.J. *Tetrahedron Lett.* **1990**, *31*, 945.

187. Kashman, Y.; Saltoun, M.; Rudi, A.; Benayahu, Y. *Tetrahedron Lett.* **1994**, *35*, 8855.

188. Solis, P.N.; Wright, C.W.; Anderson, M.M.; Gupta, M.P.; Phillipson, J.D. *Planta Med.* **1993**, *59*, 250.

189. Hammer, R.H. In *Principles of Medicinal Chemistry*, 3rd ed.; Foye, W.O. Ed.; Lea & Febiger: Philadelphia, 1989; p 394.

190. Hooper, G.J.; Davies-Coleman, M.T. *Tetrahedron Lett.* **1995**, *36*, 3265.

191. Fenical, W.; Sims, J.J.; Squatrito, D.; Wing, R.M.; Radlick, P. *J. Org. Chem.* **1973**, *38*, 2383.

192. Cimino, G.; De Stefano, S.; Minale, L. *Tetrahedron* **1973**, *29*, 2565.

193. Minale, L.; Riccio, R.; Sodano, G. *Tetrahedron Lett.* **1974**, 3401.

194. Fenical, W.; McConnell, O. *Experientia* **1975**, *31*, 1004.

195. Luibrand, R.T.; Erdman, T.R.; Vollmer, J.J.; Scheuer, P.J.; Finer, J.; Clardy, J. *Tetrahedron* **1979**, *35*, 609.

196. Djura, P.; Stierle, D.B.; Sullivan, B.; Faulkner, D.J.; Arnold, E.; Clardy, J. *J. Org. Chem.* **1980**, *45*, 1435.

197. Sarin, P.S.; Sun, D.; Thornton, A.; Müller, W.E.G. *J. Natl. Cancer Inst.* **1987**, *78*, 663.

198. Loya, S.; Hizi, A. *F.E.B.S. Letters* **1990**, *269*, 131.

199. Loya, S.; Tal, R.; Kashman, Y.; Hizi, A. *Antimicrob. Agents Chemother.* **1990**, *34*, 2009.

200. Wright, A.E.; Rueth, S.A.; Cross, S.S. *J. Nat. Prod.* **1991**, *54*, 1108.

201. Casapullo, A.; Minale, L.; Zollo, F. *J. Nat. Prod.* **1993**, *56*, 527.

202. Zubía, E.; Ortega, M.J.; Carballo, J.L.; Salvá, J. *Tetrahedron* **1994**, *50*, 8153.

203. Talpir, R.; Rudi, A.; Kashman, Y.; Loya, Y.; Hizi, A. *Tetrahedron* **1994**, *50*, 4179.

204. Bonny, M.L.; Capon, R.J. *J. Nat. Prod.* **1994**, *57*, 539.

205. De Rosa, S.; De Giulio, A.; Iodice, C. *J. Nat. Prod.* **1994**, *57*, 1711.

206. Jaspars, M.; Horton, P.A.; Madrid, L.H.; Crews, P. *J. Nat. Prod.* **1995**, *58*, 609.

207. Killday, K.B.; Wright, A.E.; Jackson, R.H.; Sills, M.A. *J. Nat. Prod.* **1995**, *58*, 958.

208. Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092.

209. Kusumi, T.; Fujita, Y.; Ohtani, I.; Kakisawa, H. *Tetrahedron Lett.* **1991**, *32*, 2923.

210. Kobayashi, M.; Chavakula, R.; Murata, O.; Sarma, N.S. *J. Chem. Research(S)* **1992**, 366.

211. Kusumi, T.; Hamada, T.; Hara, M.; Ishitsuka, M.O.; Ginda, H.; Kakisawa, H. *Tetrahedron Lett.* **1992**, *33*, 2019.

212. Rodríguez, J.; Riguera, R.; Debitus, C. *Tetrahedron Lett.* **1992**, *33*, 1089.

213. Fieser, L.F. *J. Am. Chem. Soc.* **1939**, *61*, 3467.

214. Couperus, P.A.; Clague, A.D.H.; Van-Dongen, J.P.M. *Org. Magn. Reson.* **1976**, *8*, 426.

215. Romero, D.L. *Ann. Rep. Med. Chem.* **1994**, *29*, 123.

216. Weislow, O.S.; Kiser, R.; Fine, D.L.; Bader, J.; Shoemaker, R.H.; Boyd, M.R. *J. Natl. Cancer Inst.* **1989**, *81*, 577.

217. Gulakowski, R.J.; McMahon, J.B.; Staley, P.G.; Moran, R.A.; Boyd, M.R. *J. Virol. Methods* **1991**, *33*, 87.

◆◆◆