Extractives from Six Species of South African Marine Opisthobranch Molluscs

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List of Abbreviations

Ac	acetyl
bp	boiling point
br	broad (used in conjunction with s, d or t)
n-BuLi	*normal*-Butyl Lithium
t-BuLi	*tertiary*-Butyl Lithium
CRRF	Coral Reef Research Foundation
COSY	^{1}H-^{1}H homonuclear COrrelation SpectroscopY
DABCO	1,4-diazabicyclo[2.2.2]octane
DEPT	Distortionless Enhancement by Polarisation Transfer
d	doublet
EIMS	Electron Impact Mass Spectrometry
EtOAc	ethyl acetate
EtOH	ethanol
eV	electron Volt
FABMS	Fast Atom Bombardment Mass Spectrometry
Fn	fraction
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
GLC	Gas Liquid Chromatography
hex	hexane
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherrence
HMPA	hexamethyl phosphoramide
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectrometry
HRFABMS	High Resolution Fast Atom Bombardment Mass Spectrometry
IR	InfraRed
LC	liquid chromatography

lit.	literature
m	multiplet
MeOH	methanol
mp	melting point
MTPA	α-methoxy-α-(trifluoromethyl)phenylacetic acid
NCI	National Cancer Institute (U.S.A.)
NMR	Nuclear Magnetic Resonance
NOE	nuclear Overhauser enhancement
NOEDS	Nuclear Overhauser Enhancement Difference Spectroscopy
NOESY	Nuclear Overhauser Enhancement SpectroscopY
q	quartet
qn	quintet
R	unspecified alkyl group
s	singlet
Si	silica
SCUBA	Self Contained Underwater Breathing Apparatus
t	triplet
TLC	Thin Layer Chromatography
TMEDA	tetramethyl ethylene diamine
UV	UltraViolet

Abstract

The natural product chemistry of six species of South African opisthobranch molluscs and some of their dietary marine invertebrates was investigated. Nineteen previously undescribed secondary metabolites and twelve known compounds were isolated and their structures determined by a combination of spectroscopic and chemical methods.

The circumtropical sea hares *Aplysia parvula* and *A. dactylomela* were found to contain halogenated red algal metabolites. 3*Z*-bromofucin (**120**), the *Z* analogue of a known *Laurencia* C_{15} acetogenin, was isolated from *A. parvula*. *A. dactylomela* yielded a series of novel non-aromatic cuparanes, the algoanes (**121-123**), the novel tricyclic laurane ether ibhayinol (**124**) and three known chamigrane sesquiterpenes, prepacifenol epoxide (**101**), pacif-7-enediol (**104**) and nidificene (**125**).

A variety of new octocoral sesquiterpenes were isolated from the endemic South African arminacean nudibranch *Leminda millecra* including algoafuran (**150**), cubebenone (**151**), 8-hydroxycalamenene (**152**) and a series of seven triprenylated toluquinones and toluquinols (**153-159**). *L. millecra* also yielded the known sesquiterpenes millecrones A (**142**) and B (**143**) and isofuranodiene (**149**). Twenty eight voucher specimens and eighteen crude extracts of South African octocorals collected by the Coral Reef Research Foundation were screened by GC and GC-MS and **142** was found in Alcyonium fauri, while **143**, **151** and possibly **149** were present in *Leptogorgia palma*.

An investigation of southern African chromodorids yielded the known macrocycle latrunculin B (**220**) and two new spongiane diterpenes (**221**) and (**222**) from *Chromodoris hamiltoni*, while the known spongiane diterpene (**210**) was isolated from the endemic

nudibranch *Glossodoris* sp. 4. The endemic nudibranch *Hypselodoris capensis* contained the known furanosesquiterpenes nakafuran-8 (**223**) and -9 (**224**) and the known furanosesterterpenes variabilin (**195**), 22-deoxyvariabilin (**225**) and furospinosulin (**227**) together with the new variant 22-deoxy-23-hydroxymethylvariabilin (**226**). Compounds **223** and **224** were also found in a *Dysidea* sponge, while the furanosesterterpenes **195**, and **225-227** were present in a *Fasciospongia* sponge upon which *H. capensis* specimens were found.

The *Dysidea* dietary sponge of *H. capensis* also yielded a new aromatic sesquiterpene, tsitsikammafuran (**266**), whose structure was confirmed by the synthesis of two possible regioisomers.

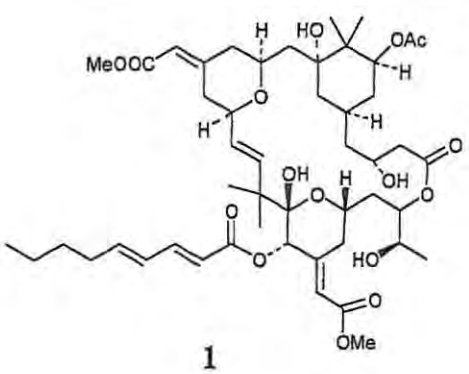
Chapter One

Introduction

For hundreds of years biologists and chemists have been retrieving clues pertaining to an alternative, chemically-mediated level on which life progresses, to which humans, without highly developed chemosenses, are not naturally privy. The chemicals (natural products) concerned are not involved in primary metabolism and therefore show an erratic distribution in living organisms. While the ecological role of most natural products is at best poorly understood, these secondary metabolites are recognized to be important for the survival of the producing organisms. Paradoxically, some of the biologically active natural products have also shown valuable potential as pharmaceuticals. Mere characterization of the phenomenal structural diversity of marine natural products in particular has been a formidable, all-consuming challenge to organic chemists for at least three decades. However, recent chemical reviews[1,2] have repeatedly alluded to a paradigm shift in the field of marine natural products chemistry. With the inevitable depletion of the stock of unprecedented molecular structures in the marine environment, and the increasingly insurmountable difficulties associated with collecting marine organisms in richly biodiverse third world countries, attention is turning away from pure structure elucidation to other challenging research areas. Among these research areas, the definition of the ecological roles of known marine secondary metabolites and scrutiny of the metabolites' interactions with biomolecular receptors to facilitate a more detailed understanding of the pharmaceutical potential of marine metabolites are proving to be very rewarding fields of endeavour.

Natural products are still considered to be important as leads for the development of new drugs.[3] However, it is somewhat surprising that, as in the past, the pharmaceutical potential of marine natural products continues to generate the bulk of the financial resource base necessary for marine natural products research. While, a number of marine metabolites, *e.g.* bryostatin 1 (**1**) and ecteinascidin 743 (**2**), have steadily progressed to Phase I/II clinical trials, and others such as eleutherobin (**3**) have proved to be exciting

leads in new drug development, no single marine metabolite is yet commercially available as a chemotherapeutic agent.[3] Conversely, an extract of the sea whip *Pseudopterogorgia elisabethae* (containing pseudopterosins) is an anti-inflammatory ingredient in the cosmetic product Resilience® marketed by Estée Lauder.[2]



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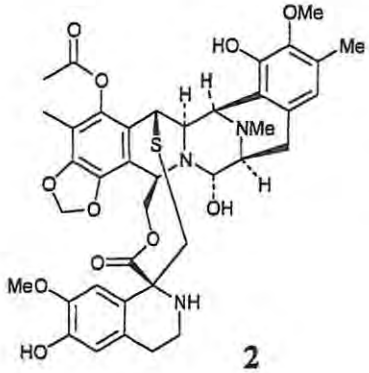

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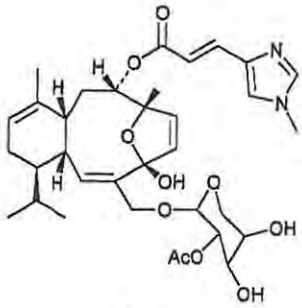

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Marine opisthobranch molluscs were among the first marine invertebrates subjected to chemical investigations, many being readily accessible in shallow tidal pools where they were observed to be remarkably free of predators in spite of the apparent vulnerability of their soft bodies. An attraction to early investigators intent on finding out why these animals were immune to predators was also the copious production of mucus by many opisthobranch molluscs when molested. In addition to fish feeding assays, early investigations by biologists therefore involved testing the acidity of the skin and secretions of opisthobranchs using pH paper and even human taste tests.[4] Accordingly many of the molluscs investigated were shown to produce acidic secretions when disturbed, although the presence of other 'defensive' secretions was noted in some cases.[4,5] Investigations of these other secretions and opisthobranch extracts containing a plethora of natural products, have since been facilitated by rapid advancements in chromatography, spectroscopy and crystallography. However, the trends in opisthobranch chemical research have remained dependent on both the technologies in hand at the time and current thinking of the leaders in the field. In 1995, Avila published a comprehensive 'biological' review on natural products from opisthobranchs,[6] which catalogued the chemical class, origin and activity of the pure natural products, extracts or secretions from all known species of opisthobranch molluscs whose secondary

metabolites have been investigated. Such a catalogue is invaluable to both biologists and chemists and revealed relationships in many instances between the order/genus of opisthobranch and the structural class of the metabolites that they either sequester or biosynthesize. However, a deficit in data on the origin and bioactivity of the natural products reported in chemical publications was also apparent and Avila emphasized the importance of consistently reporting these data in tandem with chemical structure elucidations in order to facilitate collaboration between biologists and chemists. There has been no subsequent review of this type and in the following introduction to this thesis, attention is drawn to recent developments (1995 until November 1999) in opisthobranch chemistry research in light of Avila's findings. In particular we highlight changes in the types of compounds now attracting chemists' attention, the recent increase in reports of anatomical location and biological activity testing of metabolites in chemical publications, new developments in biosynthetic studies of dorid nudibranchs and some considerations on the ecological/evolutionary role of natural products in the defense of opisthobranch molluscs.

1.1 New chemistry from opisthobranch molluscs – are new natural products harder to find?

Figure 1.1 represents a summary of the number and spread of chemical publications on natural products from five orders of opisthobranch molluscs. The nudibranchs, as the largest and most widely researched order of opisthobranchs have been subdivided into chromodorids, other dorids and remaining nudibranchs, while the anaspids are divided into *Aplysia*, *Dolabella* and other anaspids due to the large amount of literature on the former two genera. The recent decline in the number of publications describing the chemistry of opisthobranch molluscs is in line with the general trend observed across the full spectrum of marine natural product research,[7] and there appears to be a dramatic decrease in new metabolites reported from nudibranchs, particularly chromodorids, which dominated the chemical literature in the 1980's (Figure 1.1B).

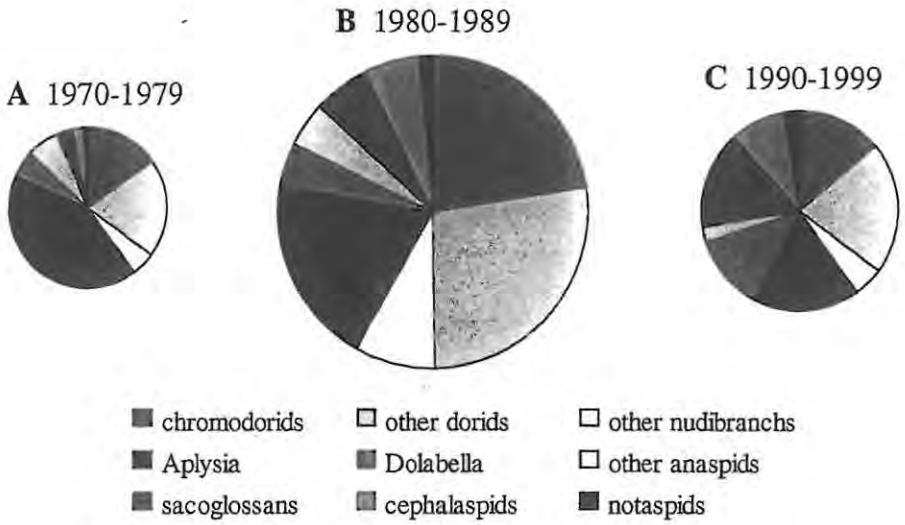
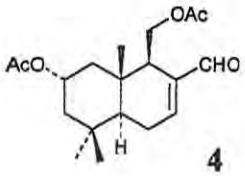


Figure 1.1 The relative numbers of chemical publications on nine groups of opisthobranch molluscs for the last three decades. The size of each piechart is proportional to the total number of publications in that decade (A = 132, B = 255, C = 163 respectively).

Most chromodorids are tropical and the vivid, potentially aposematic, coloration of many of these species, which show a remarkable skill at sequestering metabolites from a specific range of dietary organisms, led to their early collection and chemical investigation. In the last five years the reports of new chemistry from chromodorid species have yielded few metabolites with novel structural motifs and the recent literature on the genera *Hypselodoris, Chromodoris* and *Glossodoris* is included in the introductory discussion of Chapter Four. The number of publications highlighting the chemistry of other dorid nudibranchs has decreased only slightly in the 1990's. Furthermore several new species of dorids, including seven new genera, have been investigated and some of the metabolites isolated from these nudibranchs are reviewed here to illustrate the approach adopted in recent studies.

Notably many of the nudibranch species whose chemistry appears in the recent literature are from temperate or even polar climates and many of the compounds isolated are suggested to be biosynthesized *de novo*. Thus, mantle extracts of Antarctic *Bathydoris hodgsoni* specimens yielded an unusual 2-substituted drimane sesquiterpene, hodgsonal (**4**),[8] which was present in similar levels in individuals from different sites suggesting *de*

novo biosynthesis by the nudibranch analogous to *Dendrodoris* species which are known to biosynthesize drimane sesquiterpenes.[9] A skin extract of the North Sea nudibranch *Limacia clavigera* yielded the new diacylguanidine limaciamine (**5**),[10] which bears a close resemblance to triophamine (**6**) biosynthesized *de novo* by *Triopha catalinae*.[11,12]



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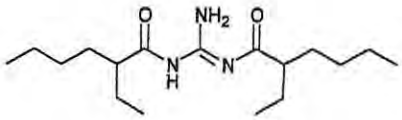

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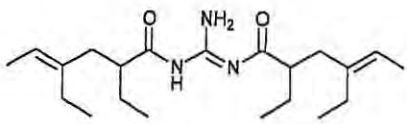

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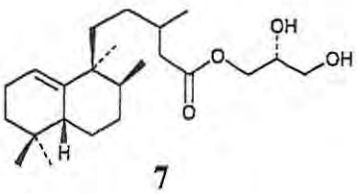
The diterpenoid glycerides isolated from dorid nudibranchs are recognized as products of *de novo* biosynthesis and exhibit significant biological activity, with some of them being potent morphogens and protein kinase C (PKC) activators. New diterpenoid glyceryl esters have been isolated from the relatively common Antarctic nudibranch *Austrodoris kerguelensis*,[13-15] including austrodorin (**7**)[13] and two unusual tricyclic diterpenoid 2'-monoglyceryl esters, austrodorins A (**8**) and B (**9**).[15] Four new tricyclic diterpenoid diacylglycerols, similar to **8** and **9**, and one diterpene (**10**) were characterized as minor metabolites from *Anisodoris fontaini* (previously reported as *Archidoris carvi*).[16]



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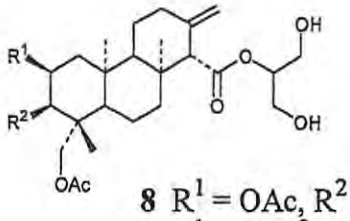
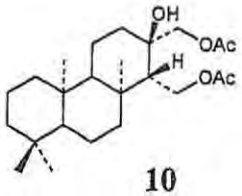
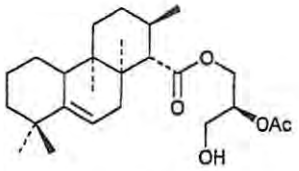


8 $R^1 = OAc, R^2 = H$
9 $R^1 = H, R^2 = OAc$



10

Verrucosin B (**11**) from the skin of *Doris verrucosa*,[17] was one of the first diacylglycerol to be used in studies on the participation of PKC in biological responses,[18] and its potent activity led to a search for similar new 1,2-*sn*-diacylglycerols. De Petrocellis *et al.*[19] have isolated five new 1,2-*sn*-diacylglycerols from *D. verrucosa* and tested these, together with several previously isolated diacylglycerols, as activators of rat brain PKC and in *Hydra* tentacle regeneration assays. Compound (**12**) proved to be more active than the other new tricyclic metabolites such as (**13**) and (**14**).



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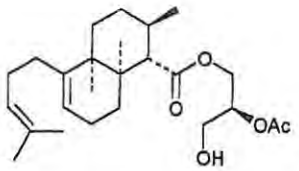

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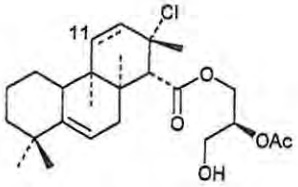
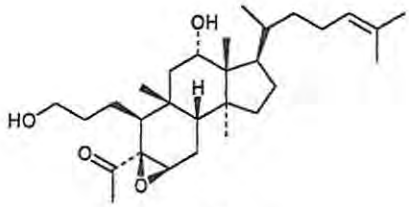


13 Δ^{11}, R = H
14 R = Cl

Other new metabolites which have been reported from previously uninvestigated dorids include lovenone (**15**), an unprecedented degraded triterpene from the skin of *Adalaria loveni*,[20] which is moderately active against human cancer cell lines and is thought to originate from a dietary bryozoan. Fontana *et al.*[21] examined the location of defense chemicals in the nudibranch *Tyrinna nobilis* whose skin contained the novel spongiane diterpene tyrinnal (**16**). *Reticulidia fungia* (F. Phyllidiidae), was found to contain four sesquiterpenes containing the rare carbonimidic dichloride functional group, of which the moderately cytotoxic reticulidins A (**17**) and B (**18**) were new.[22]



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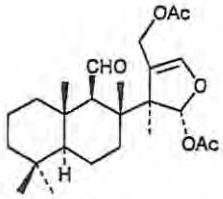

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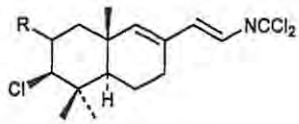


17 R = αOH
18 R = βOH

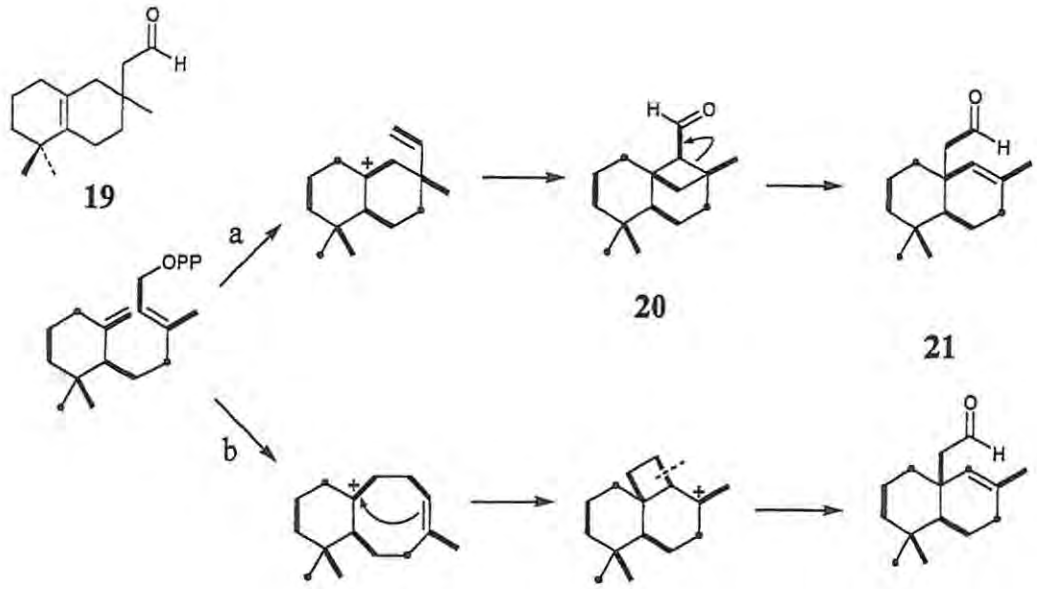
It is interesting that in the majority of recent studies on dorid nudibranchs, care has been taken to target the skin of the nudibranchs, where defense chemicals are most likely to be located. In addition some aspect of the biological activity of the novel metabolites isolated and their suggested or confirmed origin has been reported. The increase in the number of apparently *de novo* biosynthesized metabolites from temperate and polar dorid nudibranchs is in contrast to the traditional corollary of dietary derived metabolites for tropical chromodorids, and it is interesting to see whether this developing trend will become firmly established given the relative scarcity of prey species for dorids from the more extreme latitudes.[23] Not surprisingly, there has been an increase in the number of

biosynthetic studies involving opisthobranch molluscs. These are of interest not only for the confirmation of *de novo* biosynthesis that they provide but also for the stable isotope incorporation methodology employed which provides more detailed biosynthetic information than that gained from radioisotope incorporation studies.

1.2 *De novo* biosynthesis in opisthobranch molluscs

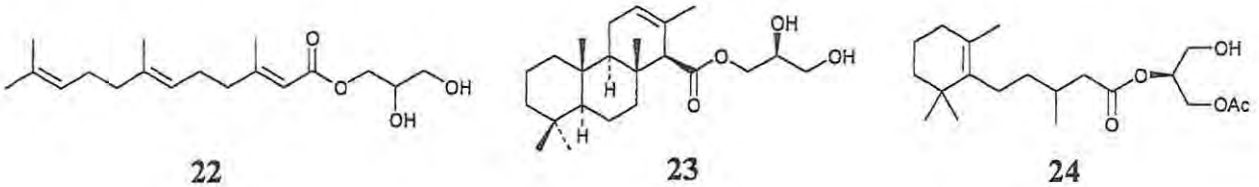
Generally, opisthobranchs capable of biosynthesis may be distinguished by a constant metabolite profile throughout their geographic range, and their secondary metabolites are absent from their dietary organisms.[24] Early studies on *de novo* biosynthesis in *Dendrodoris*, *Archidoris* and *Tethys* nudibranchs have been reviewed by Garson[9] and the metabolites concerned are also listed in Avila's catalogue.[6] All of these studies involved the use of radiolabelled precursors, which was thought to be necessary for detection of labelled products because of the very low rates of precursor incorporation typically observed in marine invertebrates.[9]

Recent biosynthetic studies of nudibranchs have shown the efficacy of using stable isotope (^{13}C) incorporation monitored by NMR spectroscopy, despite the fear that the higher levels of incorporated labelled precursor required for detection by NMR may alter the metabolic pathway being investigated. Graziani and Andersen[25] first used [1,2-$^{13}C_2$]acetate in feeding experiments to demonstrate the *de novo* biosynthesis of **19**, **20** and **21** by *Acanthodoris nanaimoensis*. An added advantage of the use of NMR spectroscopy to detect incorporation allows the location of labelled fragments within the molecule and therefore provides information about the mechanism of biosynthesis of the molecule. Hence, the expected biogenetic derivation of **21** from **20** (Scheme 1.1, path a) was disproved and a more compatible pathway (Scheme 1.1, path b) proposed. Furthermore, the use of a doubly labelled acetate precursor causes the incorporation of intact acetate units to be readily evident from the resulting doublet resonances for enriched carbons in the ^{13}C NMR spectra of labelled samples.



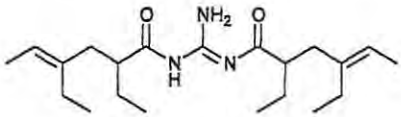
Scheme 1.1: Two biogenetic pathways (a and b) to compounds **20** and **21** proposed by Graziani and Andersen.[25]

The success of the latter study led Graziani and Andersen[26] to use stable isotopes to reinvestigate the biosynthesis of the terpenoic acid glycerides **22** and **23** by *Archidoris montereyensis and A. odhneri.* Although previous biosynthetic studies[17] on these two species using ^{14}C-labeled mevalonic acid had seemed to confirm that **22** and **23** were biosynthesized *de novo*, very low levels of incorporation of the labelled precursor were achieved and the biosynthesis of similar terpenoic acid glycerides by *Doris verrucosa* could not be demonstrated.[27] The use of [1,2-$^{13}C_2$]acetate confirmed that *A. montereyensis* and *A. odhneri* biosynthesize **22** and **23**, and this work was presented in tandem with that of Krug and Faulkner who demonstrated the biosynthesis of other terpenoic acid glycerides, the tanyolides *e.g.* tanyolide B (**24**), by *Sclerodoris tanya.*[26] The latter study used [2-^{13}C] mevalonolactone as a precursor and proved that sufficiently high levels of precursor incorporation can even be achieved for detection of singly labelled precursors, for which ^{13}C enrichment can only be detected by statistical analysis of peak heights in comparative ^{13}C NMR spectra of labelled and unlabelled products.



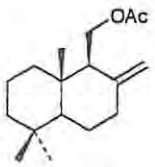
22 **23** **24**

Stable isotope incorporation has since been used to investigate the biosynthetic origin of different types of compounds in several dorid nudibranchs. The first application of stable isotopes to investigate polyketide biosynthesis[11] successfully showed that *Triopha catalinae* biosynthesizes the acyl residues of triophamine 1 (**6**) *de novo* from five intact acetate units. *Triopha catalinae* specimens invariably contained triophamine throughout their geographic range from Alaska to southern California and the absence of triophamine in possible dietary bryozoan species of *Triopha* pointed to its *de novo* biosynthesis by the nudibranch. Further biosynthetic feeding experiments[12] with [2,3-$^{13}C_2$]butyrate showed that the two ethyl branches in the acyl residues of triophamine are derived from the intact incorporation into triophamine of two butyrate units. This implied that ethyl malonate is an intermediate in polyketide biosynthesis, although ethyl malonate itself was not incorporated in sufficient amounts for detection by NMR spectroscopy.



6

Sesterterpene biosynthesis by a marine mollusc was demonstrated for the first time in *Cadlina luteomarginata*. Although, *C. luteomarginata* is well known to sequester a variety of structurally diverse terpenes from dietary sponges, British Columbia specimens of this nudibranch consistently contained albicanyl acetate (**25**), cadlinaldehyde (**26**) and luteone (**27**),[28] and the *de novo* biosynthesis of these metabolites by the nudibranch was recently demonstrated.[29] The authors observed efficient uptake of labelled precursors into **26** and **27** in spite of a low sensitivity of labelled molecules to detection. Kubanek *et al.*[29] consequently suggested that biosynthetic studies on marine invertebrates are limited by the large pool of unlabelled compound against which incorporation must be assessed, rather than inefficient incorporation of labelled precursors, even in nudibranchs which initially shed most of their accumulated unlabelled secondary metabolites when handled.



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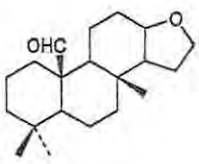

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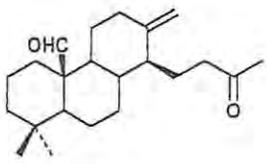
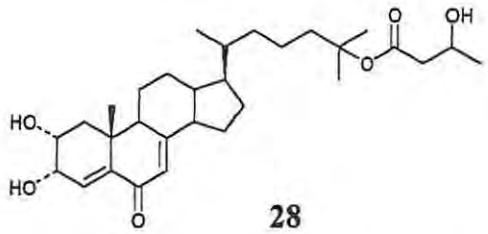


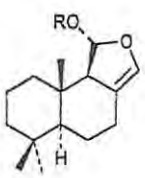
27

An attempt to extend the use of stable isotopes to the investigation of steroid biosynthesis was unsuccessful when only the butyrate substituent of diaulusterol A (**28**) from *Diaulula sandiegensis* could be shown to be biosynthesized by the nudibranch.[30] Interestingly, even incorporation of the radiolabelled precursors [2-^{14}C]mevalonate and [4-^{14}C] cholesterol into the steroid fragment of **28** could not be detected and this may be due to the close regulation of steroid biosynthesis which is thought to operate in invertebrates as well as vertebrates.[30]



28

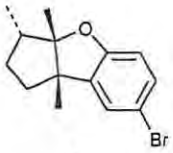
On occasion it has been found that opisthobranchs are in fact capable of biosynthesizing *de novo*, the same or similar metabolites present in potential dietary organisms. The isolation of drimanes, including large amounts of 7-deacetoxyolepupuane (**29**), from *Dysidea* sponges,[31] cast some doubt on the source of these same drimanes in *Dendrodoris* nudibranchs, even though the nudibranchs had previously been shown to be capable of *de novo* biosynthesis of drimanes.[9] Using radiolabelled mevalonate, Fontana *et al.*[32] have since confirmed the biosynthesis of drimanes in two geographically distinct populations of *Dendoris* nudibranchs. The role of **29** as a precursor to other drimanes was inferred from the results of feeding experiments at different times, which showed that the high initial level of radioactivity in **29** decreased over time, while that of the other drimanes increased. The latter study was recently extended by the use of both ^{14}C and ^{13}C labelled glucose in feeding experiments on *D. limbata* and *D. grandiflora*.[33] Specific incorporation of radiolabelled glucose into drimane compounds, and not sterols and fatty acids, demonstrated that glucose is a useful precursor for biosynthetic studies of nudibranchs while the pattern of incorporation of ^{13}C-labelled glucose into the drimane ester **30** confirmed that the drimanes in these *Dendrodoris* nudibranchs are products of the classical mevalonate pathway.



29 R = Ac
30 R = Fatty acid

1.3 Anaspids – a perennial source of new natural products

Surprisingly, investigations of anaspids continue to afford new metabolites and the number of publications on these opisthobranchs has remained consistently high, beginning in earnest in the 1970's after Yamamura's initial isolation of aplysin (**31**) in 1963.[34] Aplysin was the first of a plethora of halogenated dietary red algal metabolites to be isolated from anaspids. When the surge of interest in chromodorids tailed off in the early 1990's, *Aplysia* sea hares once again became the focus of attention with a resurgence of interest in halogenated compounds possibly generated from reports of the anticancer activity of the linear monoterpene halomon (**32**) in 1992.[35]



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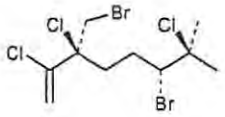
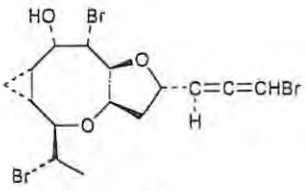


32

While there are still a substantial number of investigations of *Aplysia* sea hares being reported in the literature, far fewer halogenated algal metabolites are being reported from this genus. These include the icthyotoxic acetogenins aplyparvulin[36] (**33**) and bromoallene[37] (**34**), four new unusual acetylated linear monoterpenes **35-38** which were active against four tumour cell lines,[38] and a series of furanones, *e.g.* **39**,[39] whose bioactivity and distribution in *A. parvula* were investigated and are discussed further in Chapter Two.



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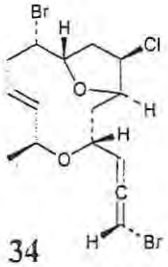

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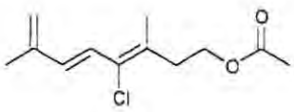

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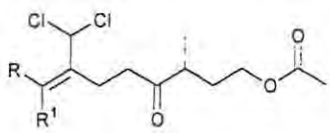
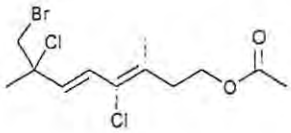


36 R = Cl, R^1 = H
37 R = H, R^1 = Cl



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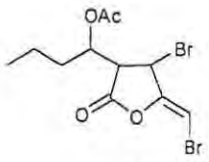
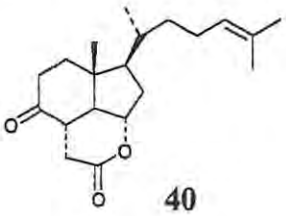


39

Importantly, these halogenated metabolites were all isolated from digestive gland or whole animal extracts of the sea hares, and a defensive role for these metabolites in sea hares is now thought to be improbable from the results of several recent ecological studies (see Chapter Two). Also, Cimino and Ghiselin[40] observed that since effective chemical defense must involve detoxification, storage and release of the dietary metabolites, it would be expected that only a specific group of compounds could be tolerated. Considering this, the varied diet of sea hares and the presence of a variety of algal products in their digestive glands is circumstantial evidence for the absence of an active chemical defense strategy involving the use of these stored metabolites.

Instead, sterols are tentatively suggested to be the thus far untraceable defense chemicals of sea hares.[41] A new degraded sterol, 3-*epi*-aplykurodinone (**40**), has been isolated from a skin extract of *A. fasciata*,[42] together with the previously reported aplykurodinone B (**41**) and aplykurodin B (**42**), while a series of five lactonized dihydroxy fatty acids similar to aplyolide A (**43**) were obtained from the skin of *A. depilans*.[43] Both sets of compounds have been shown to be icthyotoxic, and together with the few previously reported sterols, which were also isolated from skin extracts, are thought to be synthesized *de novo* by the sea hares.



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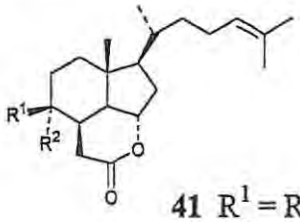
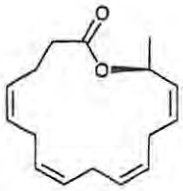


41 $R^1 = R^2 = O$

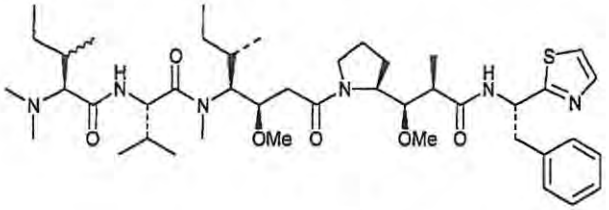
42 $R^1 = OH$, $R^2 = H$



43

Obviously the few reports discussed above do not account for the substantial amount of recent chemical literature pertaining to anaspid opisthobranch chemistry. Peptides from the sea hare *Dolabella auricularia*, which have potent antineoplastic activity, have dominated the opisthobranch chemical literature in the last five years, and perhaps precipitated a general search for peptides from opisthobranchs analogous to the well known, very promising anticancer agents dolastatins 10[44] (**44**) and 15 (**45**).[45] Work on

Indian Ocean specimens of *D. auricularia* was started by Pettit in 1972, and the range of 'dolastatins' which has since been isolated from Indian Ocean, and later Japanese, specimens of this sea hare has been reviewed in 1999.[46]



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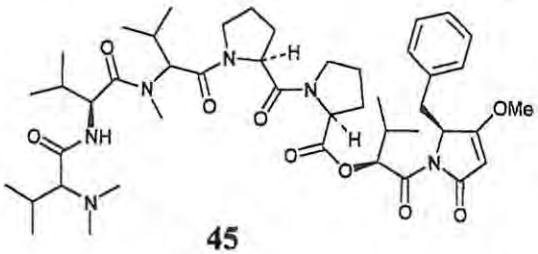
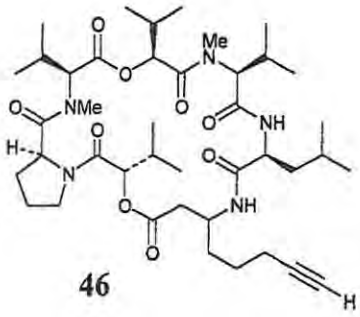


45

From Indian Ocean sea hares, the unusual cyclodepsipeptide **46** containing a new acetylenic β-amino acid, and the linear peptide **47** are the most recently reported dolastatins (17[47] and 18[48] respectively).



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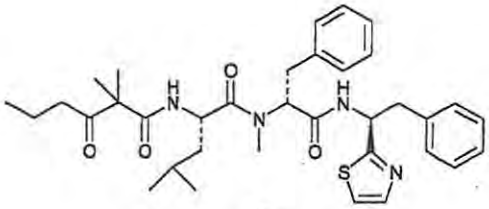
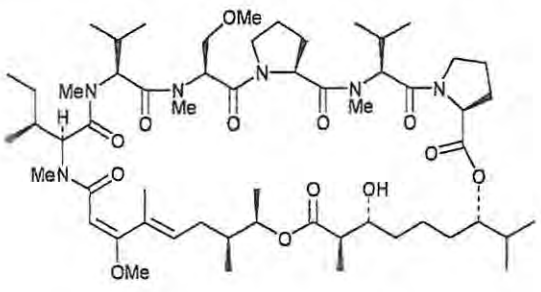


47

Japanese specimens of *D. auricularia* have yielded a different variety of 'dolastatin' peptides, including dolastatin G (**48**) of mixed peptide and polyketide biogenesis,[49] and dolastatin I (**49**),[50] which contains three kinds of five-membered heterocycles. There is a wealth of recent literature on the synthesis of dolastatins, and also analogues, which are being used in structure activity relationship studies of their anticancer activity.[46] Similarly, the potent antitumour polyketide macrolide aplyronine A (**50**) from *A. kurodai*[51] has been the focus of two recent structure activity relationship studies involving the synthesis of more than fourteen analogues.[52]



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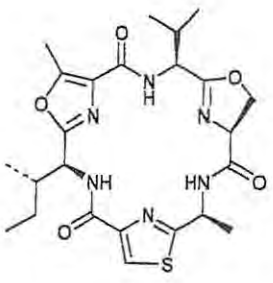

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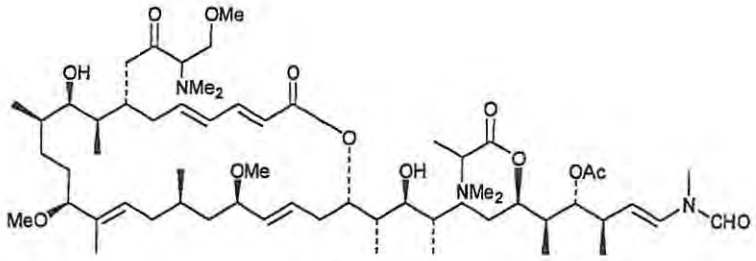
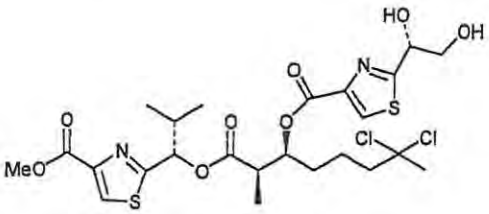


50

Besides peptides, the internal organs of Japanese specimens of *D. auricularia* have recently yielded an assortment of other novel, cytotoxic compounds including the bisthiazole metabolite dolabellin (**51**),[53] the bromotriterpene aurilol (**52**),[54] two polypropionates, auripyrones A (**53**) and B (**54**),[55] four macrolides similar to dolabelide A (**55**),[56] and a pair of macrolide glycosides, aurisides A (**56**) and B (**57**).[57]



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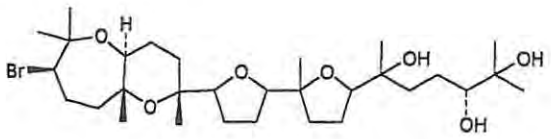

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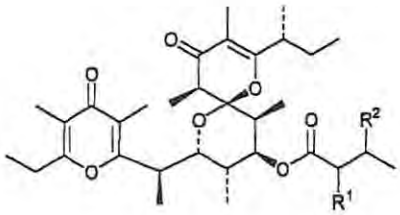
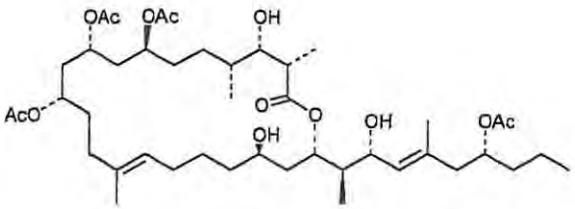


53 $R^1 = H, R^2 = CH_3$
54 $R^1 = CH_3, R^2 = H$



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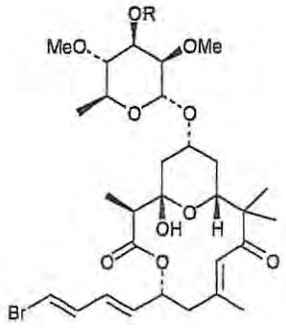
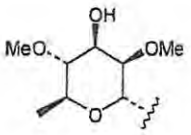
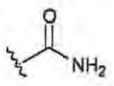


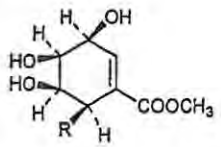
56 R =



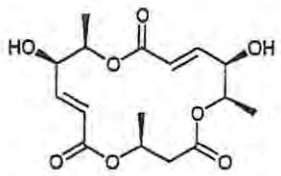
57 R =



The majority of the afore-mentioned metabolites, together with the dolastatin peptides, have been isolated from *D. auricularia* in exceedingly low yields, *e.g.* 0.5 mg auriside from 265 kg of sea hares, which together with a structural analogy to bacterial metabolites, suggests their dietary or symbiotic microbe origin. In support of this, there exist several reports of dolastatins or their analogues isolated from blue-green algae, including *Lyngbya majuscula*[58] and *Symploca hydnoides*,[59] upon which *D. auricularia* may feed. There is little direct evidence as yet for a symbiotic microbial origin of sea hare metabolites, either within the sea hare or its dietary algae. Interestingly, a fungal strain from the gastrointestinal tract of *A. kurodai*, produced the novel cytotoxic compounds pericosines A (**58**) and B (**59**) and four new macrolides similar to macrosphelide E (**60**) when cultured.[60]



58 R = Cl
59 R = OCH_3



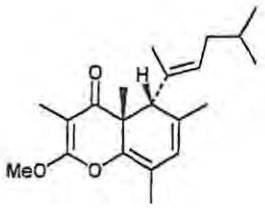
60

It is noteworthy that the peptides and several other fairly large, polar molecules from opisthobranchs, occur in minute quantities and therefore the dietary organisms, from which opisthobranchs sequester these metabolites, may be more viable sources of the compounds. In contrast it has been proposed that the concentration of diet derived terpenes by opisthobranchs facilitates the discovery of minor metabolites, which may be too 'dilute' in the dietary organism for detection by chemists.[24] Similarly, the chromodorid *Cadlina luteomarginata* was described as a useful 'surrogate' for investigations of terpenes from rare British Columbia sponges.[28] The importance of minor metabolites as potential pharmaceuticals is inferred from consideration of the hypothesis that secondary metabolites, being necessary for the survival of the producing organism, have evolved to target specific receptors in the receiving organisms.[61] Thus, according to Christophersen,[61] the major metabolites of marine invertebrates are anticipated to be most active against the receptors of biologically less developed

organisms, and a related minor metabolite is more likely to show good activity in vertebrates with highly developed receptor systems such as ourselves.

1.4 New chemistry from sacoglossans, cephalaspids and notaspids

Compared to the nudibranchs and anaspids, opisthobranchs of the orders Sacoglossa, Cephalaspidea and Notaspidea received little attention from natural products chemists in the 1970's and 1980's (Figure 1.1A and 1.1B respectively). However, Figure 1.1C shows a relative increase in the number of publications on each of these orders in the 1990's. Prior to 1995, polypropionates were widely reported from sacoglossans and to a lesser extent from cephalaspids. Accordingly, the sacoglossan *Tridachia crispata* has recently yielded the unprecedented bicyclic propionate tridachiahydropyrone (**61**),[62] a putative sunscreen, as well as a new sesquiterpene, crispatenine (**62**),[63] which occurred together with three previously reported polypropionate and sesquiterpene compounds.



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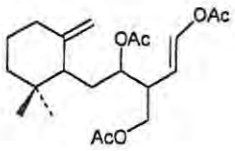
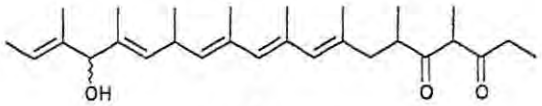


62

Similarly, the previously uninvestigated cephalaspid *Smaragdinella calyculata* yielded a new cytotoxic polypropionate, nalodionol (**63**), and an aminoalkylpyridine, naloamine (**64**),[64] while the series of new alkylphenols, *e.g.* (**65**), concentrated in the parapodia of the new cephalaspid species *Haminoea callidegenita*[65] are consistent with the previous reports of alkylphenols from *Haminoea* cephalaspids.[6]



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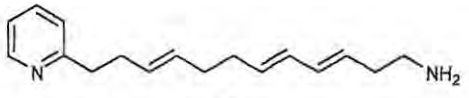

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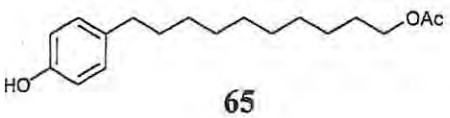
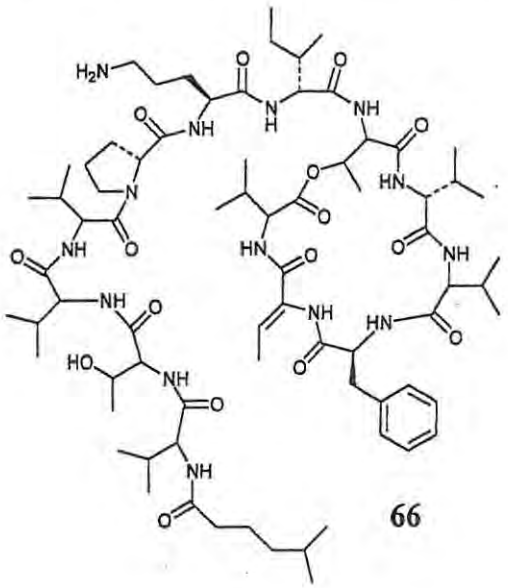


65

However, several investigations of the sacoglossan *Elysia rufescens* have yielded neither the polypropionates nor diterpenes reported previously from this genus, but a series of peptides, the kahalides,[66,67] also isolated from the dietary green alga, *Bryopsis* sp.[67,68] or possibly associated blue-green algal epiphytes of the latter alga. Of the latter mixture of linear and cyclic peptides, kahalide F[66] (**66**) was found to exhibit significant biological activity and is in development as a potential anticancer agent.



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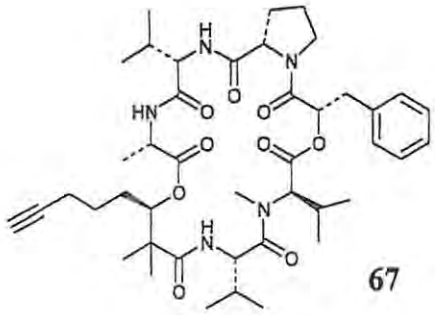
The depsipeptide kulolide (**67**),[69] characterized long after its initial isolation, was reported from the carnivorous cephalaspid *Philinopsis speciosa* and was thus anomalous in its analogy with blue-green algal metabolites. When a further five related depsipeptides, the known linear peptide pupukeamide (**68**)[70,] and the macrolide tolytoxin-23-acetate (**69**) were subsequently isolated from the same species, the presence of these compounds in a carnivorous mollusc was explained by the ready feeding of *P. speciosa* on herbivorous anaspid sea hares.[71]



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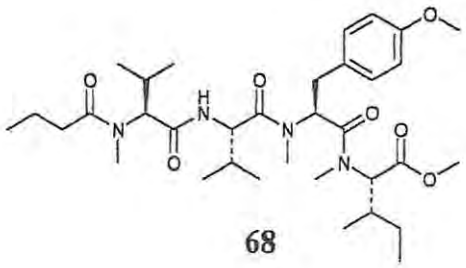

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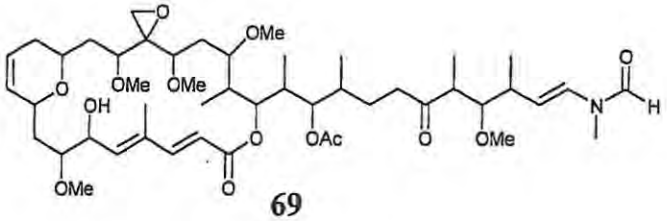
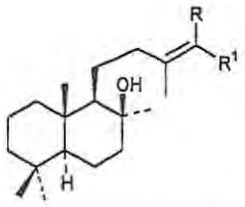
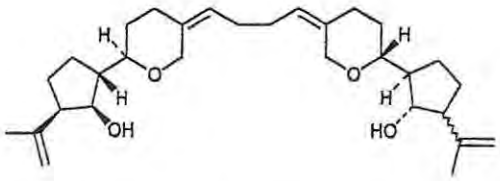


69

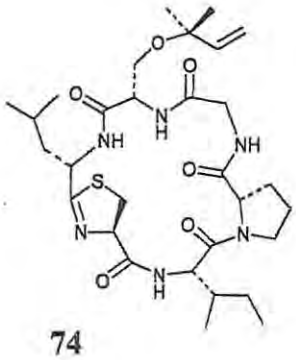
Early studies of opisthobranchs belonging to the Order Notaspidea reported strongly acidic defensive secretions by these nudibranchs and as a result notaspideans have largely been ignored, with only *Umbranculum mediterraneum* and *Pleurobranchus membranaceus* appearing in the chemical literature.[6] However, the presence of an acid based defense mechanism does not preclude the presence of alternative defense chemicals of organic origin and there has been a marked increase in the number of investigations of notaspids. A first chemical study of *P. meckelii* afforded two isomeric labdane diterpenes (**70**) and (**71**) from a skin extract and specimens collected from different geographical areas contained the same metabolites.[72] The skin and mucus secretion of *P. testudinarius* contained two interesting triterpenoids testudinariol A (**72**) and testudinariol B (**73**), the first of which was toxic to mosquito fish.[73] Another species, *P. forskalii*, yielded the bioactive cyclic peptide keenamide A (**74**).[74] In a follow up study of *Tylodina perversa*, which is known to sequester a range of isoxazoline alkaloids from *Aplysina* sponges,[75] feeding experiments showed the selective accumulation of aerophobin-2 (**75**), a putative defense chemical, in the mantle and eggs of the mollusc.[76]



70 R = CHO, R^1 = H
71 R = H, R^1 = CHO



72 10*R*
73 10*S*



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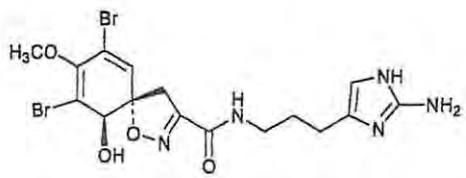


75

With the aid of pH paper, notaspids became known for their acidic secretions,[4,5] while crystallography and low field NMR spectroscopy were invaluable in the early description of halogenated metabolites from sea hares.[77] Subsequently, high field NMR spectroscopy has been instrumental in characterizing the largely terpenoid metabolites[6] from dorid nudibranchs, and most recently very high field NMR spectroscopy has enabled the structures of the diet derived peptides present in minute amounts in anaspids, sacoglossans and cephalaspids to be fully elucidated. Thus while the recent chemical literature illustrates the increased effort necessary to find new metabolites from opisthobranchs, it also shows how advancements in technology, particularly in NMR spectroscopy, have allowed the routine characterization of ever larger and more complex metabolites found in diminishing quantities.

1.5 Can natural product chemistry provide clues to opisthobranch evolution?

Within the opisthobranchs an evolutionary progression may be interpreted from the great variety of shelled, partially shelled and shell-less species. In 1983, Faulkner and Ghiselin presented evidence that chemical defense was a preadaptation that facilitated the loss of the shell in opisthobranchs and broadly discussed the evolution of chemical defense in opisthobranch molluscs with particular emphasis on dorid nudibranchs.[78] The ability of some opisthobranchs to biosynthesize defense chemicals *de novo* was viewed as an evolutionary advancement over the sequestration of dietary metabolites for defense that would alleviate complete dependence on a food source for protection. The recent review on the Sacoglossa by Cimino and Ghiselin[40] is comprehensive in its treatment of the observed chemistry and behaviour of sacoglossans in the context of the evolution and life histories of these opisthobranchs. The Sacoglossa include shelled, partially shelled and shell-less forms and the early evolution of the group is believed to have been greatly influenced by the existence of a chemical defense. Some shelled, burrowing sacoglossans, *e.g.* the primitive *Ascobulla fragilis*, sequester toxic metabolites from their algal prey species of *Caulerpa* and furthermore are able to modify these chemicals, which are released in a mucous secretion. Surface shelled forms, *e.g. Oxynoe* and *Lobiger* species, are also closely associated with *Caulerpa* algae from which they similarly

elaborate a chemical defense. Cimino and Ghiselin[40] suggest that the ability of some sacoglossans to sequester functional chloroplasts could facilitate the *de novo* biosynthesis of defensive polypropionates, from a photosynthetic carbon source, and has allowed exploitation of a greater variety of algal species as prey by sacoglossans of the family Elysiidae for example. It would be interesting to see if the biological and chemical knowledge accumulated for other orders of opisthobranchs, besides the Sacoglossa, could be similarly integrated in a coherent evolutionary saga as intimated by Cimino and Ghiselin. Although the dependence of many opisthobranchs on their distasteful dietary organisms as a refuge or source of defense chemicals is seen as a major evolutionary constraint, an obligatory association with the dietary organism may be metabolically less expensive than *de novo* biosynthesis of defense chemicals. It is remarkable that *de novo* biosynthesis of secondary metabolites, which presumably facilitates generalized feeding, appears to be a trait of both primitive, *e.g.* notaspid, and highly evolved, *e.g.* some dorid, opisthobranchs,[6] with specialist feeders, *e.g.* chromodorids, which rely totally on dietary-derived metabolites, falling somewhere in the middle of the evolutionary scale.

1.6 The chemistry of South African opisthobranch molluscs

Endemism of marine invertebrate species often signals new chemistry and in South Africa, with its range of subtropical, warm temperate and cold temperate coastal waters (Figure 1.2) a high level of endemism is evident. The highest diversity of marine inverebrates occurs along the south east coast where warm and cold waters merge as the warm, south flowing Agulhas Current is deflected offshore by the widened continental shelf (Figure 1.2). Given the marked paucity of marine natural products chemists in South Africa, there remains a wealth of largely undescribed invertebrates whose chemistry has yet to be investigated. In particular, opisthobranch molluscs have been poorly investigated, the necessity of collecting a relatively large number of individuals having resulted in their exclusion from large-scale collections made by foreign investigators *e.g.* Smithkline Beecham (1994-1996) and the National Cancer Institute (U.S.A.; 1998-2000). Thus a unique window of opportunity exists for resident scientists who have continual access to collection sites to explore the chemistry of South African

opisthobranch molluscs. Accordingly, this thesis describes the characterization of metabolites from six species of South African opisthobranch molluscs collected mainly from Port Elizabeth (Algoa Bay) and the Tsitsikamma National Park (Figure 1.2).

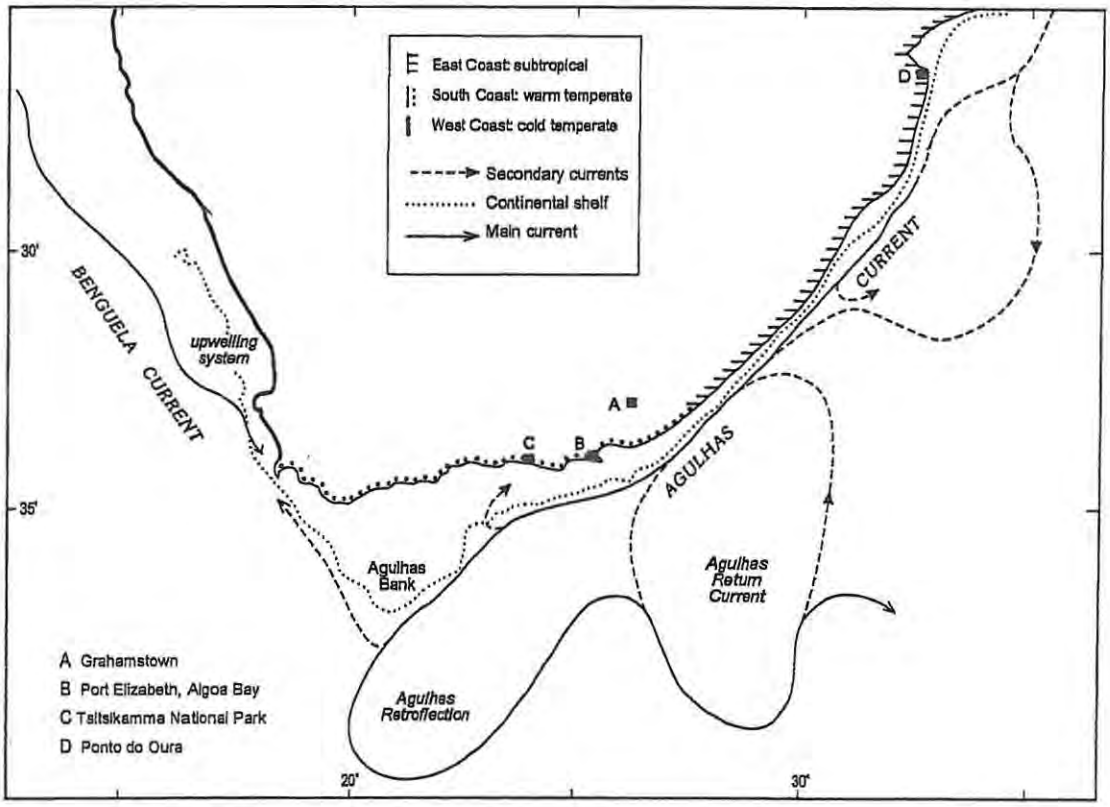


Figure 1.2 Map of the southern coast of Africa showing the different coastal climates influenced by the major ocean currents. Sample collection sites relative to Grahamstown are also shown.

Besides the role of nudibranchs in concentrating minor metabolites sequestered from their dietary organisms, we have found that the more general feeders provide valuable insights into the chemistry of the reef invertebrates and algal communities from which they are collected. Thus the chemistry of the circumtropical sea hares *Aplysia dactylomela* and *Aplysia parvula* (Chapter Two) gives probably the most extensive introduction to the chemistry of South African red algae to date, while the range of metabolites isolated from the arminacean nudibranch *Leminda millecra* (Chapter Three) affords a preview of the largely unknown South African octocoral chemistry. Sponge metabolites have been isolated from the southern African chromodorid species *Chromodoris hamiltoni*, *Glossodoris* sp. 4 and *Hypselodoris capensis* (Chapter Four). The chemistry of *H.*

capensis enabled the identification of two dietary sponges of this species, one of which yielded a new metabolite whose structure was confirmed by synthesis (Chapter Five).

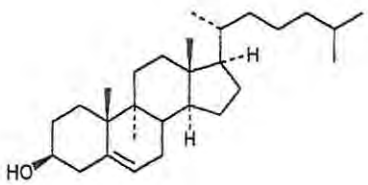
Chapter Two

The Sequestered Chemistry of South African Specimens of the Cosmopolitan Sea Hares *Aplysia parvula* and *Aplysia dactylomela*

2.1 The biology and chemical ecology of *Aplysia* sea hares

Noxious anaspid opisthobranch molluscs of the genus *Aplysia* have elicited interest since the first century A.D.[79] when they were aptly ascribed the common name of *Lepus marinus*, or sea hare, from their elongated, 'ear-like' rhinophores and humped dorsum formed from large parapodia folded over the dorsal mantle cavity.[80] *Aplysia* sea hares, which are ubiquitous in shallow marine waters worldwide, are large, fast-growing and highly fecund invertebrates with simple nervous systems and only a small internal shell plate.[79,80] In addition, although these molluscs have long-lived planktotrophic larvae, they are amenable to laboratory culture and therefore have been the subjects of many early physiological studies[81] that have resulted in a thorough investigation of their biology.[79] Thirty-seven species of *Aplysia* have been described, most of which, when disturbed, produce defensive ink and/or opaline secretions responsible for the 'venomous' nature of the specimens described in writings from the first century. The varied diet of *Aplysia* sea hares comprises red, green and occasionally brown algae,[79] and *Aplysia* ink, the exact function of which has not been unequivocally established,[82] is widely reported to contain protein bound pigments derived from tetrapyrrole chromophores of red algal biliproteins, in addition to other polypeptides of high molecular mass.[83] However, the composition of the toxic opaline secretion from subepidermal mantle glands in *Aplysia* apparently remains undetermined, although it is suggested to contain terpenes.[79] The large body of chemical literature on *Aplysia* sea hares, encompassing seventeen species, has focussed rather on a plethora of brominated and chlorinated secondary metabolites isolated from the sea hare's large specially modified digestive gland.[79] It was initially proposed that the latter, often bioactive, metabolites, which are almost entirely derived from dietary red algae,[77] were distributed from the digestive gland throughout the sea hare mantle and released into the opaline secretion as part of a defense mechanism.[84] However, recently Pennings[85] has shown that the palatability to potential predators of the

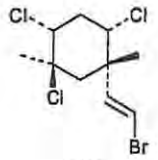
opaline secretion, mantle and eggs of sea hares is independent of the presence of red algal products in the sea hare digestive gland. *Aplysia juliana*, which feeds on green algae depauperate in secondary metabolites and does not secrete ink, is no more susceptible to predation than *A. oculifera* and *A. kurodai*, both of which sequester red algal metabolites.[85] Furthermore, the almost exclusive location of sequestered red algal metabolites in the digestive gland of sea hares is not in line with a defensive function for these toxic metabolites which would only be detected by predators that had mortally wounded their prey.[86-88] An alternative explanation for the presence of persistent, toxic red algal metabolites in the digestive glands of sea hares is that it is energetically less expensive to store the sequestered metabolites than to detoxify them for metabolism.[88] Pennings *et al.* also proposed that this may be the case for the sea hare *Dolabella auricularia*, another generalist feeder on algae.[41] Although digestive gland extracts of *D. auricularia* were very unpalatable to reef fish due to high concentrations of algal compounds, the unpalatability of extracts of ink, eggs and skin was traced to the presence (-)-7-dehydrocholesterol (**76**), rather than to halogenated algal metabolites. Similarly, bioactive fatty acids and degraded sterols have been isolated from the skin of *A. fasciata*[42] and *A. depilans*[43] and were suggested to be the defense chemicals responsible for the observed low rates of predation on sea hares. Thus, although sea hares are able to overcome the chemical defense of their algal prey species, it is uncertain whether or not these opisthobranchs actively incorporate the sequestered halogenated metabolites into their own defense systems.



76

The chemical arsenal of marine algae preyed upon by *Aplysia* includes numerous simple haloalkanes such as the commercial fumigant dibromoethane, made by Antarctic macroalgae.[89] The monoterpene telfairine (77) from *Plocamium telfairiae* resembles the

commercial pesticide lindane in structure and toxicity, while the linear monoterpene (**32**) from *Portieria hornemannii* reached preclinical trials as a potent anticancer agent.[89]



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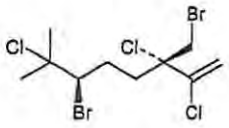
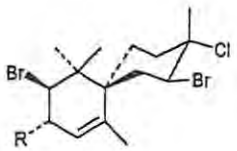
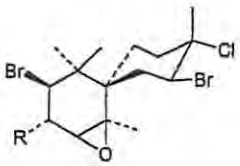


32

Laurencia red algae, which are the predominant dietary algae of *Aplysia* sea hares, are prolific producers of halogenated sesquiterpenes and C_{15} acetogenins.[90] However, although the chemistry of at least sixty six species of *Laurencia* has been investigated worldwide, the only South African species which appears in the chemical literature is *L. glomerata*, which yielded four halogenated chamigranes (**78-81**).[91] Since there are an estimated one hundred and eighty species of red algae off the eastern and southern Cape coast of South Africa,[92] which are fed upon by sea hares whose chemistry also has not been described, we embarked on an investigation of *A. parvula* and *A. dactylomela* from this region of the South African coast.



78 R = H
79 R = OH



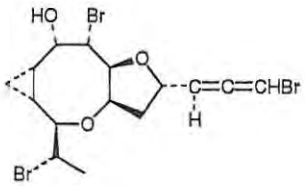
80 R = H
81 R = OH

2.1.1 Secondary metabolites isolated previously from *Aplysia parvula*

Aplysia parvula and *A. dactylomela* are two of only three truly cosmopolitan species of *Aplysia*, the third being *A. juliana*.[79] *A. parvula* (Figure 2.1) is the smallest of the *Aplysia* sea hares (typically 2-4 cm long) and has a life span of only a few months,[79] both factors which may be responsible for the paucity of chemical literature on this species. The novel, bioactive C_{15} acetogenin aplyparvulin (**33**) was the first metabolite reported from *A. parvula*[36] and is analogous to the rearranged laureatin acetogenin, laurallene (**82**), isolated from *L. nipponica*.[93]

Figure 2.1: Photograph of *Aplysia parvula* (length 3 cm) reproduced from Gosliner.[80]

Subsequent studies have focussed on the biological activity of metabolites sequestered by *A. parvula*.[39,94] Interestingly, de Nys *et al.*[39] suggest that *A. parvula* selectively concentrates the most bioactive metabolites, *e.g.* (**39**), from its host red alga *Delisea pulchra*, and although these metabolites are found in highest concentration in the digestive gland, they may be present in the sea hare skin in sufficient amounts to deter predation.



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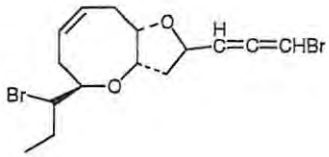

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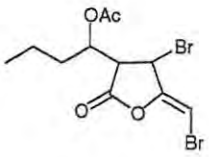

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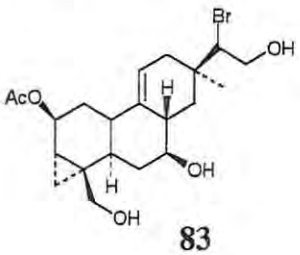
2.1.2 Secondary metabolites isolated previously from *Aplysia dactylomela*

In contrast to *A. parvula*, *A. dactylomela* (Figure 2.2) is a large sea hare, reaching up to 40 cm in length,[80] and has been the subject of many chemical investigations worldwide. The varied chemistry of *A. dactylomela* closely mirrors that of its algal prey species and even less common *Laurencia* metabolites have been reported from these sea hares. The structural diversity of *A. dactylomela* sesquestered metabolites are indicated here by the following examples.

Figure 2.2: Surface photograph of a green color variant of *Aplysia dactylomela* (length 15 cm) from Algoa Bay

Parguerene diterpenes such as **83** isolated from *A. dactylomela* from Puerto Rico[95] have also been isolated from *Laurencia* species.[96] The unusual skeleton of brominated diterpene (**84**),[97] is found in obtusadiol from *L. obtusa*,[98] while dolabellanes (**85** and **86**) found in Canary Island specimens of *A. dactylomela*,[99] are analogous to those found in *L. dictyopteris*,[100] although the former compounds are thought to be sequestered from brown algae of which they are more typical.[99]



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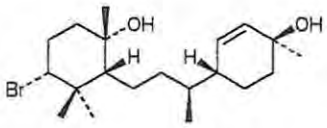

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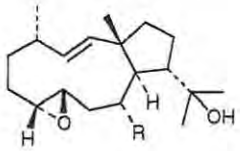
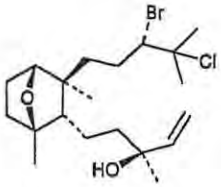


85 R = OH
86 R = OAc

Dactylomelol (**87**),[101] which arises from cyclization of the two internal double bonds of the isoprenoid precursor geranyllinalool, is another unusual diterpene which has been isolated from *A. dactylomela*. *A. dactylomela* from the northern Indian Ocean yielded the bromoindoles (**88**) and (**89**).[102] The former compound was previously found in *L. brongniartii*,[103] which has subsequently yielded further, sulfur containing bromoindoles.[104] Although bromophenols have been isolated from two *Laurencia*

species,[105] brominated diphenyl ether (**90**) isolated from *A. dactylomela* is thought to be sequestered from the green alga *Cladophora fascicularis*.[106]



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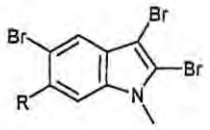
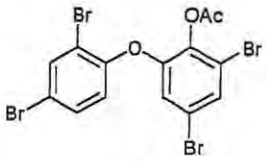
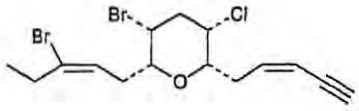


88 R = H
89 R = Br



90

As mentioned earlier, the C_{15} acetogenin class of compounds is well-represented in *Laurencia*,[90] and several of these ethers (lauroxanes) with terminal enyne or allene moieties have been isolated from *A. dactylomela*. Formation of the oxane ring is initiated by bromonium ion attack on one of the double bonds in a linear fatty acid precursor, giving rise to rings of varying sizes.[90] Lauroxixanes having a six-membered ring such as dactylyne (**91**)[107] have been isolated from *A. dactylomela* collected in the Bahamas[107,108] and Japan,[109] where *L. majuscula* was found to contain the same compounds. Nine-membered ring lauroxonanes, *e.g.* (**92**), have been isolated from Carribean specimens of *A. dactylomela*[110] and occur in several species of *Laurencia*.[111,112] The bromo-allene dactyllalene (**34**), recently isolated from Atlantic Ocean specimens of *A. dactylomela*,[113] is the C-4 epimer of obtusallene reported from *L. obtusa*.[114]



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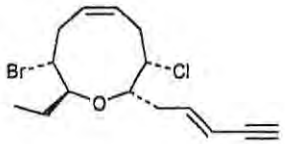

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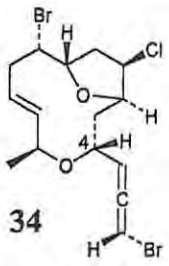
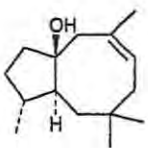


34

Bromonium-induced cyclization of six- or ten-membered monocyclic farnesanes is responsible for the numerous carbocyclic and heterocyclic skeletons of *Laurencia* sesquiterpenes,[90] which are the predominant metabolites in this genus and are therefore, not surprisingly, also most abundant in *A. dactylomela*. Derivatives of ten-membered ring intermediates include poitanes[115] such as the *A. dactylomela* metabolite dactylol (**93**),[116] and *trans*-eudesmanes[117] like (**94**) which was isolated from Indian Ocean

specimens of *A. dactylomela*.[102] Two *cis*-eudesmanes (**95** and **96**) were isolated from Hawaiian specimens of *A. dactylomela*.[118]



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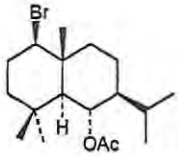

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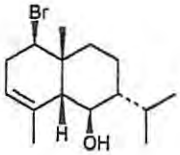

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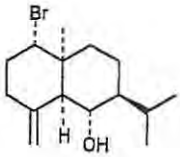
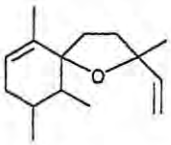


96

While the dactyloxene sesquiterpenes, *e.g.* dactyloxene B (**97**) from *A. dacylomela*[119] and *L. poitei*,[90] are uncommon derivatives of 6-membered monocyclic snyderane farnesanes, heterocyclic sesquiterpenes derived from bisabolane precursors, *e.g.* deodactol (**98**),[120] have been more frequently reported from *A. dactylomela*[120,121,109] and *L. caespitosa*.[122]



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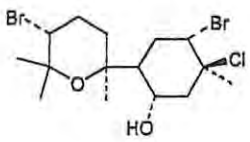
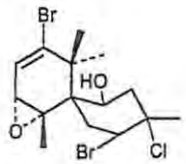


98

However, it is the spirofused chamigrane sesquiterpenes and related ring contracted aromatic cuparanes and lauranes, which are the hallmark of *Laurencia* red algae and are consequently routinely reported from *A. dactylomela*. The spirofused chamigrenes prepacifenol[123] (**99**), deoxyprepacifenol[123-125](**100**) and prepacifenol epoxide[124-127](**101**) are well known *Laurencia* metabolites which have been isolated from *A. dactylomela*.



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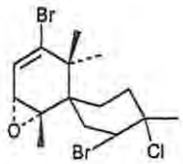

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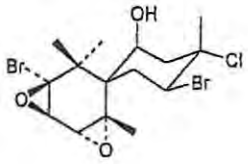
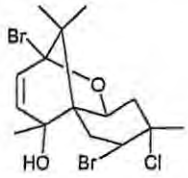


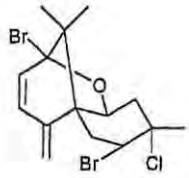
101

In *A. dactylomela*, the latter compounds are accompanied by the tricyclic chamigrene ethers pacifenol (**102**),[123,124,127] pacifidiene (**103**)[123,126] and its diol (**104**).[124,126,127] While **102** was reported from *L. pacifica* as the first natural product containing both Br and Cl atoms,[128] **103** and **104** have not been found in *Laurencia* and are probably formed from

acid attack on pacifenol[86] (**102**) and prepacifenol epoxide[124] (**101**) respectively in the sea hare digestive gland.



102



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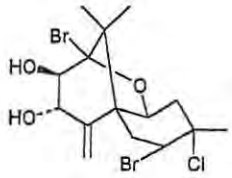
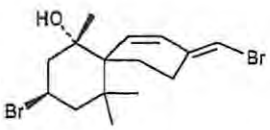


104

The interesting, unusually brominated chamigrenes (**105**),[129] (**106**) and (**107**),[130] isolated from *A. dactylomela* are similar to those reported from *L. majuscula*.[131] Ketochamigranes **106** and **107** were isolated together with further ketones (**108** and **109**),[130] which are also known from *Laurencia*.[132] In addition to the lauranes (**110**),[102] and (**111**),[123] and cyclolauranes, *e.g.* (**112**),[133] *A. dactylomela* has yielded cuparanes such as (**113**), which have bromine and oxy substituents reversed from their usual positions in ring B.[133]



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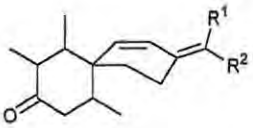
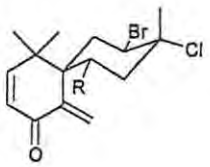
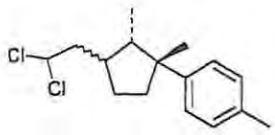


106 $R^1 = Br, R^2 = H$
107 $R^1 = H, R^2 = Br$



108 R = H
109 R = OAc



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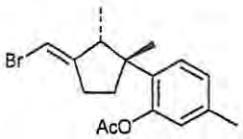

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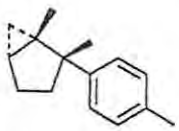

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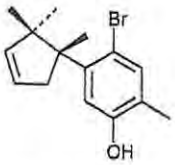
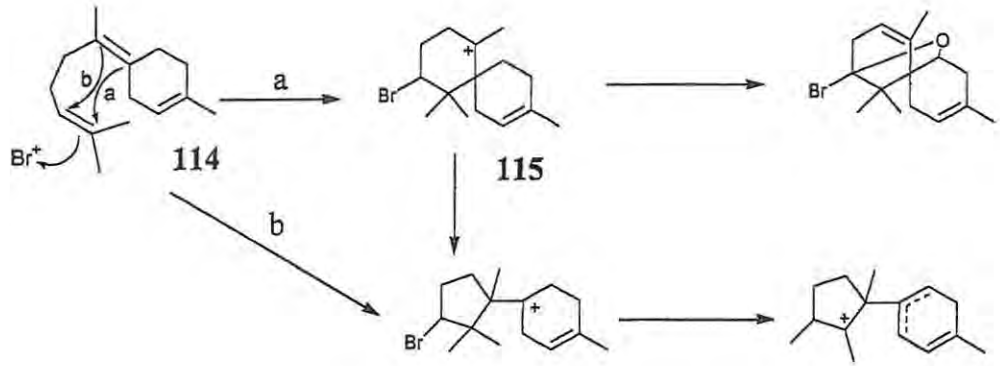


113

The co-occurrence of the spirofused chamigranes, tricyclic chamigrane ethers, cuparanes and lauranes in *Laurencia* and *Aplysia* has been invoked as evidence for their common biosynthetic origin.[90] Bromonium-induced cyclization of E-γ-bisabolene (**114**) to afford the spirofused structure (**115**) could be followed by either further cyclization to give ether linked tricycles, or a ring contraction to give cuparanes which are related to lauranes by a simple 1,2 shift (Scheme 2.1).[90] Alternatively, the five-membered ring of cuparanes and lauranes may be formed directly from E-γ-bisabolene along path b.[134]



Scheme 2.1 Biogenetic pathway from E-γ-bisabolene (**114**) to spirofused chamigranes and related tricyclic chamigrane ethers, cuparanes and lauranes.[90]

Guella *et al.*[134] considered that the bisabolene precursor to polyhalogenated chamigrenes may have a 3*R*, 4*R* or 3*S*, 4*S* configuration in the cyclohexane ring *via* the *anti* addition of halogen X^2 (Figure 2.3) to the cyclohexene olefin. Furthermore, for each of these two precursors, the initial conformation of the sidechain (Figure 2.3) determines whether cyclization results in a 6*R* or 6*S* chamigrene intermediate **115**.

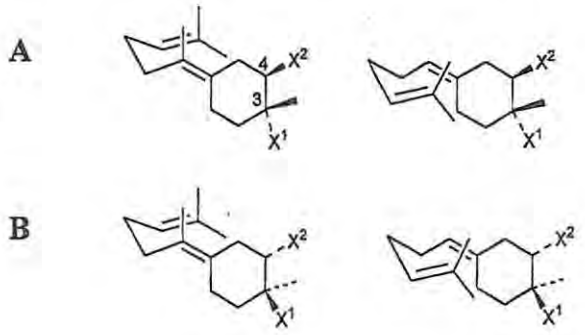
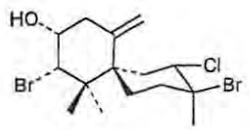


Figure 2.3 The two conformations possible for each of two bisabolene precursors [3*R*, 4*R* (**A**), 3*S*, 4*S* (**B**)].

Therefore, Guella *et al.*[134] stipulated four stereochemical types of polyhalogenated chamigrene sesquiterpenes. While three stereochemical types were known, namely obtusane (*e.g.* obtusol, **116**), iso-obtusane (*e.g.* isoobtusol, **117**) and rogiolane (*e.g.* rogiolol, **118**), the existence of a fourth 'cartilagineane' type typified by cartilagineol (**119**) could only be proposed.[134] The latter compound, isolated from a Hawaiian collection of *L. cartilaginea*, had initially been designated *allo*-isoobtusol and given the structure of isoobtusol (**117**),[135] and Guella *et al.*'s suggested reassignment of the

structure as cartilagineol (**119**) has subsequently been confirmed from both NMR and crystallographic data for the same compound isolated from a Philippine *Laurencia* species.[136]



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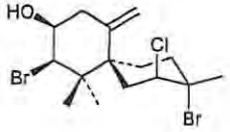

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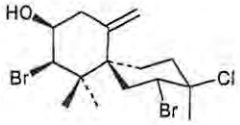

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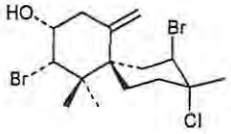
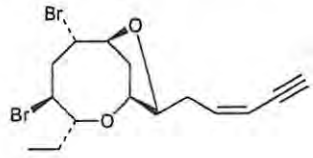
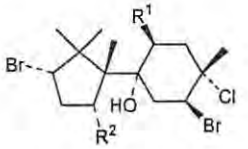


119

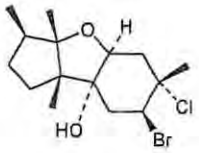
The following discussion presents the structure of the new C_{15} acetogenin (3*Z*)-bromofucin (**120**) isolated from *A. parvula* collected in the Tsitsikamma National Park, and a series of novel non-aromatic cuparanes, algoane (**121**), 1-deacetoxyalgoane (**122**) and 1-deacetoxy-8-deoxyalgoane (**123**), which we have isolated from *A. dactylomela* collected in Algoa Bay. The latter species also contained the novel tricyclic laurane ibhayinol (**124**) and the known chamigranes prepacifenol epoxide (**101**) and nidificene (**125**), and pacif-7-enediol (**104**).



120



121 R^1 = OAc, R^2 = OH
122 R^1 = H, R^2 = OH
123 R^1 = R^2 = H



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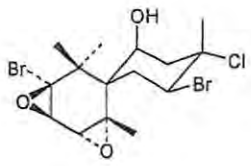

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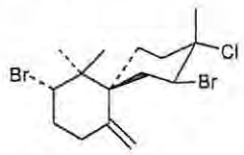

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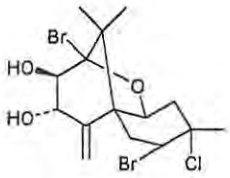
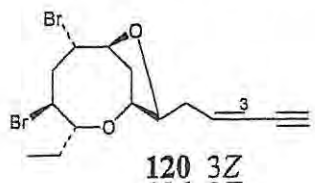


104

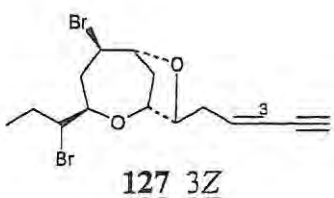
2.2 The sequestered chemistry of *Aplysia parvula* in the Tsitsikamma National Park

In February 1996 and 1997, specimens of *Aplysia parvula* were collected in an area known as 'The Gulley' in the Tsitsikamma National Park, where they were found feeding on a filamentous red alga growing in close association with a coralline alga. The specimens from the two collections were separately extracted with acetone and the two

concentrated acetone extracts were partitioned between EtOAc and water. Since the two EtOAc partition fractions were identical by ^{1}H NMR spectroscopy, they were combined and fractionated on Sephadex LH20 to afford three main fractions, the third one of which yielded (3*Z*)-bromofucin (**120**, 3.6 mg, < 0.1 mg/animal) after normal phase HPLC (hexane). The molecular formula of **120**, established as $C_{15}H_{20}Br_2O_2$ by HREIMS (389.9838, Δmmu +0.8), indicated five degrees of unsaturation, only one of which could be accounted for by two olefinic carbon resonances in the ^{13}C NMR spectrum of **120**. The absence of a broad hydroxyl absorbance in the IR spectrum of **120**, together with strong absorbances at 963 and 881 cm^{-1} suggested that the two oxygen atoms indicated in the molecular formula were present in ether rings. The remaining two degrees of unsaturation could be attributed to the presence of a terminal acetylene moiety indicated by very weak resonances at δ 80.0 and 82.0 in the ^{13}C NMR spectrum of **120** and sharp IR absorbances at 3291 (strong) and 2126 (weak) cm^{-1}. Since the only quartenary carbon present was that of the terminal acetylene moiety (C-2, δ 80.0), the contiguity of the remaining one methyl, four methylene and eight methine carbons (C-3 to C-15) was readily determined by a COSY experiment. An HMBC experiment confirmed the two and three bond H-C connectivity of **120**. Although the ^{1}H and ^{13}C NMR data for **120** were compatible with those given in the literature for compound **126** isolated from *L. implicata*,[112] significant differences between the ^{1}H and ^{13}C chemical shift values for atoms 1-5 of the two compounds were evident (Table 2.1). Furthermore, we observed reciprocal enhancements in the ^{1}H signals of H-3 (δ 5.54) and H-4 (δ 6.02) on their irradiation in 1D NOE difference experiments. Irradiation of H-4 also produced an enhancement in the ^{1}H triplet of H-6 (δ 3.79). These data indicated a *Z* stereochemistry about the double bond (Δ^3) in **120** in contrast to the 3*E* stereochemistry of **126**. Both 3*E* and 3*Z* stereochemistries are found in C_{15} acetogenins from *Laurencia* red algae, with (3*Z*)-[137] (**127**) and (3*E*)-isoprelaurefucin[138] (**128**) being two examples of compounds which are very similar to **120** and **126**.



120 3*Z*
126 3*E*

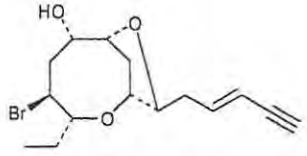


127 3*Z*
128 3*E*

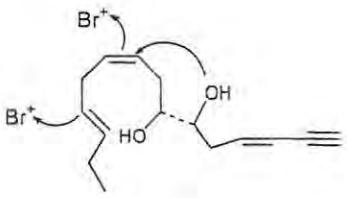
Table 2.1 ^{1}H and ^{13}C NMR data for 3*Z*-bromofucin (**120**, $CDCl_3$, 400 MHz and 100 MHz respectively) and compound **126** ($CDCl_3$, 300 MHz and 75 MHz respectively)[112]

Atom number	3*Z*-bromofucin 120		Compound 126	
	δ_H ppm (mult., J/Hz)	δ_C ppm (mult.)	δ_H ppm (mult., J/Hz)	δ_C ppm (mult.)
1	3.10 (s)	82.0 (d)	2.81 (d, 2)	76.4 (d)
2		80.0 (s)		82.1 (s)
3	5.54 (d, 11)	110.4 (s)	5.58 (ddd, 2, 4, 16)	111.4 (s)
4	6.02 (dd, 8, 10)	141.1 (d)	6.20 (ddd, 7, 8, 16)	141.8 (d)
5	2.70 (m)	30.1 (t)	2.53 (m)	32.5 (t)
	2.81 (m)			
6	3.79 (t, 5)	84.3 (d)	3.74 (ddd, 2, 7, 7)	84.3 (d)
7	4.00 (br s)	70.1 (d)	4.00 (br s)	69.8 (d)
8	2.06 (m)	33.6 (t)	2.06 (ddd, 3, 10, 16)	33.5 (t)
	2.34 (d, 15)		2.36 (d, 16)	
9	4.40 (dd, 3, 9)	79.6 (d)	4.40 (dd, 4, 10)	79.6 (d)
10	4.34 (br s)	55.3 (d)	4.33 (br s)	55.2 (d)
11	2.51 (dd, 5, 16)	38.6 (t)	2.53 (m)	38.5 (t)
	3.37 (dd, 9, 16)		3.34 (ddd, 2, 9, 16)	
12	4.50 (t, 9)	53.0 (d)	4.50 (dd, 9, 10)	52.7 (d)
13	3.91 (dt, 2, 10)	82.7 (d)	3.89 (ddd, 3, 10, 12)	82.8 (d)
14	1.65 (m)	23.0 (t)	1.68 (ddq, 7, 12, 15)	23.0 (t)
	1.99 (m)		1.99 (ddq, 3, 7, 15)	
15	1.06 (t, 7)	11.8 (q)	1.06 (t, 7)	11.8 (q)

Compounds **120** and **126** are stereoisomers of laurefucin (**129**) from *L. nipponica* and chlorofucin (**130**) from *L. snyderiae*, which have been isolated previously and are reviewed by Erickson.[90] Cyclization of *cis*-laurediol (**131**) is initiated by bromonium attack at C-12 which results in ring closure through C-7 hydroxyl attack at C-13 to give the 8-membered (lauroxocane) ring of 'laurencins', some of the most common C_{15} acetogenins[90] found in *Laurencia* red algae.



129 R = OH
130 R = Cl



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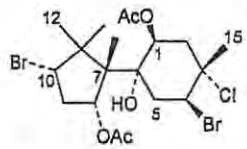
A variety of other acetylenic ethers exist which are often isolated in tandem with **120**. Although ^{1}H NMR screening of fractions obtained during the fractionation of the crude EtOAc extract of *A. parvula* revealed the presence of several further terpenes, we were unable to obtain sufficient amounts of these compounds for full structure elucidation. Unfortunately, the erratic presence of these sea hares in the Tsitsikamma National Park has precluded recollection of *A. parvula* over the past three years. The apparent periodicity in occurrence of these sea hares could simply be due to their small size and crypsis. Alternatively, recruitment may depend on larval transport from distant populations.[79] Furthermore, the difficulty of separating the filamentous red alga (a likely source of the sea hare's sequestered chemistry) from the coralline alga, and also of collecting sufficient amounts of it, has prevented a comprehensive examination of the red alga's chemistry.

2.3 The sequestered chemistry of *Aplysia dactylomela* in Algoa Bay

Of the four large specimens of *A. dactylomela* collected from the same site in Algoa Bay in March 1998, one was predominantly pale green while the remaining three sea hares were red. As we were conscious that this color variation might reflect different dietary selectivity,[79] and hence result in two different profiles of sequestered metabolites, the digestive gland of the green sea hare (Figure 2.2) was dissected out of the animal, extracted with acetone and worked up separately from those of the red specimens. After partitioning of the respective concentrated acetone extracts between EtOAc and water, Si gel chromatography of each concentrated EtOAc partition fraction, using a hexane to EtOAc solvent gradient, gave several crude fractions that were examined by TLC, ^{1}H NMR spectroscopy and the brine shrimp general bioactivity assay[139] in each case. Fortuitously, during both chromatographic separations, the major metabolite, algoane (**121**) crystallized slowly out of a chromatography fraction (8:2 hex/EtOAc) in similar high overall yield (6.6 %, calculated from the mass of the respective concentrated EtOAc partition layers).

A molecular formula of $C_{17}H_{27}Br_2ClO_4$ was established for algoane from HRFABMS data (*m/z* 489.0043 Δmmu +0.1). All seventeen carbon signals were clearly resolved in the ^{13}C NMR spectrum of **121** (Table 2.2) which, in combination with a DEPT experiment, were assigned to five quaternary, four methine, three methylene and five methyl carbon atoms. A ^{13}C NMR resonance at δ 168.8, a methyl singlet at δ 2.06 in the ^{1}H NMR spectrum (Figure 2.4) and a strong absorbance at 1721 cm^{-1} in the IR spectrum of **121** indicated the presence of a single acetate moiety. With one of the three degrees of unsaturation implied by the molecular formula thus accounted for, and no other sp^2 hybridized carbons evident in the ^{13}C NMR spectrum of **121**, a bicyclic structure was proposed for this compound. The remaining four methyl signals in the ^{1}H NMR spectrum of algoane (δ 0.91, 0.98, 1.44, 1.75) also appeared as singlets, thus placing each of these methyl groups on quaternary carbon atoms. Two bond HMBC correlations were used to define the geminal position of two methyl groups (δ 0.91 and 1.44) on C-11, and to place the remaining shielded (δ 0.98) and deshielded (δ 1.75) methyl substituents on C-7 and C-3 respectively (Table 2.2). The ^{13}C chemical shift of the quaternary carbon C-3 (δ 69.9) was consistent with a chlorine substituent at this position.[90] The two bromine atoms, evident from the molecular formula of algoane, were positioned on the similarly deshielded methine carbon signals resonating at δ 60.6 and 60.8. The only two D_2O-exchangeable resonances (δ 2.52 and 5.79) observed in the ^{1}H NMR spectrum of **121** were assigned to hydroxyl protons. Acetylation of **121** gave the diacetate (**132**), suggesting the presence of a tertiary hydroxyl group in algoane.



132

Further support for a tertiary hydroxyl moiety in **121**, and hence in **132**, was provided by a broad IR absorbance (3542 cm^{-1}), and a D_2O-exchangeable broad singlet (δ 4.32) in the ^{1}H NMR spectrum of the latter compound. A two bond correlation between the deshielded hydroxyl proton and C-6, in the HMBC spectra of both **121** and **132**,

unequivocally clinched the tertiary position of the one alcohol functionality. A strong COSY coupling between the upfield hydroxyl proton and the proton (δ 4.22) assigned to the methine carbon C-8 (δ 81.6) from an HMQC NMR experiment, was instrumental in placing the secondary hydroxyl functionality at C-8 in algoane.

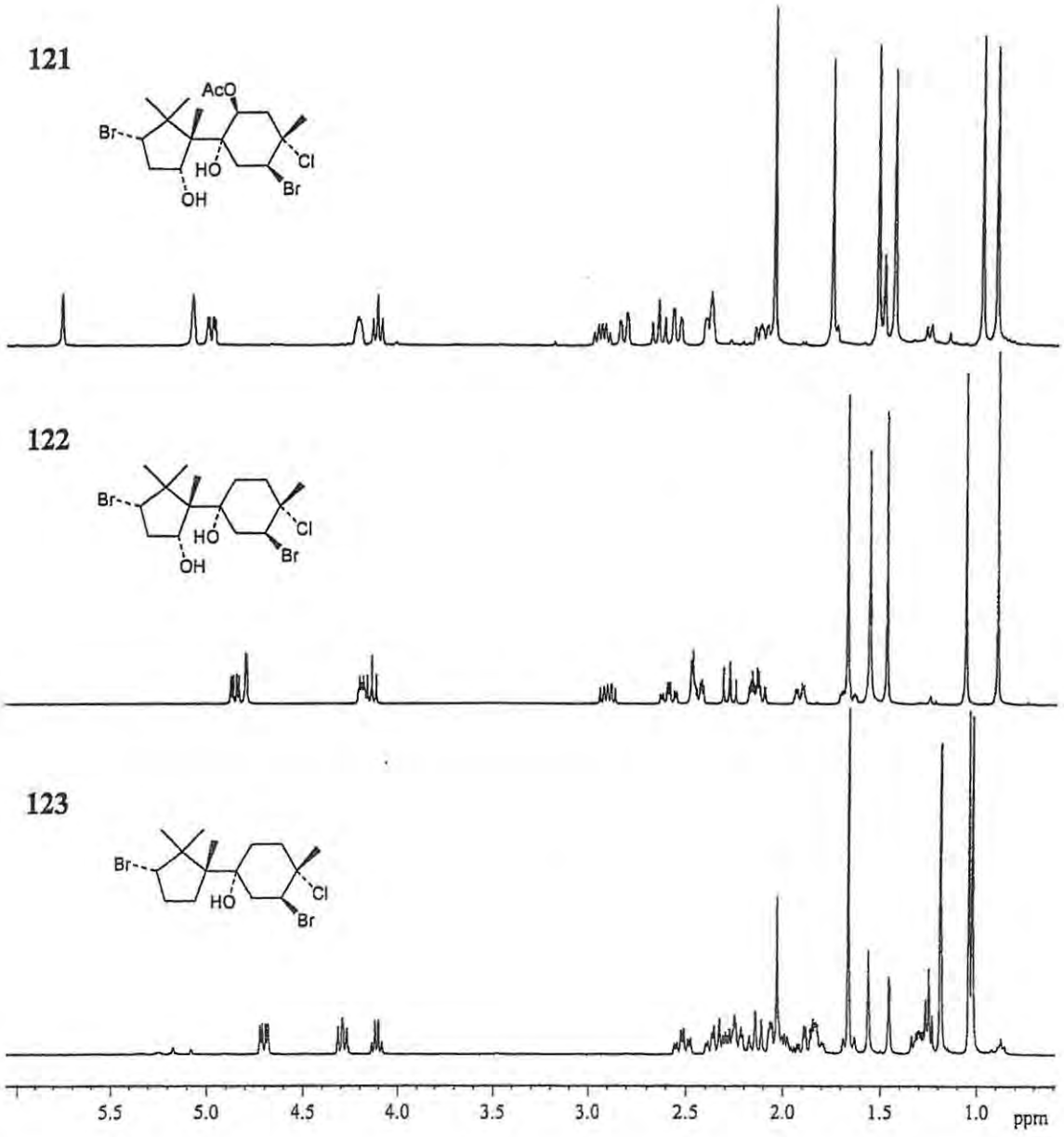


Figure 2.4 ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of the algoane compounds **121**, **122** and **123**.

Table 2.2 ^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data for compounds **121-123**

	Algoane (121)		**1-Deacetoxyalgoane (122)**		**1-Deacetoxy-8-deoxyalgoane (123)**	
Atom number	δ_H ppm (mult., J/Hz)	δ_C ppm (mult.)	δ_H ppm (mult., J/Hz)	δ_C ppm (mult.)	δ_H ppm (mult., J/Hz)	δ_C ppm (mult.)
1	5.07 (d, 3)	74.3 (d)	1.67 (m, 3) 1.92 (br d, 14)	30.3 (t)	1.83 (m) 1.89 (dd, 4, 14)	29.5 (t)
2	2.55 (dd, 3, 15) 2.83 (dd, 3, 15)	41.4 (t)	2.16 (m) 2.60 (td, 5, 14)	37.6 (t)	2.23 (dt, 3, 14) 2.52 (td, 6, 14)	38.2 (t)
3		69.9 (s)		71.1 (s)		70.8 (s)
4	4.98 (dd, 4, 12)	60.6 (d)	4.86 (dd, 4, 12)	61.5 (d)	4.70 (dd, 4, 12)	60.6 (d)
5	2.40 (m) 2.65 (t, 13)	41.3 (t)	2.29 (t, 13) 2.45 (br d, 14)	43.9 (t)	2.05 (m) 2.14 (t, 13)	43.2 (t)
6		78.9 (s)		79.8 (s)		78.9 (s)
7		52.6 (s)		52.3 (s)		50.2 (s)
8	4.22 (qn, 4)	81.7 (d)	4.20 (dt, 3, 8)	81.0 (d)	1.30 (br m) 2.36 (m)	30.7 (t)
9	2.13 (td, 3, 10) 2.94 (qn, 8)	44.1 (t)	2.13 (td, 3, 10) 2.91 (qn, 8)	44.4 (t)	1.99 (m) 2.29 (m)	30.8 (t)
10	4.12 (t, 10)	60.9 (d)	4.14 (t, 10)	61.3 (d)	4.29 (d, 8)	63.8 (d)
11		47.1 (s)		47.7 (s)		47.6 (s)
12	0.91 (s)	22.1 (q)	1.47 (s)	23.7 (q)	1.19 (s)	22.2 (q)
13	1.44 (s)	23.5 (q)	1.06 (s)	23.7 (q)	1.04 (s)	22.6 (q)
14	0.98 (s)	20.3 (q)	0.90 (s)	20.4 (q)	1.02 (s)	23.3 (q)
15	1.75 (s)	27.5 (q)	1.67 (s)	23.3 (q)	1.66 (s)	23.2 (q)
16		168.8 (s)				
17	2.06 (s)	21.5 (q)				
O<u>H</u>-6	5.79 (br s)		4.79 (d, 2)		1.44 (br s)	
O<u>H</u>-8	2.52 (d, 4)		2.39 (d, 4)			

While the use of standard 2D NMR techniques quickly facilitated entry to the substitution pattern in algoane, establishing the skeletal class of this sesquiterpene proved more challenging. Although the common, spirofused, chamigrane system of acetoxyintricatol[140] (**133**) seemed more attractive than the unprecedented, non-aromatic, cuparane **121**, crucial HMBC correlations in support of the former structure, namely two bond correlations from the methine H-1 proton and H_2-5 methylene protons to the carbon signal at δ 52.6 (C-6 in **133**), were not observed (Figure 2.5). Conversely, clear

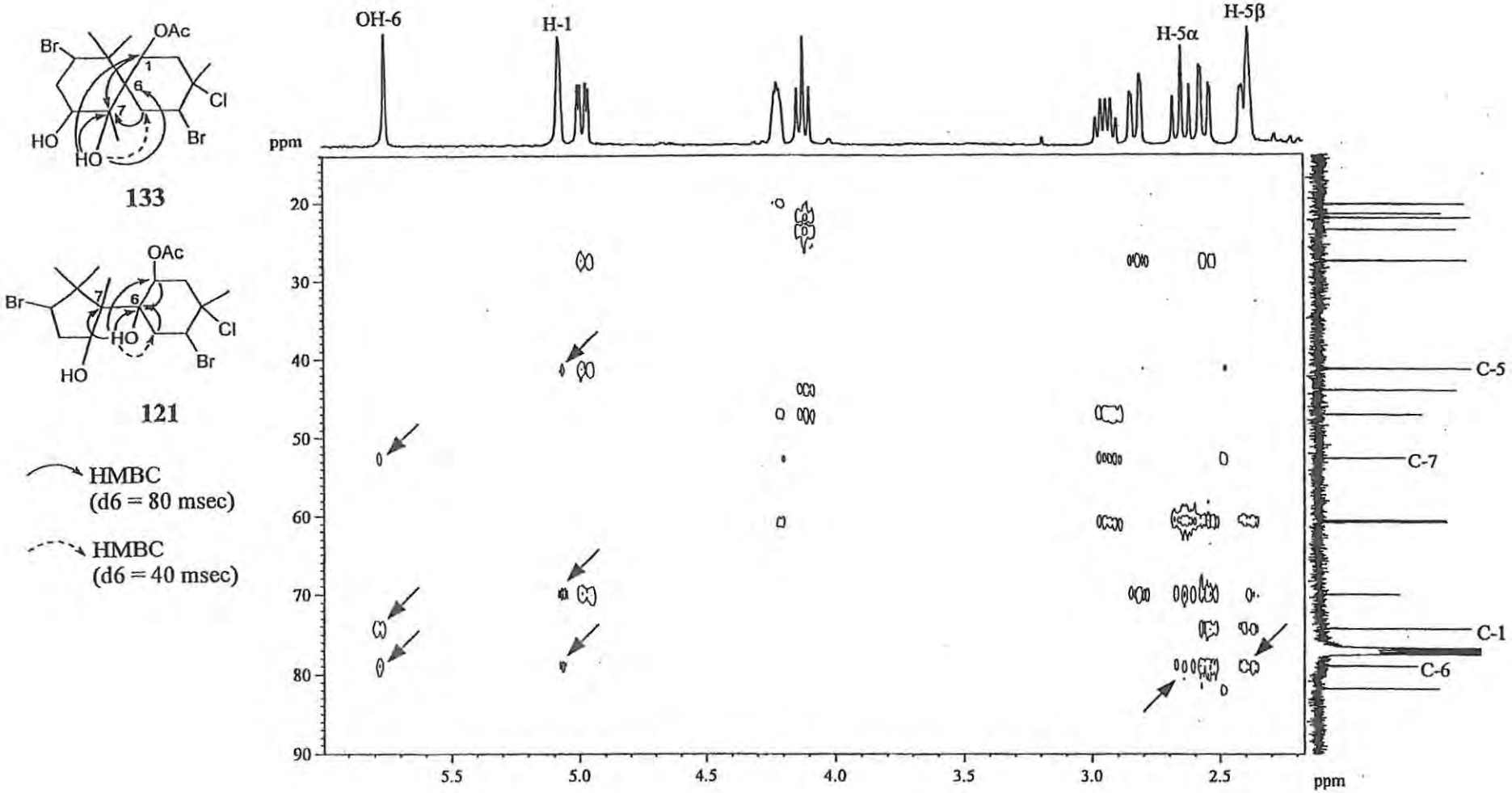


Figure 2.5: A region (F1 = δ 14 – 90 ppm, F2 = 2.2 – 6.0 ppm) of the HMBC spectrum ($CDCl_3$, 400 MHz, d6 = 80 msec) for algoane. Accompanying figure shows these correlations as applied to the spirofused chamigrane **133** and the cuparane skeleton of **121**. A three bond HMBC correlation from OH-6 to C-5 was visible in an HMBC experiment using d6 = 40 msec.

correlations between these protons and the carbon resonance at δ 78.9 (C-7 in **133** and C-6 in **121**) were evident. These data, together with the prominent two and three bond HMBC correlations from the tertiary hydroxyl group proton to the carbon resonances C-1 and C-6 strongly favored structure **121**. Furthermore, by altering the d6 delay time in the HMBC experiment, correlations to C-5 (d6 = 40 msec) and C-6 (d6 = 80 msec, Figure 2.5) were observed.

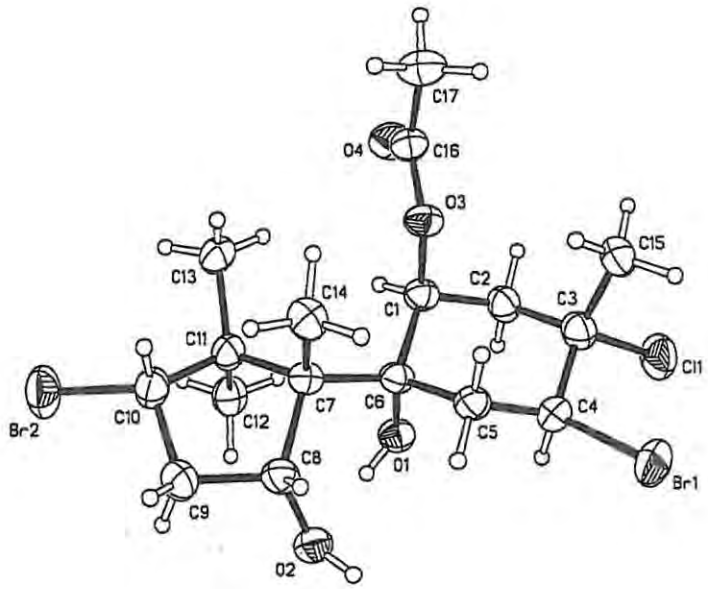
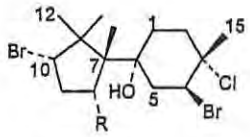


Figure 2.6: A view of a molecule of **121** from the crystal structure showing the numbering scheme employed. Anisotropic displacement ellipsoids for the nonhydrogen atoms are shown at the 50 % probability level. Hydrogen atoms are displayed with an arbitrarily small radius.

The unusual, non-aromatic, cuparane skeleton of algoane, suggested from the NMR data, was unequivocally confirmed by a single crystal X-ray diffraction study of this compound carried out at 150(2) K. The asymmetric unit contained one, fully ordered molecule (Figure 2.6) in which the five and six-membered rings adopted envelope and chair conformations respectively. In addition, the absolute configuration at each of the seven chiral centers in **121** was established unambiguously as 1(*S*), 3(*S*), 4(*S*), 6(*R*), 7(*S*), 8(*R*) and 10(*S*) by refinement of the absolute structure parameter.[141] Two hydrogen bonding interactions, one intramolecular [O1 - H1 ··· O2; distance O1 ··· O2, 2.593(4) Å] and the other intermolecular [O2 - H2 ··· O4; distance O2 ··· O4, 2.789(4) Å], were evident from

the crystal structure determination. The atomic co-ordinates of algoane are presented in Appendix 1.

The structure determination of **121** provided a valuable insight into the structures of two other crystalline compounds, **122** (mp 152-155°C; $[\alpha]^{22}_{D}$ +18.0°, *c* 0.40, $CHCl_3$) and **123** (mp 138-142°C; $[\alpha]^{22}_{D}$ +22.5°, *c* 1.15, $CHCl_3$), isolated by normal phase HPLC of more nonpolar, bioactive Si gel chromatography fractions. Interestingly, **122** was isolated from the extracts of the red color variants of *A. dactylomela* and not from the pale green color variant extract, which yielded **123** only. The ^{1}H and ^{13}C NMR data of **122** and **123** (Figure 2.4, Table 2.2) indicated that the latter compounds contained the same basic skeleton as **121**, differing only in the degree of oxygenation around the five- and six-membered rings.



122 R = OH
123 R = H

The molecular formulae of **122** and **123** were established as $C_{15}H_{25}Br_2ClO_2$ (*m/z* 430.9987 Δmmu –0.1) and $C_{15}H_{25}Br_2ClO$ (*m/z* 415.0034 Δmmu –0.5) respectively from HRFABMS data. The difference of 58 atomic mass units ($C_2H_2O_2$) between the molecular masses of **122** and **123** implied the complete loss of the acetate functionality from C-1 in the latter compound. The absence of any contrary evidence from the IR, ^{13}C and ^{1}H NMR spectra of **122**, indicative of the presence of an acetate moiety, corroborated this assumption. The emergence of a fourth methylene carbon signal (δ 30.3) and the disappearance of the C-1 oxymethine resonance (δ 74.3) in the ^{13}C NMR spectrum of **122**, gave additional support to the 1-deacetoxyalgoane structure proposed for this compound.

The ^{13}C and ^{1}H NMR data for the six-membered ring of **123** were consistent with those of **121** (Table 2.2). Therefore, from the difference of one oxygen atom between the

molecular formulae of **122** and **123**, the latter compound was identified as 1-deacetoxy-8-deoxyalgoane lacking both the acetoxy and secondary hydroxyl substituent at C-1 and C-8 respectively. In accordance with the argument presented earlier for the structure of **122**, loss of the C-8 oxymethine carbon signal and its replacement with a methylene resonance (δ 30.7) in the ^{13}C NMR spectrum of **123**, supported a deoxygenated five-membered ring in the latter compound. Furthermore, the HMBC correlations observed from the single hydroxyl proton in **123** to C-1, C-5, and C-6, and a long range COSY coupling between this proton and one of the methylene protons resonating at δ 2.14 (H-5), confirmed that the tertiary hydroxyl group at C-6 was still intact. Interestingly, the large upfield shift of the tertiary hydroxyl proton in **123** (δ 1.44), relative to the deshielded chemical shifts observed for this proton in **121** and **122** (δ 5.79 and 4.79 respectively), could be attributed to the absence of the hydroxyl moiety at C-8 in the former compound. The concomitant loss of the intramolecular hydrogen bonding interaction between the two hydroxyl groups in 1-deactoxy-8-deoxyalgoane (**123**), was confirmed by the X-ray structure analysis of **121**.

The stereochemistry of compounds **122** and **123** was shown to be the same as that for algoane from a series of 1D NOE difference experiments. The configuration around the five-membered ring in **122** was established from reciprocal NOE enhancements of the proton resonances H-8 (δ 4.20), H-10 (δ 4.14) and H_3-14 (δ 0.90). The absence of an NOE enhancement of the tertiary hydroxyl proton signal on irradiation of the latter methyl singlet tentatively confirmed the expected *trans* relationship between these two moieties. No NOE enhancement of the bromomethine signal (H-4, δ 4.86) was observed on irradiation of the vicinal methyl singlet (H_3-15, δ 1.67), thus supporting the diequatorial halogen substitution pattern implied by the axial bromomethine proton coupling constants (J = 4 and 12 Hz).[142] Similarly, NOE irradiation of the H-10 (δ 4.29) and H_3-14 (δ 1.02) resonances in **123** resulted in reciprocal enhancements, while no enhancement of the tertiary hydroxyl proton signal (δ 1.44) was observed. Diequatorial halogen substituents in the six-membered ring of **123** were also indicated by recourse to

coupling constants (J = 4 and 12 Hz) and the absence of an NOE enhancement of the bromomethine (H-4) proton signal on irradiation of the H_3-15 (δ 1.66) singlet.

The digestive gland extract from the red color variants yielded the minor new halogenated sesquiterpene, ibhayinol (**124**, $[\alpha]^{22}_D$ +9°, *c* 0.57, $CHCl_3$), which we named after the colloquial name, 'ibhayi', for Algoa Bay (pronounced ee-by-ee). Ibhayi is derived from the three main languages, Xhosa, English and Afrikaans, spoken in the eastern Cape.

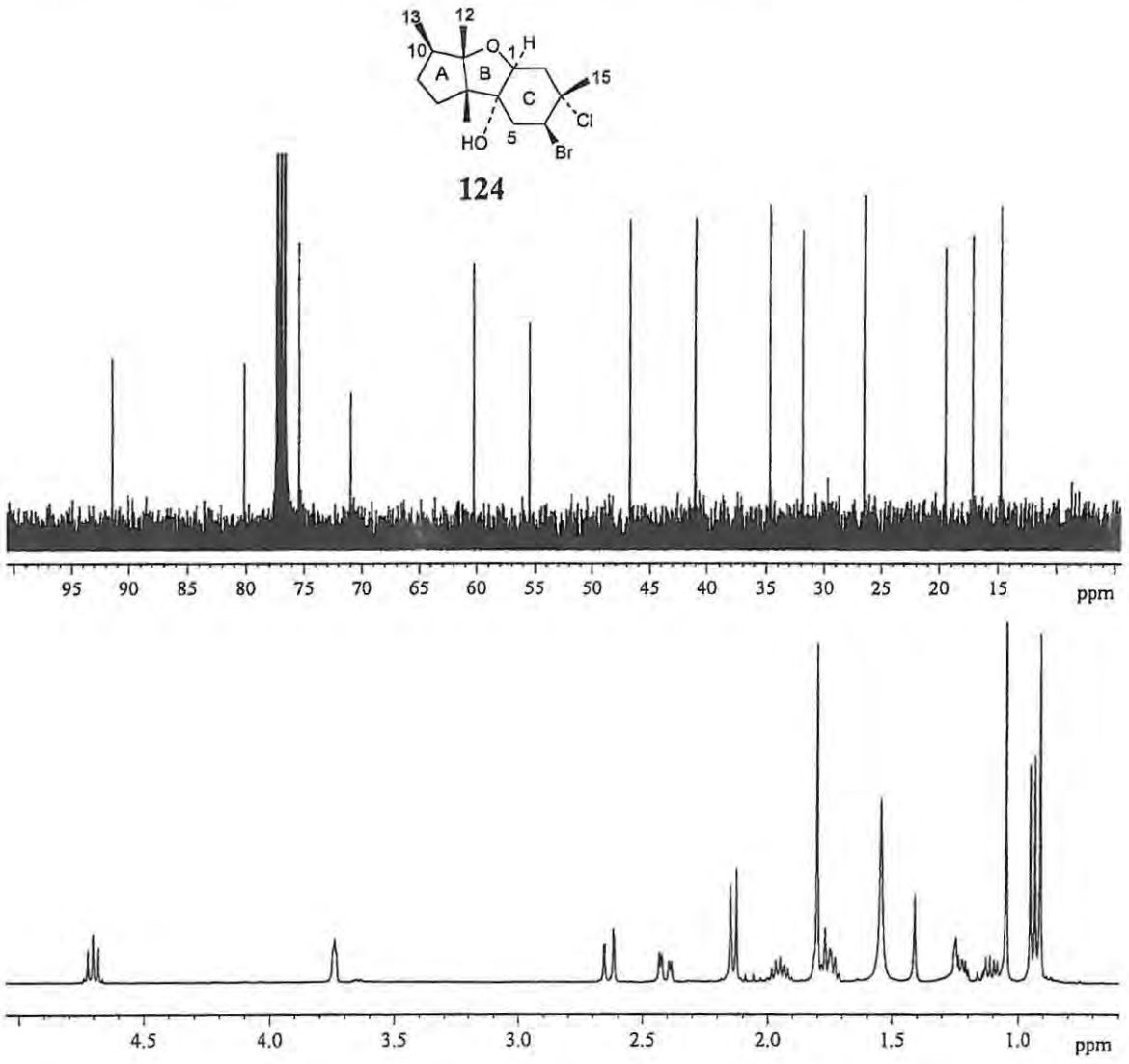


Figure 2.7 ^{13}C ($CDCl_3$, 100 MHz, upper) and ^{1}H ($CDCl_3$, 400MHz, lower) NMR spectra for ibhayinol (**124**)

The ^{13}C and ^{1}H NMR data of **124** (Table 2.3, Figure 2.7) were broadly compatible with those of the unsaturated sesquiterpenes **122** and **123**, and we initially assumed that this

compound was a fourth member of the algoane series. However, the molecular formula of **124** ($C_{15}H_{24}BrClO_2$), established from HRFABMS data (*m/z* 351.0726 Δmmu –0.1), implied three degrees of unsaturation and, with no olefinic or carbonyl signals evident in the ^{13}C NMR spectrum of **124**, required this compound to be tricylic.

From a comparison of the 1D and 2D NMR data of **124** (Table 2.3) with those of **121** – **123**, both a six- and a five-membered, unsaturated ring could be delineated for the former compound. Although the ^{13}C NMR spectrum of **124** revealed five deshielded resonances (δ 60.3, 71.0, 75.4, 80.2, 91.4) attributable to carbons attached to heteroatoms, only one exchangeable hydroxyl proton (δ 1.39) was evident in the ^{1}H NMR spectrum of this compound. The first two of these deshielded carbon signals were assigned to brominated methine and chlorinated quaternary carbon atoms respectively, in accordance with the related C-4 and C-3 ^{13}C chemical shifts established for compounds **121** – **123** (Table 2.2). A two bond HMBC correlation from the hydroxyl proton to the quaternary carbon C-6 (δ 80.3) established the tertiary position of the single hydroxyl moiety. With three of the five deshielded carbon resonances therefore accounted for, the remaining oxygen atom was placed in a cyclic ether position between C-1 (δ 75.4) and C-11 (δ 91.4) to form the third ring required by the molecular formula. The unusually deshielded, latter chemical shift is consistent with the equivalent, quaternary ether carbon chemical shift (δ 97.1) reported for the tricyclic, laurane sesquiterpene isoaplysin (**134**),[90] and suggested a similar non-aromatic, cyclic laurane ether structure for **124**. Two and three bond HMBC correlations from the methyl doublet (δ 0.94, H_3-13) to C-9, C-10 and C-11 placed this methyl group in a vicinal position relative to the ether moiety. Similarly, HMBC correlations and COSY couplings (Table 2.3) were used to position the remaining substituents and thus establish the structure of ibhayinol.

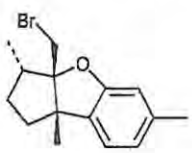


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Table 2.3. ^{1}H (400 MHz, $CDCl_3$), ^{13}C (100 MHz, $CDCl_3$) and 2D NMR data for ibhayinol (**124**)

Atom Number	δ_H ppm (mult., J/Hz)	δ_C ppm (mult.)	COSY90 coupling to	HMBC correlation to
1	3.74 (br s)	75.4 (d)	H_2-2, H_2-5	C-2/5, C-3, C-6
2	2.41 (dd, 4, 15)	41.1 (t)	H-1, H-2, H_3-15	C-1, C-3, C-15
	2.64 (dd, 2, 15)		H-1, H-2	C-1, C-3, C-4, C-6, C-15
3	-	71.0 (s)	-	-
4	4.71 (m)	60.3 (d)	H_2-5, H_3-15	C-2/5, C-3, C-6, C-15
5	2.14 (d, 9)	41.3 (t)	H-1, H-4, OH	C-1, C-3, C-4, C-6, C-7, C-15
6	-	80.2 (s)	-	-
7	-	55.5 (s)	-	-
8	1.23 (qn, 5)	34.7 (t)	H-8, H_2-9, H-10	C-7, C-9, C-10, C-11, C-14
	1.77 (m)		H-8	C-6, C-7, C-9, C-11, C-14
9	1.11 (q, 6)	31.9 (t)	H_2-8, H-9, H-10	C-8, C-10, C-13
	1.75 (m)		H_2-8, H-9, H-10	C-8
10	1.95 (sept, 6)	46.8 (d)	H-8, H_2-9, H_3-13	C-9, C-11, C-13
11	-	91.4 (s)	-	-
12	1.05 (s)	19.5 (q)	H_3-13, H_3-14	C-7, C-10, C-11
13	0.94 (d, 7)	14.8 (q)	H-10, H_3-12	C-9, C-10, C-11
14	0.92 (s)	17.2 (q)	H_3-12	C-6, C-7, C-8, C-11
15	1.80 (s)	26.5 (q)	-	C-2/5, C-3, C-4
OH	1.39 (s)	-	H_2-5	C-1, C-2/5, C-6

In determining the relative stereochemistry of ibhayinol, a NOESY experiment and the use of the NMR simulation program NMRSim to decipher the ^{1}H splitting patterns and coupling constants of H-4 (δ 4.70) and H_2-5 (δ 2.14) provided evidence for the derivation of ibhayinol from an algoane precursor. NOESY correlations between H_3-12 (δ 1.04), and H_3-13 (δ 0.94), and H_3-12 (δ 1.04) and H_3-14 (δ 0.92) placed all three methyl groups on the same side of the molecule and suggested *cis* fusion of rings A and B. No NOESY

correlations were observed between these three methyl groups and H-1 (δ 3.74). The latter proton however showed NOESY correlations to H_2-2 (δ 2.41 and 2.64), H-8α (δ 1.23), H-9α (δ 1.75) and H-10 (δ 1.95). The broad H-1 triplet (J = 2.5 Hz) indicated a gauche orientation of H-1 relative to the two vicinal protons on C-2. A significant COSY coupling from the thus equatorial H-1 to δ 2.14 could be attributed to W-coupling between H-1 and the equatorial H-5α. Since an expansion of the H-4 triplet and the H_2-5 doublet revealed satellite peaks indicative of second order coupling, NMRSim was used to reproduce the splitting pattern of the H_2-5 doublet and to differentiate the closely overlapped chemical shifts of the equatorial H-5α and axial·H-5β, giving values of 2.138 and 2.142 ppm respectively. The proximity of the latter two chemical shifts, which are separated by less than the geminal coupling $J_{5\alpha,5\beta}$, gave rise to the second order coupling observed. By considering coupling constants for analogous protons in similar brominated six-membered rings given in the literature,[90] the H_2-5 doublet could be reproduced in NMRSim using $J_{4,5\beta}$ = 12.5 Hz and $J_{4,5\alpha}$ = 5.3 Hz, with the geminal coupling $J_{5\alpha,5\beta}$ = -13 Hz. The equatorial bromine substituent and therefore axial bromomethine proton H-4 (δ 3.74) are consistent with spin systems in analogous halogenated algal metabolites and a NOESY correlation between H-4 and H-2α (δ 2.41) lends support to this assignment. Figures 2.8 and 2.9 represent energy-minimized models of ibhayinol with rings B and C *cis* fused and *trans* fused respectively. In the *cis* fused model, ring C is in a chair conformation and this model is 6.5 Kcalmol^{-1} more stable than the *trans* fused model, which would require H-1 to be axial. Unfortunately, recrystallization of ibhayinol yielded multinucleate crystals for which crystallographic data could not be obtained.[143]

Ibhayinol is possibly biosynthesized from a hypothetical 8-hydroxy-1-deacetyl-algoane precursor as follows. Loss of the equatorial bromine at C-10 followed by a 1,2 shift of the axial methyl group at C-11 would generate a planar carbocation at C-11. Nuclear attack towards the *Si* face of this planar carbocation by the hydroxyl oxygen at C-1, and concomitant loss of the hydroxyl proton, would yield ibhayinol. Therefore ibhayinol is probably a fourth member of the algoane series.

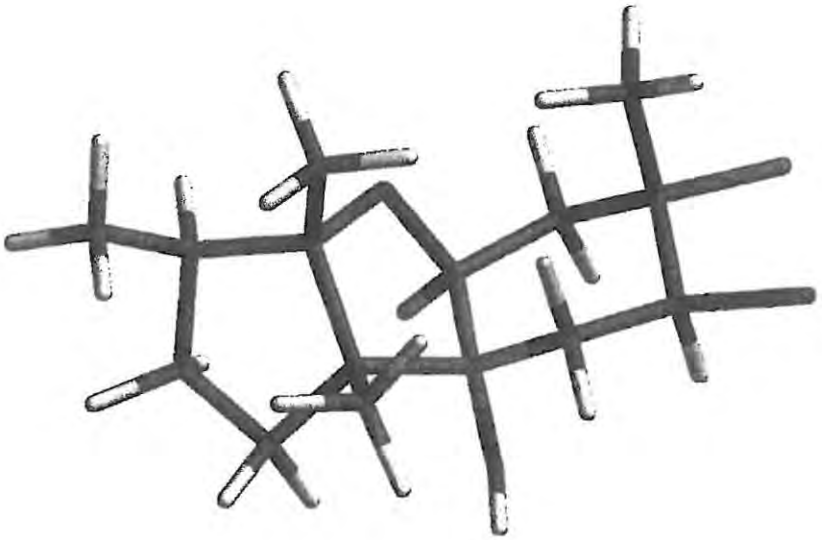


Figure 2.8 SpartanPro energy minimised model of ibhayinol (rings B and C *cis*-fused) and ring C in a chair conformation.

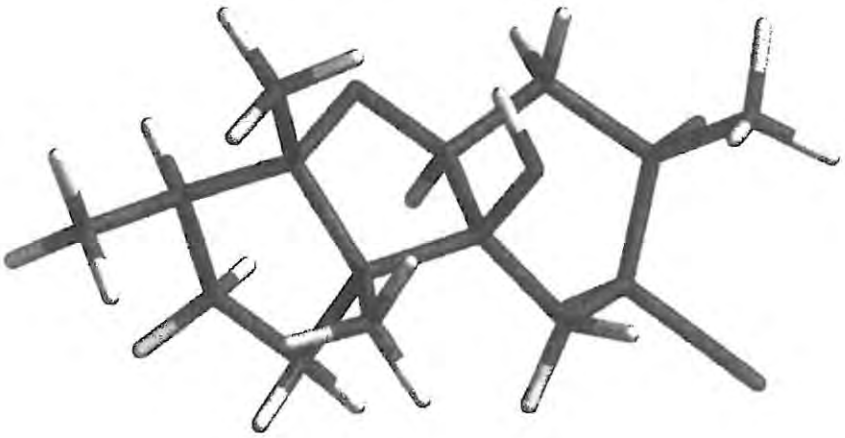
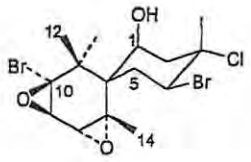


Figure 2.9 SpartanPro energy minimised model of ibhayinol (rings B and C *trans*-fused)

The known compounds **101** and **125** were obtained by exhaustive normal phase HPLC of a crude, nonpolar chromatography fraction from the green *A. dactylomela* extract.



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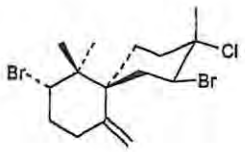
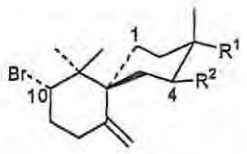


125

Compound **101** (mp 95-97°C, $[\alpha]^{22}_{D}$ +110°, *c* 0.73, $CHCl_3$) yielded a molecular formula of $C_{15}H_{21}Br_2ClO_3$ (*m/z* 441.9552 Δmmu +1.1) from HRFABMS data. The two epoxy methine (δ 55.5 and 55.6) and two quaternary epoxide carbon signals (δ 61.0 and 75.5) in the ^{13}C NMR spectrum of **101** accounted for two of the rings in this tetracyclic chamigrane sesquiterpene and provided the key to the structure of **101**. While, the ^{1}H and ^{13}C NMR data of **101** are in complete accord with those reported by Pitombo *et al.*[126] for the di-epoxychamigrane sesquiterpene, prepacifenol epoxide (mp 154-155°C, $[\alpha]^{23}_{D}$ +155°, *c* 0.1, $CHCl_3$) found in *A. dactylomela*, the melting point we obtained for our unrecrystallized compound is more compatible with that reported initially for prepacifenol epoxide (mp 98-99°C) isolated from the digestive gland of *A. californica.*[124,127]

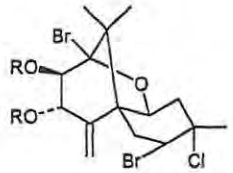
HRFABMS data secured the molecular formula of **125** ($[\alpha]^{22}_{D}$ + 27°, *c* 0.22, $CHCl_3$) as $C_{15}H_{23}Br_2Cl$ (*m/z* 395.9858 Δmmu +0.2). The exocyclic, olefinic methylene (ν_{max} 1638 and 905 cm^{-1}) carbon signal (δ 114.7) in the ^{13}C NMR spectrum of **125** accounted for one of the three double bond equivalents implied by the molecular formula and thus required **125** to be bicyclic. The IR (ν_{max} 1390 and 1380 cm^{-1}) and ^{1}H NMR (δ 0.96 and 1.14) data of **125** revealed a *gem*-dimethyl functionality. Further ^{1}H NMR signals indicated the presence of a tertiary methyl group (δ 1.69) and two halomethine protons (δ 4.43 and 4.71, J = 5, 13 Hz). A search of the marine natural product literature revealed two, isomeric β-chamigrene *Laurencia* metabolites, nidificene[144,145] and obtusane (**135**),[90] each with the same molecular formula as **125** and bearing the same array of substituents.



125 $R^1 = Cl$, $R^2 = Br$
135 $R^1 = Br$, $R^2 = Cl$

The optical rotation, IR, NMR and MS data of **125** were consistent with those reported for nidificene ($[\alpha]^{15}_D$ +30.6°, *c* 1.4, $CHCl_3$),[145] while a comparison of the ^{13}C NMR data for **125** and **135** confirmed the difference in the bromochloro substitution of ring A in each compound. In **125**, the chlorine occupies the tertiary position on the deshielded C-3 (δ 71.8 ppm), with the bromine being placed on the significantly more shielded C-4 (δ 61.4 ppm). The relative placement of the halogens in vicinal bromochlorohexanes has been a source of confusion in the past,[146] aggravated by the apparent ease with which the halogen atoms interchange during chemical transformations.[147] The ^{13}C chemical shift ranges for these systems are summarized by Erickson.[90] Extensive 2D NMR experiments allowed the previously unassigned NMR data for both compounds **125** and **101** to be assigned and were completely consistent with the structures given (Table 2.4).

A final metabolite (**104**) was isolated from the extract of the red *A. dactylomela* specimens in the same normal phase HPLC run which yielded ibhayinol (**124**). The ^1H and ^{13}C NMR data for compound **104** (mp 168-170°C) were identical to those reported by Pitombo *et al.*[126] for the diol (mp 172-173°C) obtained from Brazilian specimens of *A. dactylomela*. The successful conversion of **104** to the diacetate **136** was inferred from the presence of two additional methyl singlets (δ 2.0) in the ^1H NMR spectrum of **136** and four additional carbon resonances [δ 21.0 (q), 21.3 (q), 168.4 (s), 169.3 (s)] in the corresponding ^{13}C NMR spectrum of **136**. While HRFABMS surprisingly failed to afford an M^+ peak for **104**, a molecular formula of $C_{15}H_{26}Br_2ClO_5$ for **136** was confirmed from HRFABMS data (526.982998, Δmmu –0.6). The diol **104** was initially obtained from chemical transformations of johnstonol (**137**)[148] and prepacifenol epoxide (**101**)[124,127] under acidic conditions, and has also been reported as a minor natural product from *A. californica*.[124,127]



104 R = H
136 R = Ac

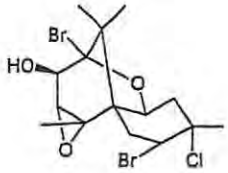


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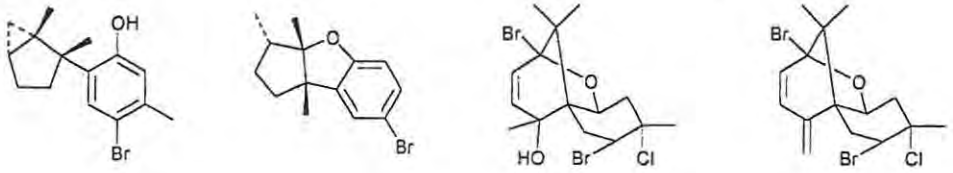
Table 2.4 ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C ($CDCl_3$, 100 MHz) NMR data for compounds **101** and **125**

	Prepacifenol epoxide (**101**)		Nidificene (**125**)	
Atom Number	δ_H ppm (mult., *J*/Hz)	δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)	δ_C ppm (mult.)
1	4.03 (sept, 3)	69.8 (d)	1.72 (br d, 14) 1.97 (m)	25.4 (t)
2	2.52 (m)	47.2 (t)	2.44 (dd, 4, 14) 2.14 (m)	38.7 (t)
3		71.5 (s)		71.8 (s)
4	4.73 (dd, 4, 13)	62.1 (d)	4.71 (dd, 5, 13)	61.4 (d)
5	2.14 (m) 2.54 (t, 14)	33.9 (t)	1.94 (br d, 14) 2.14 (m)	38.6 (t)
6		50.2 (s)		51.1 (s)
7		61.0 (s)		145.6 (s)
8	3.06 (s)	56.7 (d)	2.31 (dd, 5, 14) 2.14 (m)	33.5 (t)
9	3.65 (s)	55.6 (d)	2.02 (dd, 5, 13) 2.25 (dd, 6, 19)	35.8 (t)
10		75.5 (s)	4.43 (dd, 5, 13)	63.6 (d)
11		46.8 (s)		43.8 (s)
12	0.98 (s)	24.3 (q)	0.95 (s)	17.5 (q)
13	1.53 (s)	27.2 (q)	1.14 (s)	23.6 (q)
14	1.48 (s)	22.1 (q)	4.86 (s) 5.25 (s)	114.7 (t)
15	1.91 (s)	28.0 (q)	1.69 (s)	24.1 (q)

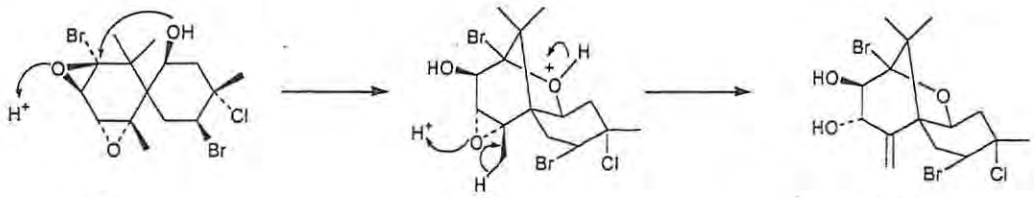
To the best of our knowledge, no non-aromatic cuparanes like those which we have reported here have been found in any species of *Aplysia* or red algae, although we assume that the algoanes **121-123** and ibhayinol (**124**) are sequestered from dietary *Laurencia* species in Algoa Bay along with chamigranes **125** and **101**. It is unlikely that the proposed biotransformation of a chamigrane precursor to give the algoanes *via* ring

contraction (Scheme 2.1) may have occurred from the contact of the sea hare extracts with Si gel, which has been found to cause changes in other halogenated chamigranes,[132,149] since examination of the ^{1}H NMR spectra of the crude digestive gland extracts revealed signals characteristic of the algoane compounds, implying their existence in the extracts prior to Si gel chromatography. However, it is uncertain whether the algoanes are dietary red algal metabolites or whether they have been formed from chamigrane precursors in the sea hare digestive glands. Stallard and Faulkner[86] have shown that the gut contents of *A. californica* are at a pH of 5.0 – 6.1 which was deemed sufficiently acidic for the transformation of laurinterol (**138**) to aplysin (**31**) and also pacifenol (**102**) to pacifidiene (**103**).



138 **31** **102** **103**

Indeed, acid catalysed transformation of prepacifenol epoxide (**101**) in the sea hare digestive gland is the most likely source of the diol **104** which we obtained from the red specimens of *A. dactylomela* (Scheme 2.2).



101 **104**

Scheme 2.2 Possible acid catalysed conversion of **101** to **104** in the digestive gland of *Aplysia dactylomela*

The myriad of red algal sesquiterpenes and acetylenic ethers has presented a challenge to natural products chemists attempting to characterize new structures and differentiate between very similar compounds using spectroscopic techniques. Highly halogenated cyclic molecules of low mass do not lend themselves to ready structure elucidation by ^{1}H

NMR spectroscopy and also, halogens may be labile under the conditions required for mass spectrometry,[77] as we have found in dealing with the algoanes.[150] Obviously there is a paucity of full NMR data for compounds first characterized by crystallography or chemical derivatization twenty five to thirty five years ago and this makes dereplication of known compounds very difficult. Reassignments of structures and especially stereochemistries are commonplace. Nevertheless, careful analysis of the chemistry of *Laurencia* has become of paramount importance in unravelling the complex taxonomy of this genus. Guella *et al.*[134] propose that the four stereochemical types of polyhalogenated chamigrenes (see earlier) found in *Laurencia* red algae reflect phylogeny and represent four lineages within the genus. Furthermore, while most species of *Laurencia* have many minor metabolites in common, they can be characterized by at least one compound unique to that species.[151] Thus, within some species such as *L. nipponica*, morphologically indistinguishable chemical 'races' have been described,[152] each of which is characterized by a specific major secondary metabolite, either acetylenic ether or sesquiterpenoid, whose biosynthesis is not apparently influenced by environmental factors. The syntheses of C_{15} acetylenic ethers and sesquiterpenes are thought to be along different metabolic pathways requiring different sets of enzymes and thus the diversity of halogenated secondary metabolite synthesis in *L. nipponica* is taken to represent the first step in speciation.[152]

Whether or not the great diversity of halogenated metabolites in *Laurencia* is a source of potential defense chemicals for *Aplysia* is unresolved. Although it is well proven that *Aplysia* sea hares contain bioactive metabolites,[153] not all red algal extracts show significant biological activity.[154] Since most *Laurencia* species are characterized by a few major metabolites, one might expect that the *Aplysia* sea hares would be specialist feeders on those *Laurencia* species that provide significant amounts of the potential defense metabolites. However, for the most part, *Aplysia* sea hares are generalist feeders on *Laurencia* and other red algal species.[79] Those *Aplysia* species described as specialist feeders are often the less well studied sea hares.[79] From feeding experiments using *A. californica*, Stallard and Faulkner[84] concluded that the sequestered halogenated

metabolites persist for no more than three months in the sea hare digestive glands. However, three months is a significant period of time considering the approximately annual life cycle of *Aplysia* species. In addition, de Nys *et al.* found that the sequestered metabolites in *A. parvula* were present in much the same concentrations as in the sea hare's dietary red alga.[39] The above observations could indicate that *Aplysia* sea hares simply accumulate toxic metabolites, which they are unable to metabolize, in their large specially modified digestive glands. Obviously more ecological studies are necessary to determine not only whether the sequestered metabolites are bioactive at their natural concentrations per animal, but also whether these metabolites are present in the sea hare mantle in concentrations sufficient to deter predators.

Chapter Three

The Sequestered Chemistry and Diet of the Arminacean Nudibranch *Leminda millecra* in Algoa Bay, South Africa

3.1 The chemical ecology of arminacean nudibranchs

The endemic South African nudibranch *Leminda millecra* (Family Lemindidae, Suborder Arminacea) is a translucent pink nudibranch with a blue-edged mantle that is expanded into well-developed parapodia (Figure 3.1). The species, which lacks external gills or cerata and has a distinct internal morphology, was described in 1985 and represents a single genus in a new family of arminacean nudibranchs.[80]

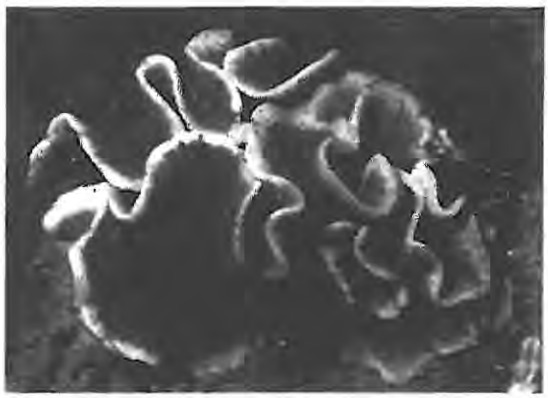
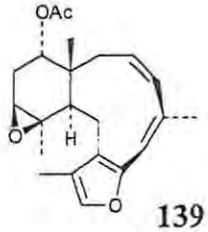


Figure 3.1 *Leminda millecra* photographed in Algoa Bay

Compared to dorid nudibranchs, little is known about the biology of the Arminacea, the smallest suborder of nudibranchs, which also includes families Arminidae and Janolidae. Nudibranchs belonging to family Arminidae feed mostly on soft corals and sea pens (pennatulaceans), while janolids are commonly associated with bryozoans.[80] Whereas arminids have no obvious physical defense, janolids possess well-developed cerata which contain extensions of the digestive gland in several species.[80] Presumably, these highly visible autotomizable structures have a defensive function, although whether they contain chemical deterrents or merely serve as a physical decoy is unknown. No chemical deterrent storage glands analogous to the mantle dermal formations of chromodorids have been described for any arminaceans. However, the few chemical investigations of arminacean nudibranchs show that, like dorids, they may contain secondary metabolites

of dietary origin. Several investigations of the chemistry of the nudibranch *Armina maculata* and its pennatulacean prey, *Veretillum cynomorium*, have revealed the presence of briarane diterpenes,[155] *e.g.* verecynarmin A (**139**), and the cembranoid precursor, preverecynarium (**140**),[156] in both organisms. The Mediterranean nudibranch *Janolus cristatus* yielded a toxic tripeptide, janolusimide (**141**) of unknown origin.[157]



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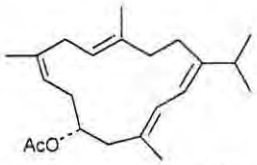

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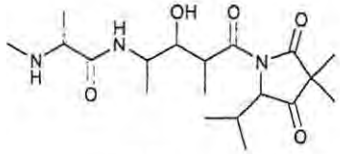

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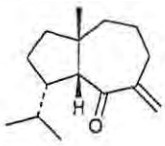
Recently, the first evidence of an octocoral diet for *Leminda millecra* was provided by Pika and Faulkner,[158] who found soft coral spicules of *Alcyonium foliatum, A. valdiviae* and *Capnella thyrsoidea* in the digestive tract of four specimens of *L. millecra*. These individuals, collected at the northern limit of *L. millecra*'s range in the Transkei on the East coast of South Africa, yielded millecrones A (**142**) and B (**143**), and the millecrols A (**144**) and B (**145**).



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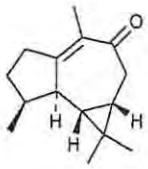

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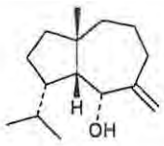

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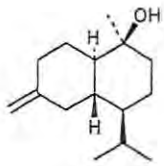

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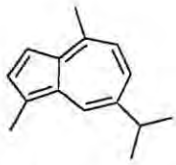
To the best of our knowledge, millecrone A and millecrol A are the only sesquiterpene examples of the carotane skeletal type isolated from a marine source. The guaiane skeleton of millecrone B is found in metabolites from soft corals belonging to the families Alcyoniidae[159] and Neptheiidae,[160] and in one *Xenia* species.[161] Unlike millecrone B, none of these previously reported guaiane sesquiterpenes are oxygenated at C-9. Gorgonian octocorals have yielded several aromatic guaianoid metabolites[162-165] including guaiazulene (**146**),[163] amines,[165] *e.g.* **147**, and the dimer **148**.[164] The *trans* ring fusion of millecrol B is unusual among the similar cadinane type compounds found in soft corals[166,167] and brown algae.[168]



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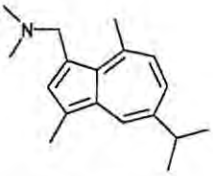

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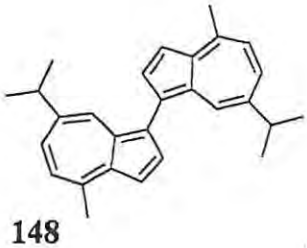

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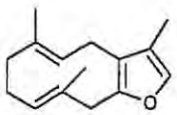
Hampered by a paucity of nudibranch material, Pika and Faulkner[158] noted the presence of several minor metabolites in their nudibranch extract, in insufficient amounts for structure elucidation. Fortuitously, *L. millecra* appears to be far more common in Algoa Bay, south of the Transkei, where we have observed this species on a variety of gorgonian octocorals at depths of 20 to 40 m. Therefore, given the impetus provided by the unfinished work of Pika and Faulkner, and the potentially interesting terpenoid chemistry[169] of the octocoral prey of *L. millecra*, we investigated the chemistry of *L. millecra* specimens collected from Algoa Bay. Known sesquiterpenes isolated from extracts of the Algoa Bay specimens of *L. millecra* included the millecrones A (**142**) and B (**143**) and isofuranodiene (**149**), while the new compounds obtained were algoafuran (**150**), cubebenone (**151**), 8-hydroxycalamenene (**152**), and a series of seven triprenyltoluquinones and -quinols (**153-159**).



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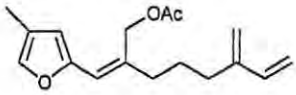

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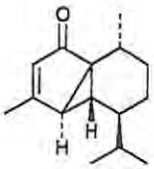

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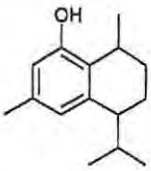
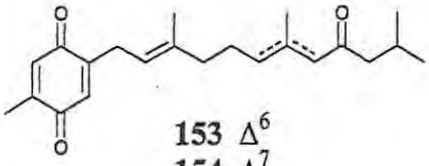
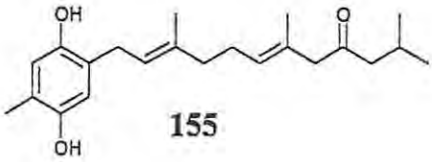


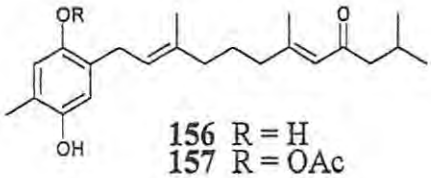
152



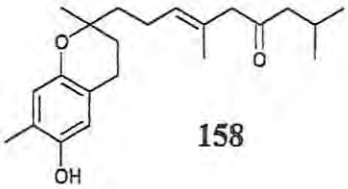
153 Δ^6
154 Δ^7



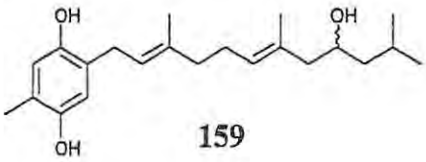
155



156 R = H
157 R = OAc



158

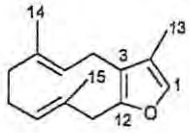


159

3.2 Isolation and characterization of *Leminda millecra* metabolites

Two collections of *Leminda millecra* were made in October 1998 and February 1999 in collaboration with the CRRF, who were making large-scale contract collections of marine invertebrates from Algoa Bay for the NCI (USA). The two separate collections of *L. millecra* yielded acetone extracts whose EtOAc partition fractions were identical by ^{1}H NMR spectroscopy. Chromatography of the combined EtOAc fractions on Si gel gave seven crude fractions, of which fractions 1, 3 and 4 were selected for further investigation from their interesting ^{1}H NMR spectra and relatively high sample mass (Scheme 3.1).

The first (hexane) fraction consisted mainly of millecrone A (**142,** 93 mg, 2.9 mg/animal), isolated previously from *L. millecra,*[158] and identified by comparison of its spectral data and optical rotation with those reported. Normal phase HPLC (hex/EtOAc 39:1 and 79:1) used to purify millecrone A also yielded the known soft coral metabolite isofuranodiene (**149**, 11.0 mg, 0.3 mg/animal).[170] The bicyclic structure of **149** was indicated from the six degrees of unsaturation implied by its molecular formula of $C_{15}H_{20}O$, established from HRFABMS data (Δmmu –0.02), and the presence of eight olefinic resonances in the ^{13}C NMR spectrum of **149**. Four of the latter ^{13}C resonances [δ 149.7 (s), 136.0 (d), 121.9 (s) and 118.9 (s)] together with an aromatic proton singlet (δ 7.06) in the ^{1}H NMR spectrum of **149**, suggested the presence of a furan moiety in **149**. Although our ^{1}H NMR data were almost identical to those reported for furanodiene (**160**),[171] as well as isofuranodiene (**149**), two vinyl methyl resonances (δ 16.2 and 16.5) in the ^{13}C NMR spectrum of **149**, were consistent with *E* geometries for both of the ring olefins as found in isofuranodiene. Bowden *et al.*[170] suggest that the *E, Z* geometry of furanodiene (**160**), initially established without the aid of ^{13}C NMR, was incorrect since corresponding vinyl methyl ^{1}H resonances for **149** and **160** differ by no more than ±0.02 ppm, which would indicate that the two compounds are identical. Four additional bicyclic furanodecanes, which are double bond positional isomers of **149**, have been isolated from xenid soft corals,[170] the neptheid soft coral *Lemnalia africana,*[172] and one gorgonian species.[173]



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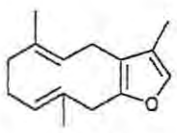
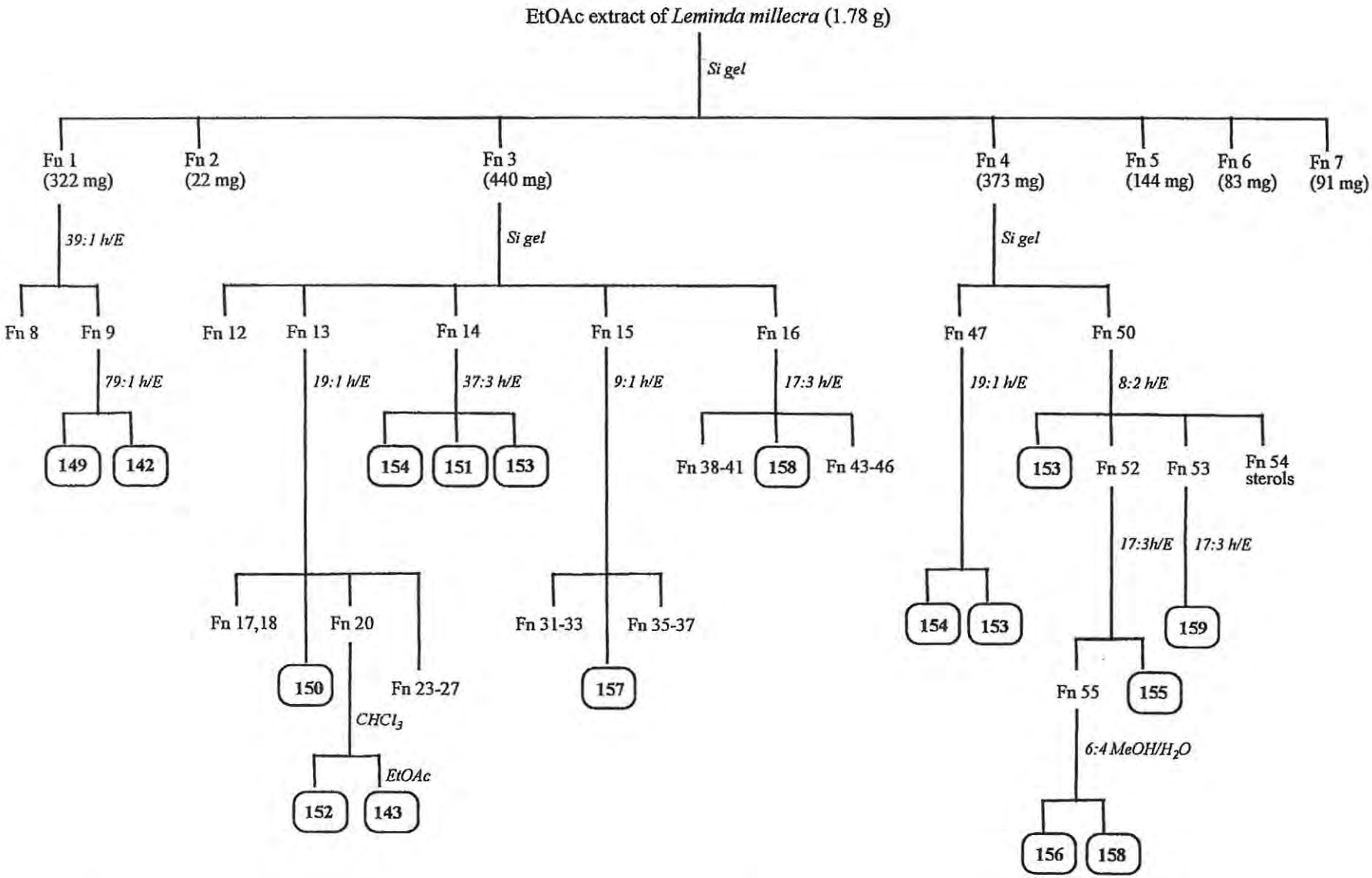


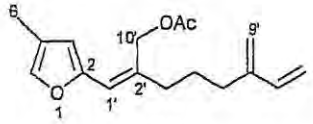
160



Scheme 3.1 Chromatography protocol used to isolate *L. millecra* metabolites. Solvent systems given are for semi-preparative normal phase and reversed phase HPLC separations (h = hexane, E = EtOAc)

Millecrone B (**143**, 19 mg, 0.6 mg/animal), concomitantly isolated with **142** by Pika and Faulkner,[158] was obtained in significant amounts from the main chromatography fraction 3, together with the new sesquiterpenes algoafuran (**150**, 1.5 mg, 0.05 mg/animal), cubebenone (**151**, 129 mg, 4 mg/animal) and 8-hydroxycalamenene (**152**, 16 mg, 0.5 mg/animal) by following the chromatographic sequence presented in Scheme 3.1.

HRFABMS data yielded a molecular formula of $C_{17}H_{22}O_3$ (274.156705, Δmmu –0.2) for algoafuran (**150**), which, in conjunction with a carbonyl carbon resonance (δ 171.1) in the ^{13}C NMR spectrum of **150**, and a deshielded methyl singlet (δ 2.07) in the corresponding ^1H NMR spectrum, suggested that **150** was a sesquiterpene monoacetate. Deshielded singlets possibly attributable to a furan ring were evident at δ 7.13, 6.16 and 6.36 in the ^1H NMR spectrum of **150**. Besides this, additional downfield ^1H signals (~ 5-6.5 ppm), nine resolved olefinic carbon resonances in the ^{13}C NMR spectrum of **150**, and only seven double bond equivalents calculated from the molecular formula, implied that **150** was an unsaturated linear substituted furan. While the ^1H and ^{13}C NMR data for **150** were broadly compatible with those of compound **161**,[174] the absence of a vinyl methyl singlet at δ 1.62, and the presence of a deshielded methylene singlet (δ 4.98, H_2-10') in the ^1H NMR spectrum of the former compound, led to placement of the acetate group (IR absorbance 1739 cm^{-1}) at C-10' in **150**. The presence of the latter methylene acetate moiety at C-2' was confirmed by two and three bond HMBC correlations from the H_2-10' protons to C-2' (δ 134.6), C-1' (δ 118.0) and C-3' (δ 35.6) as well as to the acetate carbonyl carbon C-11' (δ 171.1). Compound **161** was isolated from the Mediterranean soft coral *Alcyonium palmatum*,[174] and several other linear 2,4-disubstituted furanosesquiterpenes, lacking a terminal diene moiety, have been reported from species of *Sinularia* soft corals.[175] To the best of our knowledge, no linear furanosesquiterpenes have been isolated from gorgonian octocorals.



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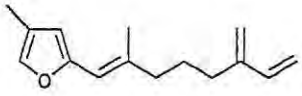
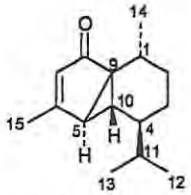
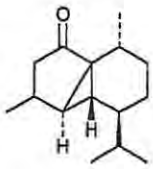


161

A molecular formula of $C_{15}H_{22}O$ (*m/z* 219.174802, M+1, Δmmu –0.1) for **151** was established from HRFABMS data. A strongly deshielded quartenary resonance (δ 209.0) in the ^{13}C NMR spectrum of **151**, together with two further sp^2-hybridized carbon resonances at δ 123.4 (d) and 177.9 (s), immediately suggested the presence of an α,β-unsaturated carbonyl moiety in **151**, which was confirmed by catalytic hydrogenation of the double bond to give **162**. Both vinyl carbon resonances were absent from the ^{13}C NMR spectrum of **162**, which contained additional methylene and methine carbon resonances (δ 40.9 and 29.6 respectively), while the IR absorptions of the carbonyl group in **151** (1694, 1607 cm^{-1}) were shifted down to 1721 cm^{-1} in the IR spectrum of **162**.



151



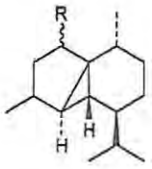
162

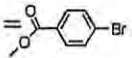
A tricyclic structure for **151** was indicated from the remaining three (of five) double bond equivalents implied by the molecular formula. The combination of decalin and cyclopropane rings, although not unprecedented, is unusual and was the only tricyclic structure compatible with the spectral data. The isopropyl and methyl substituents of the cyclohexane ring were defined by a methine proton octet coupled to two closely overlapped methyl doublets (δ 0.84, 0.88, J = 7 Hz), and a methine proton septet coupled to a methyl doublet (δ 0.89, J = 6 Hz) respectively in the ^1H NMR spectrum of **151** (Table 3.1). The latter methyl doublet showed two and three bond HMBC correlations to C-1 (δ 26.3), C-2 (δ 30.5) and C-9 (δ 42.8), placing the methyl substituent at C-1, while the methine isopropyl proton octet was correlated to C-4 (δ 45.2) and C-3/C-10 (δ 26.3) and the isopropyl group was thus positioned at C-4. The remaining methyl substituent (δ 2.10, H_3-15) was positioned on the olefinic quartenary carbon C-6 (δ 177.9) from a two bond HMBC correlation from the methyl protons to C-6 and three bond HMBC correlations to C-5 (δ 35.5, d) and C-7 (δ 123.4, d). Therefore, the absence of a methyl substituent on the only sp^3 quartenary carbon (δ 42.8) dictated the placement of the latter

carbon at the junction of the three rings in **151**. The connectivity of the three rings could be established by two and three bond HMBC correlations from methine proton H-10 (δ 1.31) to C-1/3, C-5, C-6, C-8, C-9 and C-11, while the oxygenated ring was delineated by HMBC correlations from vinylic proton H-7 (δ 5.31) to C-5, C-6, C-8, C-9 and C-15 (Figure 3.2).

The expected diequatorial stereochemistry of the isopropyl and methyl groups in **151** (Figure 3.3) was confirmed by 1D and 2D NOE experiments. Selection of the more shielded of the two H-2 resonances (δ 0.64, H-2α) in a 1D gradient selective NOESY experiment produced enhancements in the resonances of H-3α, H-4, H-5 and H_3-14 (Figure 3.4) and thus placed H-4 axial and assigned the isopropyl group as β–equatorial. Selection of H-2β (δ 1.74) resulted in the enhancement of the resonance at δ 2.44 (H-1). Since no enhancement of this resonance was seen on selection of the axial H-2α, H-1 was assigned as β. The consequent α orientation of H_3-14 could be confirmed by an enhancement of its proton signal (δ 0.89) on irradiation of H-5 (δ 1.89). While irradiation of H-2α, H-4 and H-5 produced no enhancement in the H-10 doublet, reciprocal enhancements in the H-10 and isopropyl methine proton signal (H-11) indicated a β orientation for H-10 (Figure 3.3). The 1D NOE enhancements observed were supported by corresponding cross peaks in a 2D NOESY experiment (Figure 3.4).

In an attempt to assign the absolute stereochemistry of **151**, we prepared the *p*-bromobenzoate derivative **163** from which we were hoping to obtain crystals suitable for X-ray crystallography. Compound **151** was completely reduced with sodium borohydride to afford the non-crystalline alcohol **164** and its crystalline epimer **165** in a 3:1 ratio. The alcohols were easily separated by HPLC and the relative stereochemistries of the alcohol moieties established from NOE data.



163 R = 

164 R = α OH
165 R = β OH

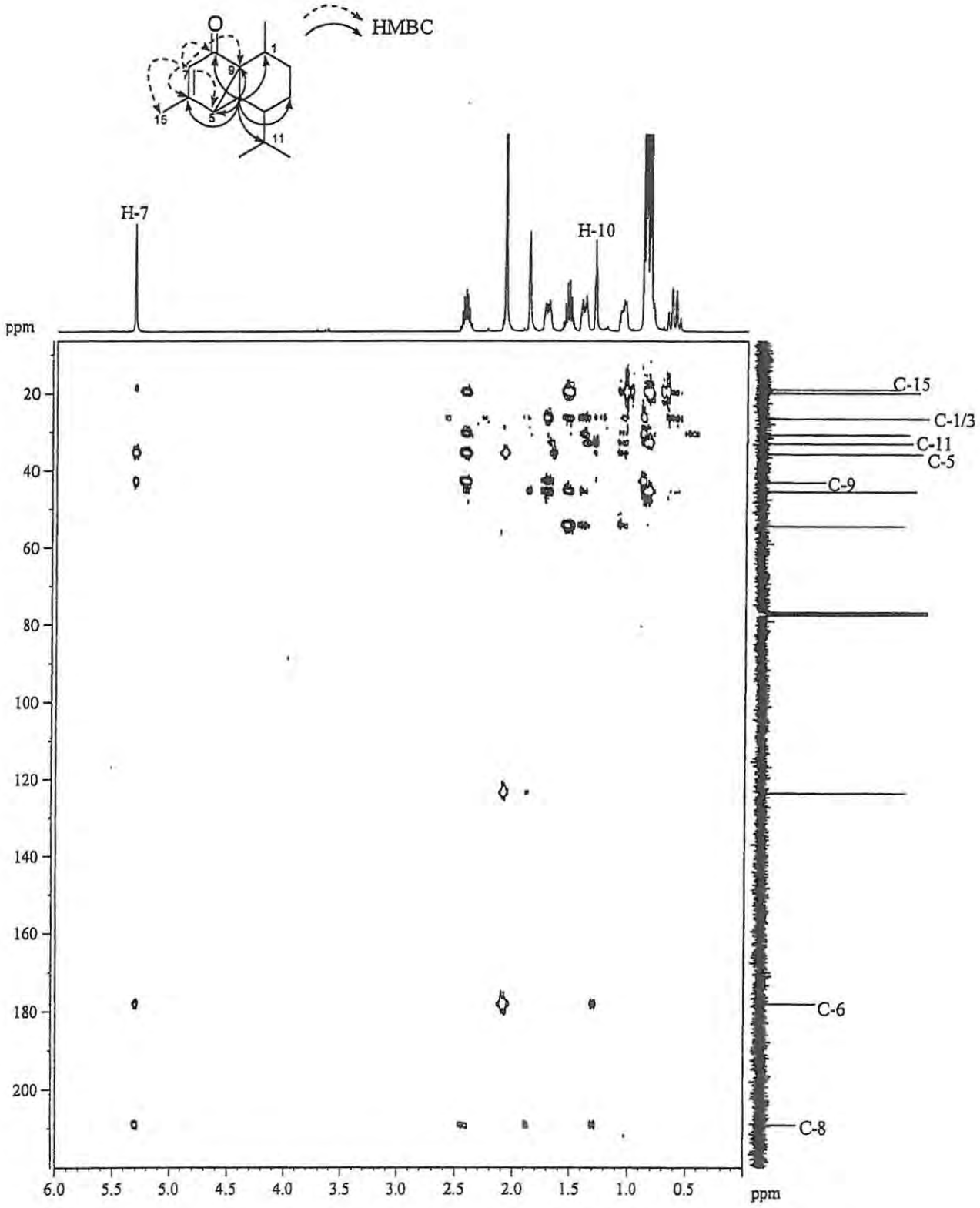


Figure 3.2: The HMBC spectrum ($CDCl_3$, 400 MHz, delay time d6 = 80 msec) for cubebenone (**151**). Accompanying figure represents key HMBC correlations used to establish the connectivity of the three rings.

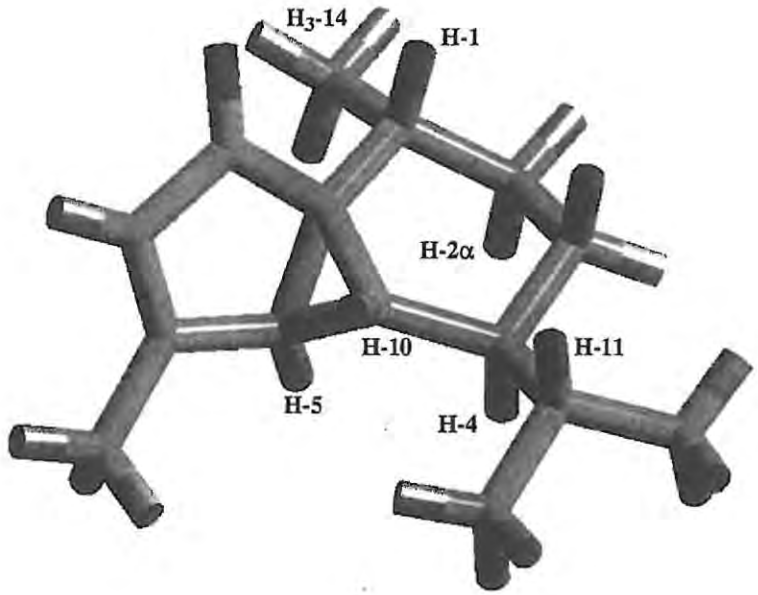


Figure 3.3 An energy minimized molecular model of cubebenone (**151**)

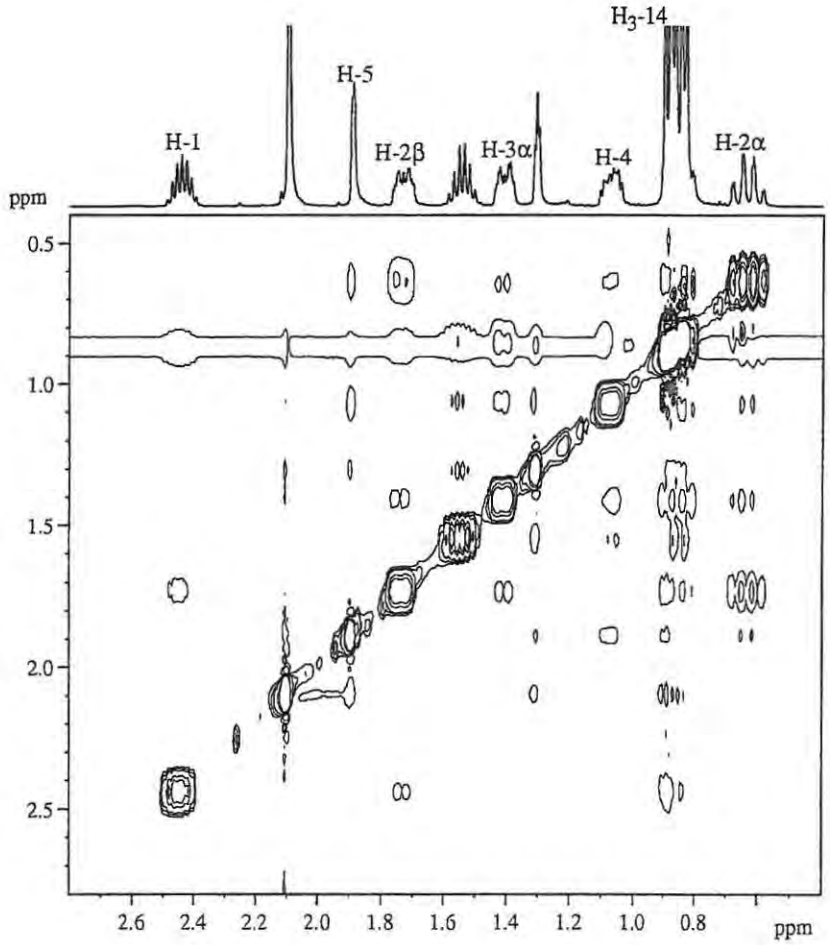
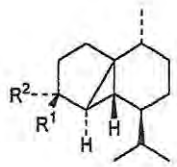
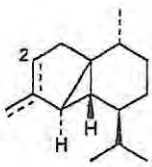


Figure 3.4: The upfield region (δ 0.4 – 2.8 ppm) of a gradient NOESY spectrum ($CDCl_3$, 400 MHz, d8 = 1 sec) for cubebenone (**151**).

Treatment of the major alcohol **164** with *p*-bromobenzoylbromide in the presence of pyridine under anhydrous conditions afforded the desired cubebane-*p*-bromobenzoate (**163**) and the inseparable crystalline *p*-bromobenzoic anhydride, which was subsequently decomposed by refluxing the reaction mixture after the addition of water. Frustratingly, attempts to recrystallize **163** (amorphous white solid) from numerous solvent combinations including EtOAc/hex, benzene/hexane, petroleum ether, pentane and chloroform continually produced fine 'feathery' needles or denticulate crystals which were unsuitable for X-ray crystallography. Unfortunately, the small amount of epimer **165** we had in hand precluded a similar preparation of the *p*-bromobenzoate derivative of this compound in sufficient amounts for recrystallization.

The skeletal structure of cubebenone is analogous to that of cubebol (**166**), first isolated in 1926,[176] and subsequently found in the terrestrial plant *Piper cubeba*.[177] More recently **166** was isolated from an Australian *Cespitularia* species of soft coral[178] (later reclassified as *Heteroxenia*[9]) while its C-6 epimer **167** was reported from two species of brown algae.[179,180] The cubebane skeletal structure is found in few gorgonian secondary metabolites and to the best of our knowledge only α-cubebene (**168**) has been isolated, from *Pseudoplexaura porosa*.[181]



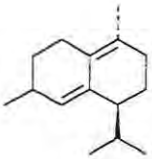


166 $R^1 = CH_3, R^2 = OH$
167 $R^1 = OH, R^2 = CH_3$

168 Δ^2
169 Δ^3 (exocyclic)

Epicubebol (**167**) was isolated for the first time as a natural product when Suzuki *et al.*[179] reinvestigated the brown alga *Dictyopteris divaricata*, steam distillation of an extract of which had previously yielded cadinane type compounds, and α-cubebene (**168**). Epicubebol and the cubebenes **168** and **169** were obtained from a fresh algal extract by rapid chromatography at temperatures lower than 35°C. To investigate the effect of temperature on **167**, Suzuki *et al.* heated the latter compound at *ca.* 130°C for five hours,

which afforded the cubebenes **168** and **169**, δ-cadinene (**170**) and *trans*-calamenene (**171**). Therefore we wondered whether our nudibranch metabolite 8-hydroxy-calamenene (**152**) was derived from thermal degradation of cubebenone (**151**). However, no **152** was formed on prolonged heating (6 h, 135-150°C) of a sample of **151**, as judged by periodic GC analysis of the sample. Interestingly, **151** was found to occur consistently with millecrone B (**143**), rather than **152**, in the extracts we obtained from individual nudibranchs and the gorgonian *Leptogorgia palma* in the screening program described later.



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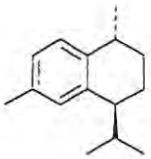
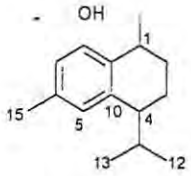


171



152

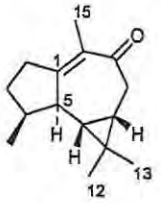
Compound **152** co-eluted with millecrone B (**143**, 19:1 hex/EtOAc) during the normal phase HPLC separation of the Si gel chromatography fraction 3 (Scheme 3.1). After an unsuccessful attempt to separate the two compounds using hexane, in which the compounds did not elute at all, we experimented with solvent combinations of hexane and $CHCl_3$. A solvent eluent of 1:1 hex/$CHCl_3$ gave Si gel TLC R_f values of 0.17 and 0.36 respectively for **143** and **152**, which hinted that this solvent system was suitable for effecting the separation on our HPLC system. However, most surprisingly, neither compound eluted off the column in this mobile phase. Finally, we serendipitously discovered that pure $CHCl_3$, which gave TLC R_f values of 0.48 and 0.66 for **143** and **152** respectively, eluted only **152**, while we were able to obtain pure **143** by subsequently washing the column with pure EtOAc. A comparison of the ^{13}C NMR data obtained for 8-hydroxycalamenene, **143**, with those of 8-methoxycalamene[180] (**172**) reported in the literature revealed the absence of a methoxy methyl group in **152**, and a significant difference only in the shifts of C-7, C-8 and C-9 for the two compounds (Table 3.1).

Table 3.1 ^{13}C (100 MHz, $CDCl_3$) and ^{1}H (400 MHz, $CDCl_3$) NMR data for compounds **151** and **152** and ^{13}C NMR (22.63 MHz, $CDCl_3$) literature values for compound **172**.[180]

	Cubebenone (151)		**8-Hydroxycalamenene (152)**		**8-Methoxycalamenene (172)**
Atom number	δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)	δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)	δ_C ppm (mult.)
1	26.3 (d)	2.44 (sept, 6)	26.6 (d)	3.06 (qn, 6)	26.6 (d)
2	30.5 (t)	0.64 (qd, 2, 13) 1.74 (m)	27.2 (t)	1.97 (m)	27.2 (t)
3	26.3 (t)	0.88 (qd, 2, 14) 1.41 (m)	19.1 (t)	1.80 (br m)	19.2 (t)
4	45.2 (d)	1.07 (m)	43.1 (d)	2.45 (sext, 3)	43.1 (d)
5	35.5 (d)	1.89 (d, 2)	123.0 (d)	6.58 (s)	122.6 (d)
6	177.9 (s)	-	135.0 (s)	-	134.6 (s)
7	123.4 (d)	5.31 (s)	113.3 (d)	6.42 (s)	108.6 (d)
8	209.0 (s)	-	153.0 (s)	-	157.2 (s)
9	42.8 (s)	-	126.0 (s)	-	128.6 (s)
10	54.2 (d)	1.31 (t, 3)	141.2 (s)	-	140.7 (s)
11	32.8 (d)	1.54 (octet, 7)	33.2 (d)	1.99 (m)	33.3 (d)
12	19.6 (q)	0.88 (d, 7)	19.6 (q)	0.82 (d, 7)	19.2 (q)
13	19.6 (q)	0.84 (d, 7)	22.1 (q)	0.97 (d, 7)	19.6 (q)
14	19.7 (q)	0.89 (d, 6)	21.2 (q)	1.19 (d, 7)	22.1 (q)
15	18.8 (q)	2.10 (s)	21.1 (q)	2.24 (s)	21.5 (q)
$O\underline{C}H_3$					55.6 (q)

A molecular formula of $C_{15}H_{20}O$ for **152** was confirmed from HREIMS data (218.1679, Δmmu +0.8), while a broad hydroxyl absorption band centred at 3437 cm^{-1} in the IR spectrum of **152** supported the presence of an alcohol functionality in this compound. The two methyl substituents H_3-12 and H_3-13 could be assigned from the NOE enhancements of these signals on irradiation of H_2-3 (δ 1.80) and H-5 (δ 6.58). In a 2D NOESY experiment, H_3-12 showed a strong correlation to H_2-3, but only a small correlation to H-5, while the reverse was true for H_3-13. Unfortunately, overlap of the proton signals of H-2β (δ 1.97) and H-11 (δ 1.99), and the two H-3 protons (δ 1.80), prevented unambiguous assignment of the relative stereochemistry of **152**. Trans-calamenene (**171**) was found in the gorgonian *Pseudoplexaura porosa*,[181] while Kashman[180] reported the two substituted calamenenes **172** and **173** from the Red Sea

gorgonian *Subergorgia hicksoni*. More recently, eleven calamenenes, many highly oxygenated, were isolated from the soft coral *Lemnalia cervicornis*.[166]



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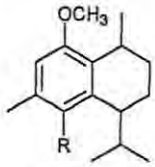


172 R = H
173 R = OH

In common with the cubeb compounds, the calamenenes were well known terrestrial plant products before they were isolated from marine sources.[182] The coexistence of a mixed assemblage of endosymbiotic photosynthetic algae, *Symbiodynium microadriaticum*, with the coral polyps of gorgonians is a well-documented phenomenon, and has led to some debate as to the origin of the terpenes found in some octocorals. Scheuer[183] noted the co-occurrence of cembranoid diterpenes in octocorals and *Tobacco* plants, and surmised that the cembranes in some octocorals are produced by endosymbionts. However, conflicting evidence is presented from biosynthetic studies of the incorporation of radiolabelled acetate precursors into terpenes (*e.g.* cubebol, **166**) by gorgonians.[184] Furthermore, the consistent variation in the GLC 'finger-prints' of a multitude of soft coral species led Kashman[185] to conclude that the sesquiterpenes of interest were true octocoral metabolites, since the symbiont assemblage of the species investigated was apparently uniform.

The remaining seven compounds (**153-159**), isolated from both main chromatography fractions 3 and 4 by chromatography as outlined in Scheme 3.1, are all new triprenyltoluquinones and -quinols related to the triprenylquinol, rietone (**174**),[186] previously obtained from the South African soft coral *Alcyonium fauri*. All seven metabolites, whose ^{1}H NMR spectra are shown in Figure 3.5, differ from rietone in that they possess a methyl, not a methylene carboxy, substituent at C-4' in the quinone/quinol ring, and they do not have an hydroxy or acetoxy substituent α to the sidechain carbonyl group. In this respect compounds **153-159** are analogous to the glycoside toluquinone, moritoside (**175**), isolated from a Japanese *Euplexaura* gorgonian.[187]

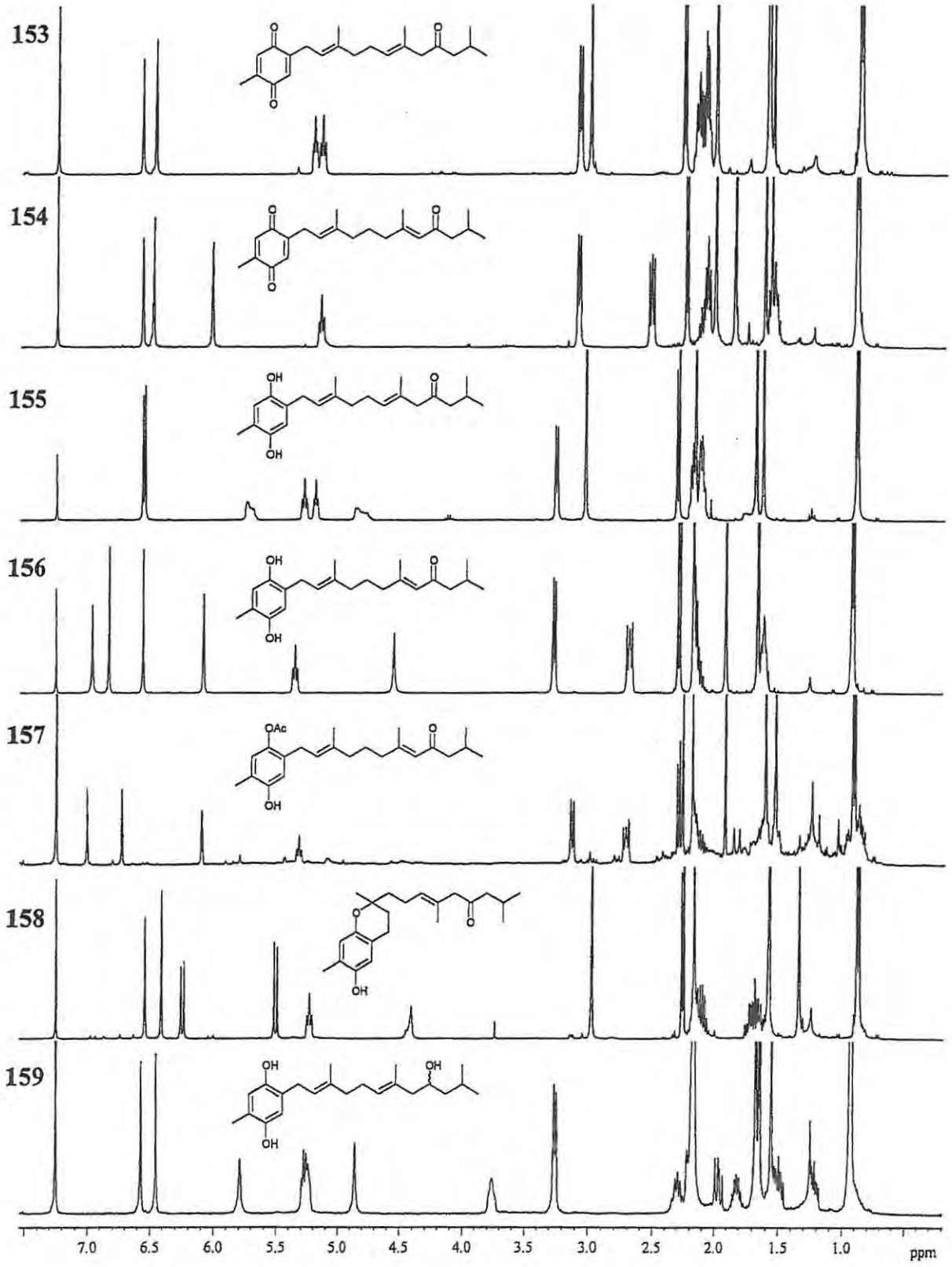
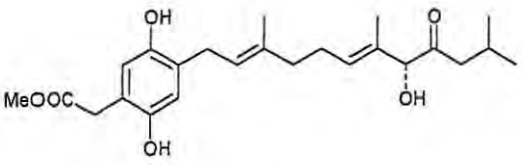
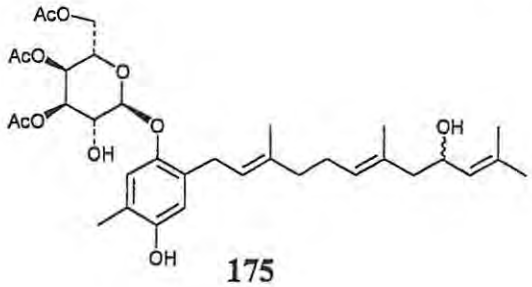


Figure 3.5 ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of triprenylated aromatic compounds **153-159**.



174



175

The least polar, bright yellow compounds **153** (36 mg, 1.1 mg/animal) and **154** (15 mg, 0.5 mg/animal), obtained from both crude chromatography fractions, displayed UV (250 and 252 nm, respectively) and IR (1657 and 1656 cm^{-1}, respectively) absorptions characteristic of quinones.[188] HREIMS data established a molecular formula of $C_{22}H_{30}O_3$ (342.2198, Δ mmu +0.5) for **153**, while the ^{13}C NMR spectrum of **153** showed 22 carbon resonances (Table 3.2) of which eight were vinylic, two were typical of quinone carbonyls (δ 187.8 and 188.3),[188] and one was a ketone resonance (δ 209.3). With seven of the eight double bond equivalents suggested by the molecular formula thus accounted for, we proposed a substituted quinone structure for **153**. The farnesyl sidechain of **153**, containing the carbonyl moiety (IR absorption, 1712 cm^{-1}), was delineated by two and three bond HMBC correlations from the ^1H NMR signals of the regularly spaced methyl groups to the ^{13}C resonances of their three nearest carbon neighbours (Figure 3.6). Two and three bond HMBC correlations from the H_2-1 proton doublet (δ 3.10) to C-1', C-2' and C-3' positioned the farnesyl sidechain at C-2' of the quinone ring, while the methyl group of the quinone ring (H_3-7') could be placed at C-5' by two and three bond HMBC correlations from the methyl singlet (δ 2.02) to C-5', C-4' and C-6' (Figure 3.6). The ^{13}C chemical shifts of the olefinic H_3-14 and H_3-15 (δ 16.4 and 16.0 respectively) indicated an *E* geometry around the two exocyclic double bonds (Δ^2 and Δ^6).[186]

The structure of the second isomeric quinone **154** ($C_{22}H_{30}O_3$, HRFABMS, 343.227298, M+1, Δmmu –0.02) could readily be solved by comparison of its very similar ^{13}C NMR data with those of **153** (Table 3.2). A slightly shielded ^{13}C resonance for the sidechain carbonyl carbon (C-9, δ 200.6) in **154** compared to that for C-9 in **153** (δ 209.3), as well as significant differences in the chemical shifts of C-6, C-7, C-8 and H_3-14 (Table 3.2)

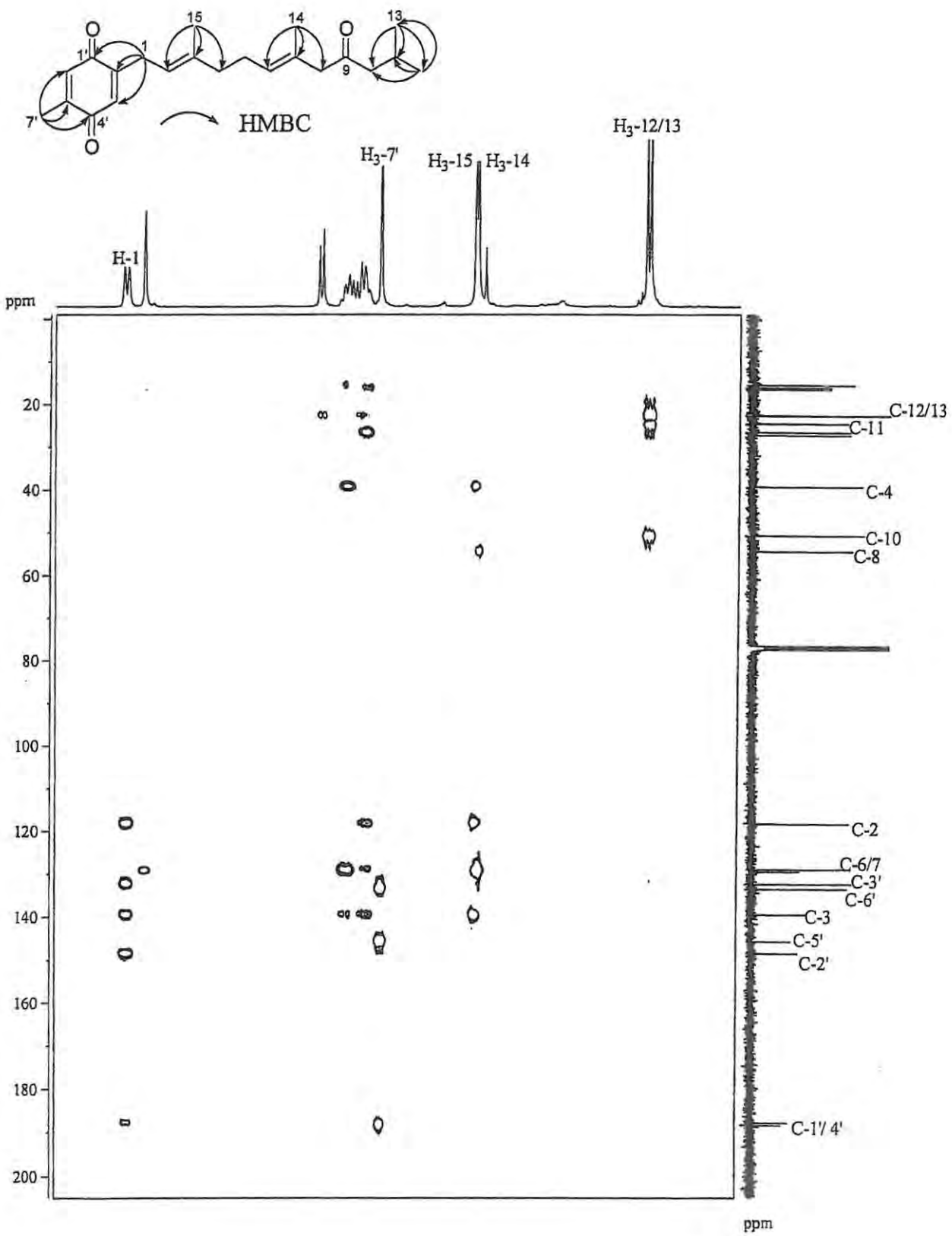


Figure 3.6: A region (F1 = δ 0-205 ppm; F2 = δ 0.5-3.4 ppm) of the HMBC spectrum ($CDCl_3$, 400 MHz, delay time d6 = 60 msec) of quinone **153**. The accompanying structure shows the key HMBC correlations used to delineate the farnesyl sidechain of **153** and position the substituents on the quinone ring.

indicated a double bond shift from Δ^6 in **153** to Δ^7 in **154**. The presence of an α,β-unsaturated carbonyl in **154** was confirmed by a strong IR absorption at 1682 cm^{-1} (*cf* 1712 cm^{-1} for **153**).

Table 3.2. ^{13}C NMR ($CDCl_3$, 100 MHz) data for compounds **153-159**

	Compound						
Carbon	**153**	**154**	**155**	**156**	**157**	**158**	**159**
1	27.1 (t)	27.1 (t)	28.3 (t)	27.5 (t)	27.0 (t)	122.4 (d)	28.0 (t)
2	118.3 (d)	118.0 (d)	122.2 (d)	122.4(d)	122.3 (d)	129.5 (d)	123.1 (d)
3	139.5 (s)	139.8 (s)	136.8 (s)	137.3 (s)	137.4 (s)	77.9 (s)	136.7 (s)
4	39.2 (t)	39.7 (t)	39.0 (t)	39.8 (t)	39.9 (t)	40.6 (t)	39.1 (t)
5	26.5 (t)	26.5 (t)	25.8 (t)	25.4 (t)	25.1 (t)	22.9 (t)	25.3 (t)
6	128.9 (d)	33.5 (t)	128.8 (d)	33.1 (t)	33.0 (t)	129.4 (d)	128.4 (d)
7	129.4 (s)	158.6 (s)	128.9 (s)	161.0 (s)	161.7 (s)	129.2 (s)	132.4 (s)
8	54.3 (t)	124.3 (d)	53.7 (t)	123.9 (d)	123.8 (d)	54.4 (t)	48.2 (t)
9	209.3 (s)	200.6 (s)	211.1 (s)	202.8 (s)	203.6 (s)	209.6 (s)	66.5 (d)
10	50.7 (t)	53.5 (t)	51.1 (t)	53.7 (t)	53.8 (t)	50.6 (t)	46.0 (t)
11	24.4 (d)	25.1 (d)	24.6 (d)	25.7 (d)	25.9 (d)	24.4 (d)	24.7 (d)
12	22.5 (q)	22.7 (q)	22.5 (q)	22.6 (q)	22.6 (q)	22.5 (q)	*23.3 (q)
13	22.5 (q)	22.7 (q)	22.5 (q)	22.6 (q)	22.6 (q)	22.5 (q)	*22.3 (q)
14	16.4 (q)	25.5 (q)	17.0 (q)	25.2 (q)	25.1 (q)	16.3 (q)	16.2 (q)
15	16.0 (q)	16.1 (q)	15.9 (q)	15.8 (q)	15.7 (q)	26.1 (q)	15.6 (q)
1'	187.8 (s)	187.8 (s)	147.1 (s)	148.8(s)	141.1 (s)	146.7 (s)	146.9 (s)
2'	148.4 (s)	148.5 (s)	125.3 (s)	124.8 (s)	130.4 (s)	119.5 (d)	125.3 (s)
3'	132.3 (d)	132.4 (d)	115.8 (d)	115.4 (d)	115.1 (d)	112.5 (d)	115.4 (d)
4'	188.3 (s)	188.4 (s)	148.0 (s)	146.4 (s)	153.3 (s)	147.5 (s)	148.2 (s)
5'	145.6 (s)	145.6 (s)	122.4 (s)	122.3 (s)	122.7 (s)	124.6 (s)	122.7 (s)
6'	133.5 (d)	133.5 (d)	117.8 (d)	117.7 (d)	123.5 (d)	118.1 (d)	118.2 (d)
7'	15.4 (q)	15.4 (q)	15.5 (q)	15.5 (q)	15.6 (q)	15.9 (q)	15.5 (q)
8'					170.0 (s)		
9'					20.8 (q)		

* Values are interchangeable

In addition to the quinones **153** and **154**, their aromatic, dihydro analogues, **155** (154.7 mg, 4.8 mg/animal) and **156** (9.8 mg, 0.3 mg/animal), were isolated from main chromatography fraction 4 (Scheme 3.1). HRFABMS established a molecular formula of $C_{22}H_{32}O_3$ for the latter isomeric compounds (344.235138, Δmmu –0.01; 344.235132, Δmmu –0.01 respectively), which both gave hydroxyl (3396 and 3369 cm^{-1} respectively)

and aromatic (1420 cm^{-1}) IR absorptions. A comparison of the ^{13}C NMR spectral data for the major metabolite **155** with those for **153** revealed significant differences only in the chemical shifts of the six ring carbons for each compound, which were compatible with those expected for a quinol as opposed to a quinone ring structure. While no quinone carbonyl ^{13}C resonances (δ 187.8 and 188.3 for **153**) were evident in the spectrum of **155**, two deshielded olefinic resonances (δ 147.1 and 148.0) and four relatively shielded resonances at δ 125.3 (s, C-2'), 115.8 (d, C-3'), 122.4 (s, C-5') and 117.8 (d, C-6') were consistent with an aromatic quinol moiety (Table 3.2). Furthermore, inspection of the ^{1}H spectrum of **155** revealed the presence of two broad D_2O-exchangeable hydroxyl proton peaks (δ 4.83 and 5.72). As in quinone **153**, the ring methyl group H_3-7' could be positioned at C-5' in **155** on the basis of two and three bond HMBC correlations from the methyl protons (δ 2.16) to C-5', C-4' and C-6'. The H_2-1 proton doublet (δ 3.26) of the farnesyl sidechain showed strong HMBC correlations to C-2', C-1' and C-3', placing the sidechain at C-2'. A similar argument could be applied to compound **156** with respect to quinone **154**, with hydroxyl proton peaks evident at δ 6.08 and 4.54 in the ^{1}H NMR spectrum of **156**.

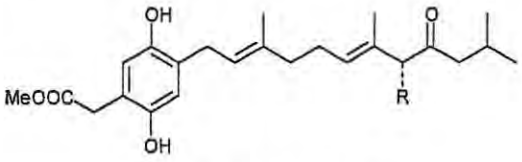
Three minor dihydroquinone derivatives were also isolated. The second crude chromatography fraction investigated yielded **157** (3.1 mg, 0.1 mg/animal) and **158** (8.2 mg, 0.3 mg/animal). A molecular formula of $C_{24}H_{34}O_4$ (386.245669, Δ mmu –0.04) for **157** was established by HRFABMS. A methyl singlet at δ 2.27 in the ^{1}H NMR spectrum of **157**, which showed an HMBC correlation to a ^{13}C resonance at δ 170.0, together with a strong absorbance at 1760 cm^{-1} in the IR spectrum of **157** implied the presence of an acetate moiety in this compound. A comparison of the ^{13}C NMR data for **157** with those of **156** (Table 3.2) indicated that the farnesyl sidechain of **157** was identical to that of **156**, while inspection of the ^{1}H NMR spectrum of **157** revealed a single hydroxyl proton signal (δ 8.13) which showed a two bond HMBC correlation to C-5' (δ 122.7). Thus the molecular mass discrepancy of 58 atomic mass units ($C_2H_2O_2$) between **157** and **156** was attributed to the presence of an acetate moiety at C-1' in the former compound.

HRFABMS established a molecular formula of $C_{22}H_{30}O_3$ (342.219402, Δmmu –0.1) for **158**, whose ^{13}C NMR data were in good agreement with those of **155** except for marked discrepancies between the chemical shifts of sidechain carbons C-1, C-2, C-3 and C-15 in the two compounds (Table 3.2). Since ten olefinic carbons and one carbonyl moiety, evidenced in the ^{13}C NMR spectrum of **158**, accounted for only six of the eight degrees of unsaturation inferred from the molecular formula, the presence of a second ring, arising from cyclization of the initial portion of the farnesyl sidechain was indicated. The chromenol structure of this compound was confirmed by a comparison of the ^{1}H and ^{13}C NMR chemical shifts for the proposed bicyclic portion of the molecule with those given in the literature for a similar bicyclic system.[189]

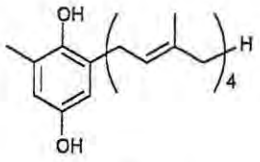
The ^{13}C NMR spectrum of **159** (3.6 mg, 0.1 mg/animal) was distinct from those of the other quinols in that it lacked a ketone carbon resonance (~ δ 200 ppm). A third D_2O-exchangeable signal (δ 1.25) in the ^{1}H NMR spectrum of **159**, and a hydroxy methine carbon resonance (δ 66.5) in the ^{13}C NMR spectrum were consistent with a reduced sidechain carbonyl moiety in **159**. Placement of the hydroxy methine carbon at C-9 was confirmed by strong COSY couplings from the corresponding hydroxy methine proton (δ 3.76) to H_2-8 (1.96 and 2.21) and H_2-10 (1.22 and 1.49). Unfortunately, a paucity of material prevented the assignment of the absolute stereochemistry about C-9 employing Mosher's standard MTPA ester procedure. The ^{13}C NMR data for **159** were otherwise in agreement with those of **155**, and HRFABMS confirmed a molecular formula of $C_{22}H_{34}O_3$ for the former compound (346.250742, Δmmu –0.05).

Linear polyprenyl quinones and quinols are not common in octocorals. With the exception of the triprenylated rietone compounds (**174**, **176**, and **177**) from *Alcyonium fauri*,[186] only tetraprenyltoluquinols and quinones have been isolated from soft corals, in particular from species of the genera *Sinularia*[190,191] and *Nepthea*.[189,190] Two investigations of *Euplexaura* gorgonians have yielded six triprenyltoluquinols bearing a glycoside substituent at C-1', *e.g.* moritoside (**175**).[187,192] While all of the prenylated

quinones and quinols reported from octocorals have a methyl derived substituent at C-5' as evident in the *Leminda* compounds (**153-159**) described here, analogous polyprenylated toluquinone and quinol metabolites from brown algae have the methyl substituent at C-6', *e.g.* **178**.[193] Therefore, although prenylated toluquinones are ubiqitous in brown algae,[194] and relatively rare in octocorals, we speculate that the 2, 5 alkylated quinones and quinols isolated from octocorals are true octocoral metabolites and not algal symbiont products.

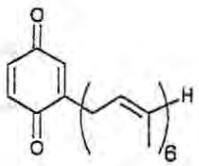


174 R = OH
176 R = OAc
177 R = H



178

Marine sponges are the source of an overwhelming majority of structurally varied sesquiterpene quinones and quinols that include compounds having linear, monocyclic, bicyclic and tricyclic sesquiterpene subunits, *e.g.* quinol **179**,[195] panicein A (**180**),[196] ilimaquinone (**181**)[197] and cyclospongiaquinone-1 (**182**)[198] respectively. The drimane sesquiterpene quinol avarol[199] (**183**) and the corresponding quinone avarone[199] (**184**) elicited a surge of interest in sesquiterpene quinones and quinols when the anti-HIV activity of these two *Dysidea* sponge metabolites was discovered.[200]



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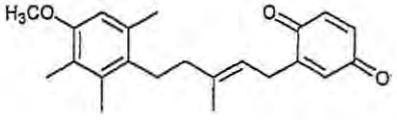

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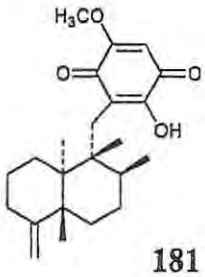

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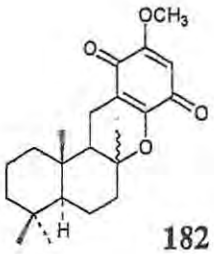

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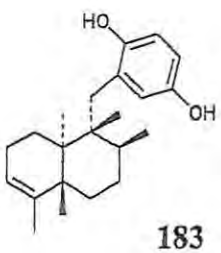

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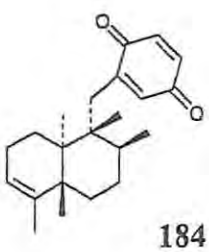
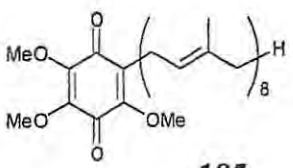


184

Although avarol and avarone alter the biochemistry of HIV-infected cells, recently the actual function of the two compounds as anti-HIV agents has been disputed.[201] Nevertheless, the sesquiterpene quinones/quinols, products of mevalonate and shikimate metabolism,[202] generally show moderate biological activity. The bioactivity is attributed to the quinone/quinol ring, and interesting structure-activity relationship studies have been carried out on twenty-three linear 2-prenyl-1, 4-quinones and quinols.[203] While quinones were more active than the corresponding quinols, di- and tri-prenylated compounds were the most potent in antimicrobial, brine shrimp lethality and mosquito fish lethality assays.[203]

While terpenoid quinones and quinols, are often considered to be secondary metabolites, their ubiquitous occurrence in both marine and terrestrial organisms technically precludes their allotment to the class of secondary metabolites,[202] which formally only includes compounds of restricted distribution among organisms. Furthermore, many terpenoid quinones and quinols participate in primary metabolism, in the electron transfer reactions that facilitate ATP production. Thus the ubiquinones, *e.g.* **185**, are vital components of respiratory chain phosphorylation and bacterial photophosphorylation, and are absent from only few classes of living organisms, most notably Gram-positive bacteria and blue-green algae.[202] The plastoquinones of higher plants and algae, *e.g.* **186**, take part in the light reaction of photosynthesis, while the methylated phylloquinone **187** is a vitamin (vitamin K). Bacteria and fungi produce menaquinones, including vitamin K_2 (**188**). The tocopherols, *e.g.* **189**, which constitute vitamin E, derive from cyclization of **178** which has been isolated from brown algae.[193]



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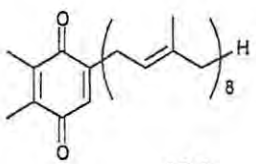

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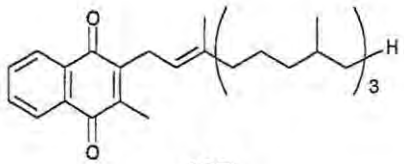

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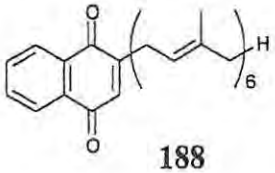

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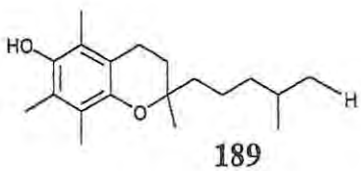


189

3.3 Identification of dietary octocorals of *Leminda millecra*

Although we have only observed and photographed Algoa Bay specimens of *L. millecra* on two species of gorgonians (Figure 3.7), the previous work on this species, suggests that *L. millecra* also feeds on *Alcyonium* and *Capnella* soft corals.[158] In addition, in the preceding discussion of the metabolites isolated from the Algoa Bay nudibranchs, we have touched on numerous octocoral sources of analogous compounds.

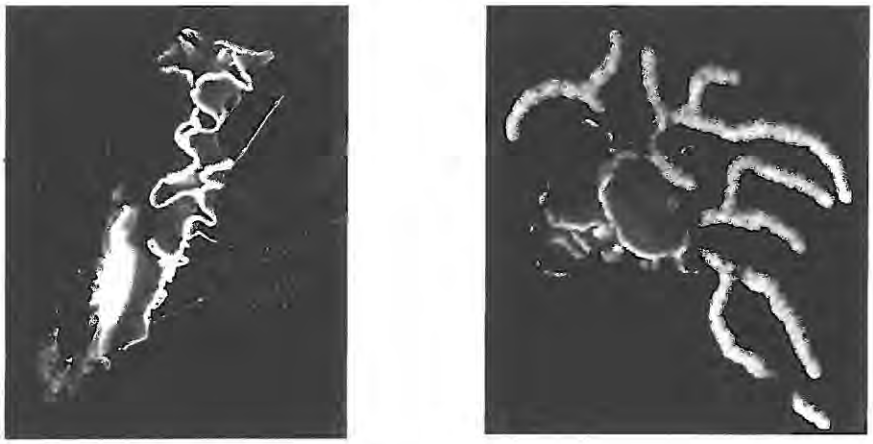


Figure 3.7 *Leminda millecra* photographed feeding on two species of gorgonians

The large scale marine invertebrate collections made by the CRRF in September 1998 and February 1999 provided us with a unique opportunity to access numerous octocoral voucher specimen extracts from octocorals collected randomly at the same time as our targeted collections of *L. millecra*. Thus, in November 1999, extracts of twenty-eight octocoral voucher specimens from both collections were screened by GC for the presence of millecrones A (**142**) and B (**143**), cubebenone (**151**), 8-hydroxycalamenene (**152**) and the prenylated chromenol **158**. The GC protocol used clearly separated all five compounds, which exhibited retention times of 20.17, 25.25, 21.50, 24.92 and 41.11 min respectively. Interestingly, GC analysis of the crude *L. millecra* extract under the same conditions showed that these compounds were major volatile components of the extract (Figure 3.8). Unfortunately, the furanosesquiterpenes isofuranodiene (**149**) and algoafuran (**150**) had degraded by this time, and the remaining toluquinols/quinones were not amenable to GC using any of the low polarity, medium polarity or high polarity GC columns we had available.

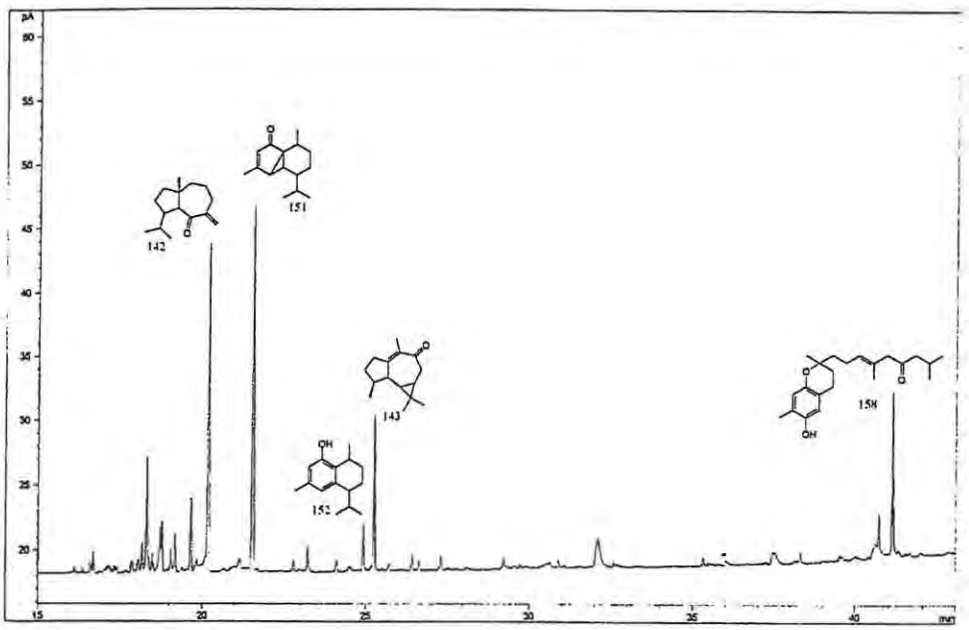


Figure 3.8 GC trace of the crude *Leminda millecra* extract.

It was not surprising that this screening exercise initially met with limited success given that only twenty eight of the large number of different potential octocoral prey species in Algoa Bay were screened, and the voucher specimens of these octocorals had been standing in EtOH at room temperature for several months, both conditions unsuited to labile sesquiterpenes. Our investigation of the voucher specimens from the second collection (November 1999) yielded a single match: millecrone A (**142**) was present in the voucher specimen of *Alcyonium fauri* (OCDN 6385). No matches emerged from the screening of the octocoral voucher specimens from the first collection. Subsequently we were grateful to receive crude extracts of the eighteen octocorals acquired during the first Algoa Bay collection from the NCI. The extraction and storage protocol used by the NCI ensures that the loss of volatile components from the extracts is minimized. Two of these crude extracts, from an *Alcyonium* species (OCDN 6174) and *A. fauri* (OCDN 6176) contained **142**. To confirm the results of our GC analyses of the extracts and voucher specimens, the three samples that contained **142** were subjected to GC-MS. In each instance, the selected peak gave a mass fragment pattern identical to that obtained for pure **142** isolated from *L. millecra*.

As mentioned earlier, GC proved unsuitable for the screening of the triprenyltoluquinones and –quinols and in an attempt to find a source for the more stable of these compounds, we used reverse phase analytical HPLC to reinvestigate the eighteen crude organic extracts received from the NCI. Major compound **155** and the similar quinol **156** were used as standards on a Wakosil microbore column (7:3 MeOH/H_2O). Although two crude octocoral extracts (OCDN 6004 and OCDN 6167, both *Leptogorgia palma*) piqued our interest, spiking of these samples with compounds **155** and **156** revealed a mismatch of the relevant retention times.

The metabolites reported here were obtained by combining many individuals collected from a variety of sites in Algoa Bay at different times. While combining nudibranch specimens is convenient for a standard natural product chemistry investigation, it provides no information about the dietary selectivity of individual specimens of *L. millecra*. Therefore, we decided to look at extracts of individual *L. millecra* specimens using gas chromatography to establish whether all five compounds (**142**, **143**, **151**, **152** and **158**) could be detected in individuals, or if not, which metabolites always occurred together and therefore may be from the same octocoral source. Establishing the co-occurrence of cubebenone (**151**) and 8-hydroxycalamenene (**152**) could be useful in ascertaining whether **152** could be a 'degradant' of **151**, as discussed earlier. Accordingly, eight *L. millecra* specimens were collected from White Sands in Algoa Bay (March 2000), together with three pieces of different gorgonians upon which three of the nudibranchs were found. Each of the eight nudibranch acetone extracts contained **151** as a major constituent by GC, with a small, although significant, amount of **143** present. In addition to cubebenone (**151**) and millecrone B (**143**) peaks (retention times 21.50 and 25.25 min respectively), all of these extracts showed a prominent GC peak at 18.33 min of varying intensity (Figure 3.9). In three of the extracts, this compound was present in greater or equal quantity to **151**. We suggest that this compound was isofuranodiene (**149**), since a ^{1}H NMR spectrum of one of the nudibranch extracts showed the presence of signals consistent with the furan moiety and the vinyl protons of **149** (Figure 3.10).

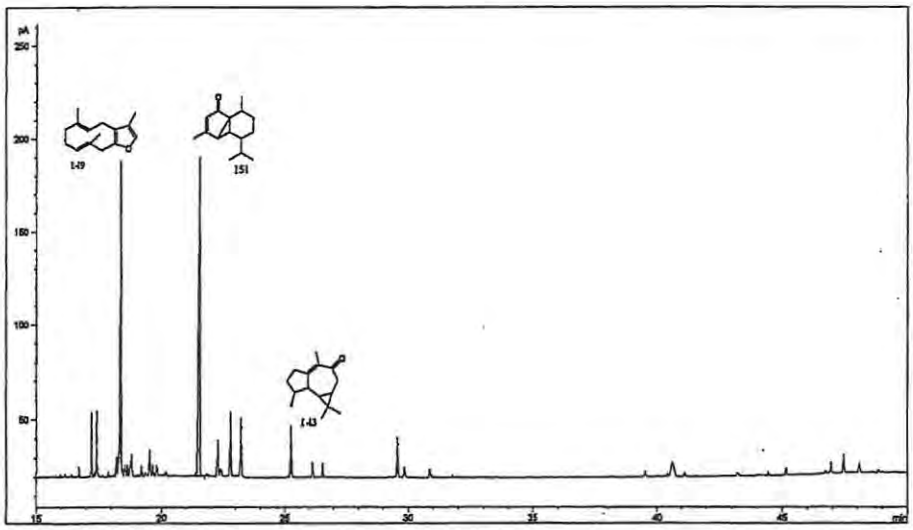


Figure 3.9 GC trace of the crude extract of a single *Leminda millecra* specimen suggested to contain isofuranodiene (**149**) as well as compounds **143** and **151**.

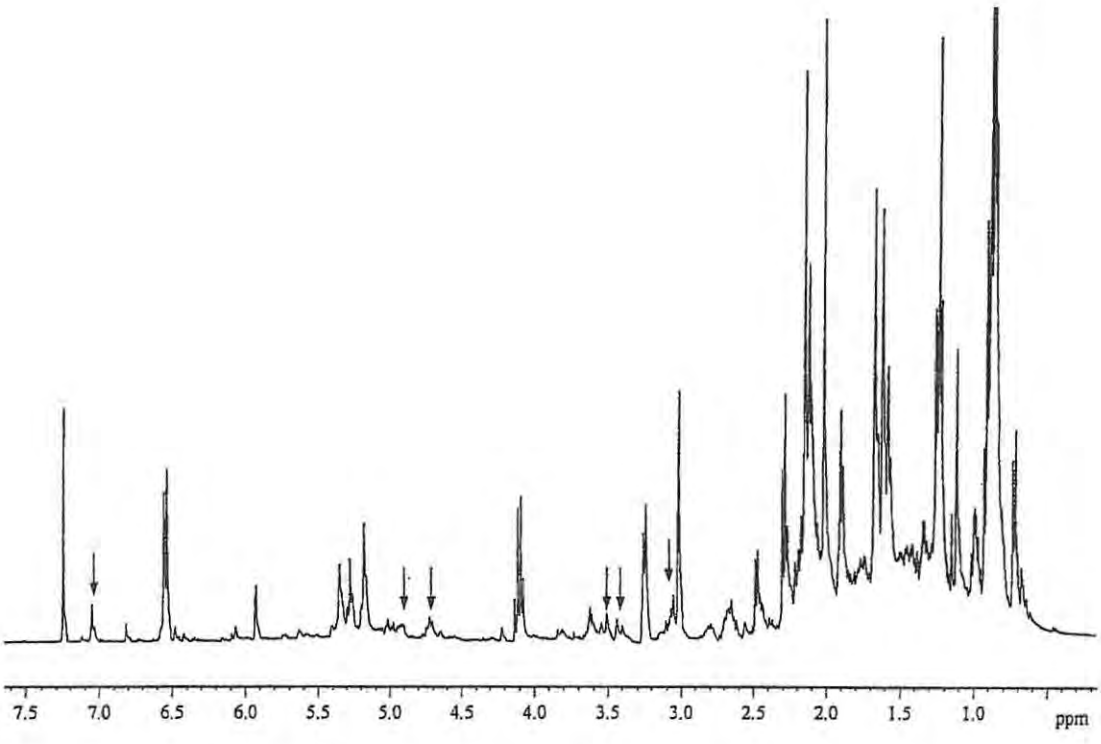


Figure 3.10 ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of the crude EtOAc extract of a single *Leminda millecra* specimen. Arrows indicate probable isofuranodiene (**149**) signals.

Unfortunately, no mass spectrum for the compound could be obtained, since by the time the sample was injected onto the GC-MS instrument three days later, the prominent GC peak at 18.33 min had collapsed to several small peaks. This observation in itself would seem to confirm that the unidentified GC peak is due to a labile furanoterpene. Of the three octocoral extracts, only that from the small piece of *Leptogorgia palma* (Figure 3.11) matched the nudibranch extract profiles, with prominent GC peaks at 18.33, 21.50 and 25.25 min. Once again, the GC-MS data of the latter two peaks confirmed the presence of **143** and **151** in the extracts. The absence of **152** in the *L. palma* extract would suggest that **152** is not derived from **151** (see page 78).

Figure 3.11 The *Leptogorgia palma* specimen extracted for GC analysis (actual size).

In conclusion, the diverse array of secondary metabolites characterized from *L. millecra* and the discovery of **142**, **143**, **151** and possibly **149** in different Algoa Bay octocorals is indicative of the varied diet of *L. millecra* nudibranchs, and supports the supposition that they are indiscriminate feeders on octocorals. Octocorals are recognised producers of allelochemicals for the purposes of moderating competition for space on marine reefs and protection from predators,[169] and thus *L. millecra*, like other nudibranchs, is specialized in being able to deal with the toxins of its octocoral prey. How far *L. millecra* may have evolved towards using the acquired chemicals in their own defense is uncertain and it would be necessary to investigate the chemical content of the mantle and digestive gland separately to determine whether any of the sequestered chemicals are dispersed throughout the mantle.

Chapter Four

The Sequestered Chemistry of Three Species of Nudibranchs belonging to the Family Chromodorididae

4.1 The chemical ecology of chromodorid nudibranchs

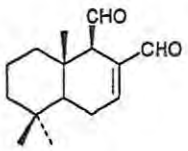
Opisthobranch molluscs show immense variation of form and color in keeping with the perception that the evolutionary loss of a prominent calcareous shell has allowed adaptive radiation of these aspects of their biology.[5,204] In as colorfully diverse a habitat as a marine reef, it is hardly surprising that shell-less, colorful opisthobranchs apparently conform to their surroundings. However, some species of nudibranch are easily distinguished and appear to flaunt their presence as they wander openly across the reef. It is often very difficult to unequivocally establish whether a particular sea slug is demonstrating crypsis or aposematic coloration, a fundamental problem being our inability to know how these animals are perceived by their predators.[204] In the absence of definitive measures of such parameters, it is only with the acquisition of large amounts of data over time that we see patterns of crypsis *versus* aposematism emerging.

A recurrent trend is the striking coloration of highly conspicuous members of the family Chromodorididae. Often, this coloration may not be matched with the dietary sponges of these nudibranchs or some common substrate. Rather, similar color patterns are reported to have emerged across different genera, in a convergent evolution, which is invoked as evidence of aposematic coloration.[204] An example of this proposed Mullerian mimicry is the group of blue and gold chromodorids to which *Chromodoris hamiltoni* (Figure 4.2, p92) belongs. In establishing the existence of aposematism, a most important criterion, and perhaps the most readily investigated one, is obviously the presence of the chemical deterrents that are thought to be advertised by the striking coloration. Thus the chemistry of chromodorids has been well investigated in the last twenty five years.[6] In this thesis the sequestered chemistry of three southern African chromodorid nudibranchs, *Chromodoris hamiltoni*, *Glossodoris* species 4 and *Hypselodoris capensis*, is presented. Given the myriad of metabolites isolated from chromodorids and also the complex

taxonomy of this family of nudibranchs, the discussion in this chapter will focus specifically on the three genera *Chromodoris*, *Hypselodoris* and *Glossodoris*.

4.1.1 The chemical defense of chromodorid nudibranchs

Although ecologically relevant toxicity and antifeedant assays of nudibranch metabolites have not routinely been carried out,[6] there is a significant body of circumstantial evidence for the defensive role of the metabolites isolated. Some nudibranch metabolites such as polygodial (**190**) from *Dendoris* species[6] and dendrolasin (**191**) from *Hypselodoris* species[6] were already known as effective plant and/or insect antifeedants[205,206] which would suggest an analogous defensive role in the nudibranchs.[207]



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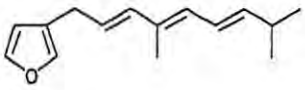

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The evolutionary schematics of sponges may also give clues to the chemical defense of nudibranchs,[78] as chromodorids are generally spongivorous and many investigations have successfully matched the chemistry of the subject nudibranchs and their sponge prey. The metabolites isolated in these investigations are thus often referred to as predator-prey markers.[6] There is a tendency towards a reduced spiculose skeleton in more highly evolved sponges[208] and it has been noted that sponges without spicules tend to contain significant amounts of deterrent chemicals.[78] When these metabolites are accumulated by nudibranchs, it may be inferred that they similarly play a defensive role. The heightened importance of the opisthobranch diet in a dual role of providing both nourishment and protection is construed from the diversity of radular form in opisthobranchs compared to prosobranch molluscs which rely on their calcareous shells for protection. As pointed out by Faulkner and Ghiselin,[78] although it is not impossible that some diet-derived metabolites might be present incidentally, many nudibranchs release these antifeedant compounds in a mucous secretion when disturbed, and compounds stored within the exposed dorsum seem to be strategically deployed for defense.[209] High concentrations of

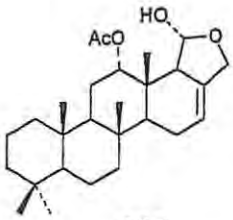
biologically active terpene compounds have been located in mantle dermal formations (MDF's)[210-212] which are present to varying degrees in *Hypselodoris*, *Chromodoris* and *Glossodoris* species.[213] These specialized 'white glands', consisting of highly vacuolate cells, may differ in number and position on the mantle border between individual specimens, possibly in accordance with nudibranch size.[211] However, they are consistently strategically concentrated in the region of the head, rhinophores and gills in line with a defensive function. Alternatively, the localization of toxic metabolites along the mantle border may be viewed as physiologically important to the nudibranch in avoiding autotoxicity.[209] Although much work has been done on MDF's,[210-212,214,215] especially those of *Hypselodoris* nudibranchs, which have the most prominent glands, the mechanism by which sequestered toxic metabolites are transferred intact from the digestive gland to the MDF's has not been rationalized.

The liberation of the opisthobranch body form resulting from the loss of the hard and relatively heavy calcareous shell that comprises a physical defense in other gastropods is moderated in chromodorids by their reliance on diet for the acquisition of a chemical defense. It follows that nudibranchs of a particular species collected from different locations may contain different metabolites due to geographical variation in the chemistry of the single sponge prey species or to variation in the species of dietary sponges themselves.[24] Similarly, different nudibranch species, or even genera, may feed on the same sponge species in a given area, and so have identical secondary metabolite patterns.[24] Therefore, as the occurrence of specific secondary metabolites in a nudibranch is dependent on the nudibranch's diet, it is impossible to predict precisely the chemical constituents of any given chromodorid. However, certain general trends are evident. For instance, nudibranchs of the genera *Chromodoris* and *Glossodoris* contain diterpenes, often of the spongiane type or rearrangements thereof, while *Hypselodoris* nudibranchs are reported to contain almost exclusively furanosesquiterpenes.[6,210] These observations hold over wide geographical ranges, in spite of significant variation in the dietary sponges present at different locations, and have led to the assumption that potential defense

chemicals, of the relevant structural or functional type, act as feeding cues for chromodorid nudibranchs.[6,212,213]

4.1.2 The sequestered chemistry of *Hypselodoris*, *Chromodoris* and *Glossodoris* nudibranchs

The consistent sequestration of furanosesquiterpenes by *Hypselodoris* nudibranchs is readily evident from perusal of the comprehensive catalogue compiled by Avila[6] which clearly assigns the metabolites isolated from each nudibranch species to the appropriate terpenoid class, although the chemical structures themselves are unfortunately lacking in this biological review.[216] The only exceptions among the *Hypselodoris* nudibranchs studied to date are *H. orsini*,[210,217] *H. ghiselini*,[218] and as discussed later, *H. capensis*. Perhaps more surprising than the fact that *H. orsini* (originally *Glossodoris tricolor*[217]) sequesters sesterterpenes, is the fact that of these two scalarane compounds (**192** and **193**), the furanoterpene **193** is a minor constituent of both the nudibranch and its dietary sponge *Cacospongia mollior*, which contains scalaradial (**194**) as the major metabolite.



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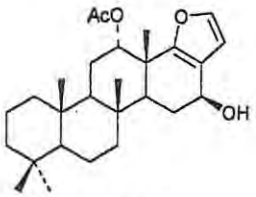

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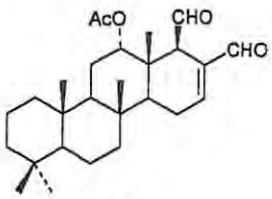

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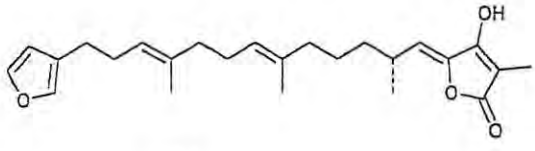
Although we have found sesterterpenes in *H. capensis* (see later), they are all β-substituted furanosesterpene tetronic acids such as (18*R*)-variabilin (**195**), and thus their presence in *H. capensis* supports the selection of furanoid metabolites as defense chemicals by *Hypselodoris* nudibranchs.[212,213] Similarly, *H. ghiselini* from the Gulf of California contained a furanoditerpene epoxide, ghiselinin (**196**),[218] together with two furanosesquiterpenes and a related butenolide.



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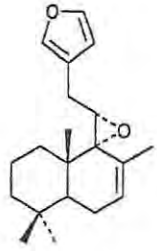

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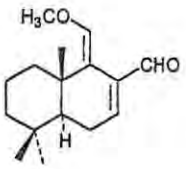
The observation that *Chromodoris* nudibranchs predominantly rely on sequestered spongiane-derived diterpenes for their chemical defense is also apparent from Avila's review,[6] and the subsequent literature.[219,220] However, this trend is less defined than that for *Hyselodoris* nudibranchs. There are several reports of sesquiterpenes from *Chromodoris* nudibranchs,[207,221-225] all furan-containing (or butenolide derivatives thereof) except for the drimane pu'ulenal (**197**) isolated from Hawaiian specimens of *C. albonotato*[207] and an unidentified *Chromodoris* species.[225] More notably, scalarane sesterterpenes are found in specimens of *C. youngbleuthi*,[226] *C. inornata*,[227] *C. funerea*,[228] *C. sedna*[229] and *C. splendida*.[229] Scalarane sesterterpenes are relatively uncommon terpenes which have been reported from several dictyoceratid sponges,[230,231] and in line with this, *C. youngbleuthi* feeds on *Spongia oceania*, while *C. inornata* is suggested to sequester scalaranes and further sesterterpenes, such as inorolide A (**198**), from *Hyrtios* sponges. To the best of our knowledge, no dietary sponge was identified for *C. sedna*, *C. splendida* or *C. funerea*.



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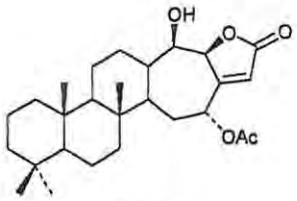

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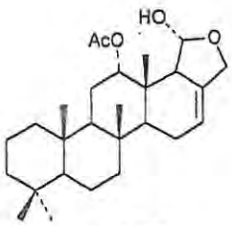
Scalarane sesterterpenes have similarly been found in three species of *Glossodoris*, *G. pallida* (**192** and **194**), *G. cincta* (**199** and **200**) and *G. hikeurensis* (**199** and **200**).[230] Indeed, the only instances of diterpenes from *Glossodoris* nudibranchs are the five new (**201-205**) and four known (**206-209**) spongianes from *G. atromarginata* reported recently by Fontana *et al.*,[232] and 6α, 12β, 15α, 16α-tetraacetoxyspongian (**210**) from the South African *Glossodoris* sp. 4 which we present in this chapter for the first time.



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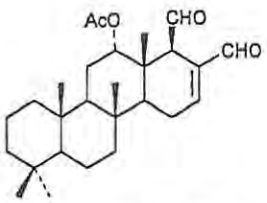
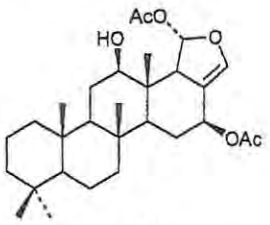


194 R = α OAc
199 R = β OAc



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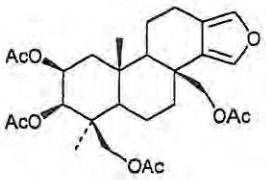

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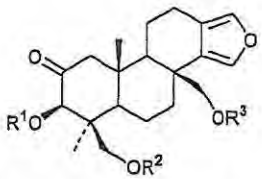
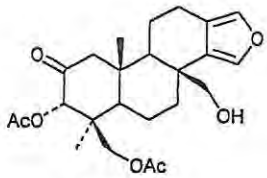


202 $R^1 = H, R^2 = R^3 = Ac$
203 $R^1 = Ac, R^2 = R^3 = H$
204 $R^1 = R^2 = Ac, R^3 = H$
206 $R^1 = R^2 = R^3 = Ac$
207 $R^1 = R^2 = R^3 = H$



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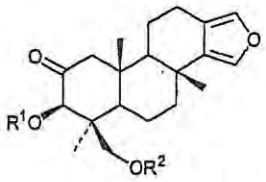
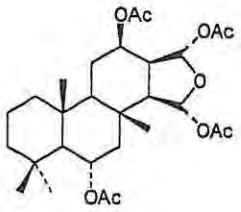


208 $R^1 = Ac, R^2 = H$
209 $R^1 = R^2 = H$



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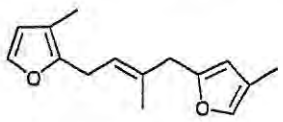
4.1.3 The taxonomy of chromodorid nudibranchs

In the literature, the chemistry of *Glossodoris* species has been complicated by the difficult taxonomy of this group. For example, the identification of *Glossodoris valenciennisi* as *Hypselodoris webbi*[213] is consistent with the isolation of the furanosesquiterpene longifolin (**211**) from the digestive gland and mantle of this nudibranch. However, the reassessment of *G. tricolor* as *H. orsini*[210] is anomalously incompatible with the scalarane sesterterpene chemistry of this species.



211

It has been suggested that both the *Glossodoris* and *Chromodoris* nudibranchs which feed on sponges containing sesterterpenes, together with the anomalous *Hypselodoris orsini*, could represent a new distinct Chromodorididae genus.[210] This interesting possibility draws attention to the complex taxonomy of the three chromodorid genera discussed here. At one stage, the names *Hypselodoris* and *Glossodoris*, were used interchangeably with *Chromodoris* to describe one enormous genus of chromodorid nudibranchs.[214]

Subsequently the names *Hypselodoris* and *Chromodoris* were applied to distinguish the two distinct radula types characteristic of chromodorids.[214] However the use of these names was considered merely a convenient way of dividing the enormous number of chromodorid species. Rudman[214] appears to have resolved the controversy surrounding the three generic names *Hypselodoris, Chromodoris* and *Glossodoris* in his taxonomical review of the family Chromodorididae by using the total anatomy, and not only the radular morphology, of approximately 100 nudibranch species to define genera. In doing this, he outlined a possible phylogenetic arrangement of the genera within the Chromodorididae (Figure 4.1) which indicates that there are significant anatomical differences between *Hypselodoris*, *Chromodoris* and *Glossodoris* nudibranchs.

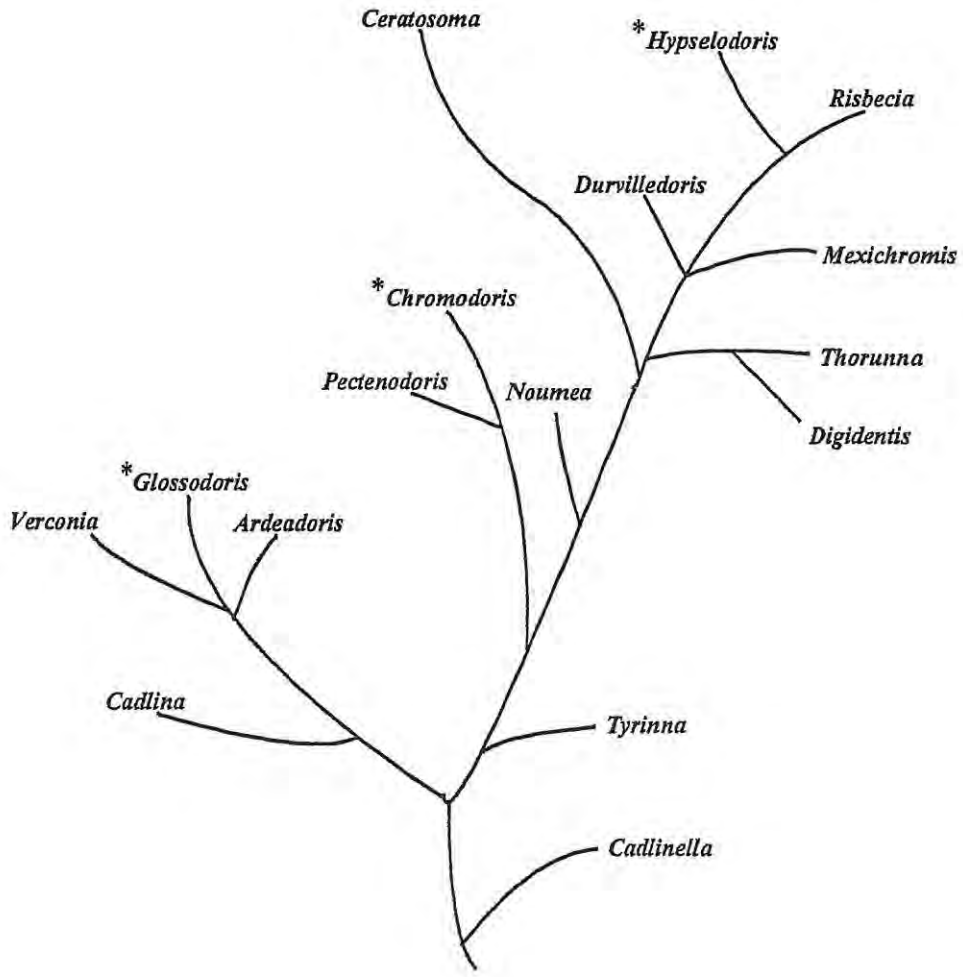


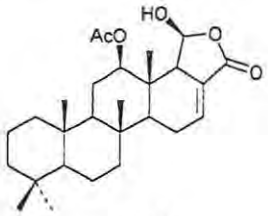
Figure 4.1 The phylogeny of chromodorid nudibranchs as proposed by Rudman[214]

For this reason, it seems unlikely that the many different species of nudibranchs that sequester sesterterpenes, predominantly scalaranes, have been consistently assigned to more than one wrong genus. One would think that some recurrent morphological anomaly common to the various *Hypselodoris*, *Chromodoris* and *Glossodoris* species that contain sesterterpenes would have been noted. In addition, the relatively few chemical investigations of *Glossodoris* nudibranchs[230,232] have revealed a preponderance of sesterterpenes (see above), not diterpenes as would be implied by the assignment of the sesterterpene-sequestering nudibranchs to a new genus. It is of interest to see whether such a trend persists as further chemical studies of the genus *Glossodoris* are carried out.

4.1.4 The modification of sequestered metabolites by chromodorid nudibranchs

It is accepted that some chromodorid genera have diverged to such an extent that nudibranchs belonging to different genera select different structural types of compounds as defense chemicals. However, the extent to which nudibranchs are able to actively select a particular defense chemical over other metabolites present, and whether the targeted compounds are chosen for their relatively reduced or increased toxicity is debatable. In a few instances, modification of toxic sponge metabolites has been demonstrated. Whether this process is a passive decomposition in the digestive gland or is actively mediated by enzymes is also debatable. Presumably passive decomposition in the digestive gland would indicate a detoxification of the sequestered metabolites, while enzyme catalyzed modifications of metabolites could result in more or less toxic products. Rogers and Paul[230] found that three species of *Glossodoris* nudibranchs from Guam did not passively sequester metabolites from their dietary sponges. Some metabolites, *e.g.* scalarin (**212**) were not sequestered, while others, *e.g.* scalaradial (**194**), were modified, with the result that extracts of these nudibranchs were less toxic than extracts of the sponges that they fed upon. Indeed, specimens of *G. pallida*, were more readily eaten by predatory fish in field assays than pieces of their dietary sponge. It was later demonstrated that *G. pallida* is confined to the bases of its host *Cacospongia* sp., avoiding the much higher concentrations of desacetylscalaradial (**213**) found in the tips of this branching sponge.[233] Thus, although the nudibranch sequesters small amounts of

desacetylscalaradial (**213**) together with scalaradial (**194**), apparently it is unable to deal with the former compound at high concentrations and thus is not immune to the chemical defense of its only dietary sponge.



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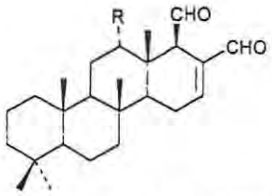
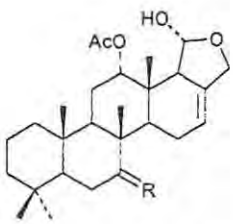
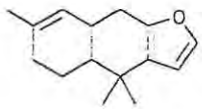


194 R = OAc
213 R = H

Therefore it has been concluded that *G. pallida* does not incorporate sponge metabolites into an effective chemical defense system of its own, but avoids predators by associating with a noxious sponge, whose defense chemicals the nudibranch must detoxify or avoid. It may be inferred that *G. pallida* is not highly evolved enough to possess the sophisticated mechanism for processing a range of toxic metabolites, as some other chromodorids do, which would liberate it from its dependence on a single dietary sponge for protection. *Hypselodoris orsini* has been shown to reduce scalaradial (**194**) to deoxoscalarin (**192**), which it then converts to 6-keto-deoxoscalarin (**214**) that is specifically accumulated in the MDF's of the nudibranch.[210] However, it is not known whether this compound is more or less toxic than scalaradial. *Chromodoris funerea* from Palau oxidises furodysins, *e.g.* furodysinin (**215**), to produce unstable compounds such as (**216**).[223] These singlet oxygen oxidation products were shown to have biological activities an order of magnitude greater than those of the furodysins in fish feeding assays.



192 R = H_2
214 R = O



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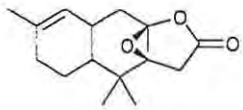

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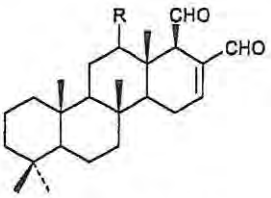
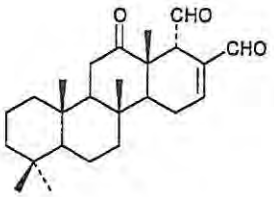
The presence of two new deacetylscalaradials (**217** and **218**) as major metabolites in *C. youngbleuthi* was taken as an indication that *C. youngbleuthi* is capable of modifying a minor metabolite, 12-deacetylscalaradial (**219**).[226] The latter compound, together with scalaradial (**194**) was present in the nudibranch's dietary sponge, *Spongia oceania*. In several other instances, the slight variations between sponge and nudibranch metabolites as been attributed simply to a failure, on the chemist's part, to locate the correct dietary sponge.[229,234,235]



217 R = β OH
219 R = α OH

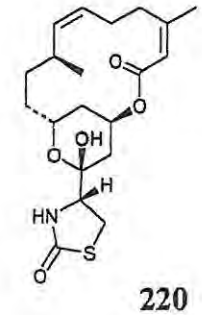


218

The direction in which chromodorid nudibranchs may be evolving is unclear. Some authors have suggested that a particular nudibranch may select one of two toxic compounds as closely related as the nakafurans, assuming that both compounds are present in the sponge specimen preyed upon.[235] However, such selectivity may impose severe restrictions on the nudibranch, confining it to the geographical distribution of a particular dietary sponge and might be viewed as an evolutionary precursor to the development of an ability to biosynthesize the desired defense chemical *de novo*. It is more usually suggested, and often proved, that the metabolite content of a particular collection of nudibranchs is directly related to that of the specific sponges that the nudibranchs have been feeding on.[6] By maintaining the ability to sequester a broad range of related metabolites with similar activity, chromodorids would both relieve the necessity to locate a specific dietary sponge, and possibly be equipped to deal with a wider range of predators.

The following discussion describes the isolation of the known alkaloid latrunculin B (**220**) and two new spongiane diterpenes (**221** and **222**) from *Chromodoris hamiltoni*, and the known spongiane diterpene **210** from *Glossodoris* sp. 4.[80] Unfortunately, dietary

sponges were not identified for either species. We have also examined the predator-prey relationship between the nudibranch *Hypselodoris capensis* and two sponges, a *Fasciospongia* species and a *Dysidea* species, on which the nudibranchs were observed during excursions to the Tsitsikamma National Park. *H. capensis* yielded the known compounds (18*R*)-variabilin (**195**), nakafuran-8 (**223**) and -9 (**224**) and 22-deoxyvariabilin (**225**), together with a new variant, 22-deoxy-23-hydroxymethylvariabilin (**226**), while the *Fasciospongia* sponge, collected concomitantly, yielded furospinosulin-1 (**227**) in addition to compounds **195**, **225** and **226**. On returning to our collection site in the Tsitsikamma National Park the *Dysidea* sponge was collected, and was found to contain **223** and **224**.



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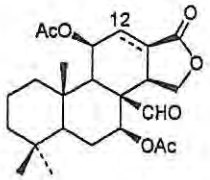
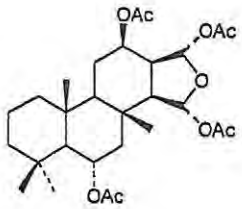


221
222 Δ^{12}



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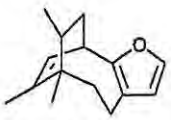

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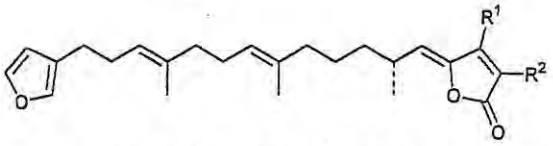
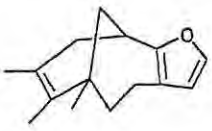


195 $R^1 = OH, R^2 = CH_3$
225 $R^1 = H, R^2 = CH_3$
226 $R^1 = H, R^2 = CH_2OH$



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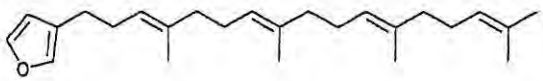

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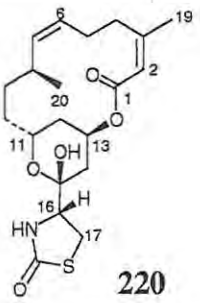
4.2 Two new spongiane diterpenes and a known macrolide from *Chromodoris hamiltoni*

In September 1995, fifteen small specimens of *Chromodoris hamiltoni* were collected by hand, using SCUBA equipment, from Malangaan Reef (-27 m) off Ponto do Oura on the border of South Africa and Mozambique. *C. hamiltoni* (Figure 4.2) is the most common

of the sixteen known South African species of *Chromodoris*, and like most of its congeners is confined to the tropical reefs of the North coast of South Africa.[80] The whole animals were steeped in acetone for four months and subsequently re-extracted with further acetone. The combined acetone extracts were concentrated and partitioned between EtOAc and water to afford a crude EtOAc partition fraction (150 mg) which was purified by normal phase HPLC.

Figure 4.2 Surface photograph of *Chromodoris hamiltoni*

The initial HPLC separation (3:2 EtOAc/hex) yielded the macrolide latrunculin B (**220**, 4.2 mg), as determined by comparison of its ^{1}H and ^{13}C NMR data with those reported in the literature.[236] A molecular formula of $C_{20}H_{29}NO_5S$ was confirmed by HREIMS (395.176584, Δ mmu –0.1). The presence of this compound prompted the dissection of five specimens of the nudibranch and the examination of the gut contents, a process which revealed acanthorhab spicules typically found in latrunculid sponges. Latrunculin B and its homologue, latrunculin A (**228**), were reported as the first 2-thiazolidinone-bearing marine macrolides[237] and proved to be responsible for the extreme toxicity to fish of the sponge *Latrunculia magnifica*, from which they were initially isolated.



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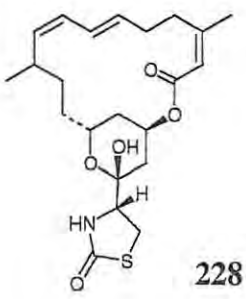
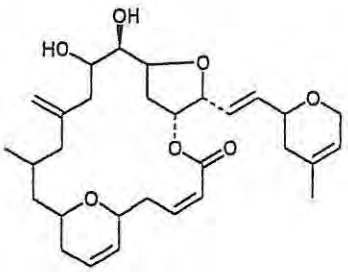


228

Interestingly, the latrunculins cause disruption of the microfilament organisation in cells[236] and this activity coupled with the novelty of their structures has prompted their total syntheses.[238] Different collections of *L. magnifica* from different sites were reported to yield either latrunculin A or latrunculin B.[236] Thus our finding of only latrunculin B in the *C. hamiltoni* specimens collected is consistent with the existence of a dietary sponge which contains only latrunculin B. However, both latrunculins were found in *C. hamiltoni* specimens collected from the Kwazulu Natal coast (500 km south of our collection site) in an earlier investigation by Pika and Faulkner.[219] It is interesting that several species of chromodorid nudibranchs contain these and other nonterpenoids. Latrunculin A was the only metabolite isolated from *C. elisabethina* and *C. willani*.[6] Indonesian specimens of *C. lochi* contained latrunculin B together with two polyketide macrolides, laulimalide (**229**) and an isomer (**230**).[239] Latrunculin B has also been isolated from *Glossodoris quadricolor*.[240] Although the dietary sponges of *C. hamiltoni* have not been identified in the field, it would appear that latrunculid sponges are a common source of defense chemicals for *C. hamiltoni* along the south east coast of Africa, just as they provide these metabolites for chromodorids from other locations.



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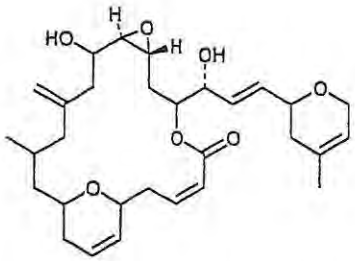

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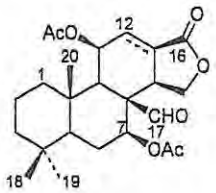
Further purification (7:3 hex/EtOAc) of a less polar fraction obtained from the intial HPLC separation of the *C. hamiltoni* extract yielded two new spongiane diterpenes, 7β, 11β-diacetoxy-16-oxospongian-17-al (**221**, 2.1mg, 0.14mg/animal) and 7β, 11β-diacetoxy-16-oxospongi-12-en-17-al (**222**, 1.9mg, 0.13mg/animal) as colorless oils.



221
222 Δ^{12}

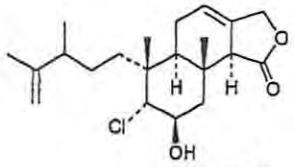
A molecular formula of $C_{24}H_{34}O_7$ for **221** was deduced from NMR data and confirmed by HREIMS (*m/z* 434.2287, Δmmu –0.9). The carbonyl groups of a lactone functionality, an aldehyde and two acetate moieties, indicated in the ^{13}C NMR spectrum of **221** by resonances at δ 176.3, 201.0, 169.2 and 169.6 respectively, accounted for four of the eight degrees of unsaturation calculated from the molecular formula. The remaining four degrees of unsaturation therefore implied a tetracyclic substructure for **221**. The NMR data for **221** were consistent with published data for a basic tetracyclic spongiane skeleton[241] and were also supported by characteristic spongiane diterpene fragment ions observed at *m/z* 201, 123 and 109 in the LREI mass spectrum of **221**.[242] Two and three bond HMBC correlations from the aldehyde proton singlet (δ 10.19) to carbon resonances at δ 53.8 (C-8) and 52.3 (C-14), together with a W-coupling to H-14 (δ 1.93) in the COSY90 spectrum of **221**, placed the aldehyde moiety at C-8. A three bond HMBC correlation from the deshielded proton doublet H-7 (δ 4.78) to the acetate carbonyl carbon at δ 169.6 positioned one acetate functionality at C-7. The second acetate moiety could be unequivocally placed at C-11 only by consideration of the deshielded shift of the H-11 proton signal (δ 5.59) together with COSY couplings from this signal to H-9 (δ 1.24) and to the multiplets at δ 1.46 and 2.35 (H_2-12). Three bond HMBC correlations from H-11 to C-13 (δ 35.3) and C-8, and from H-9 to C-14 (δ 52.3) provided conclusive evidence for the connectivity of rings B and C. The five membered lactone ring (ring D) was also delineated from the HMBC data where correlations were observed between the H_2-15 proton triplets (δ 4.46 and 4.04) and the ^{13}C resonances at δ 176.3 (C-16), 35.3 (C-13) and 52.3 (C-14).

The relative stereochemistry of **221** was determined from a series of 1D NOE difference experiments. Irradiation of the H-13 proton signal (δ 3.47) resulted in the enhancement of the H-14 doublet (δ 1.93), confirming the *cis* fusion of rings C and D as found in previously reported spongiane diterpenes.[242] The C-7 acetate moiety was assigned a β orientation since irradiation of H-7 produced enhancements in the proton doublet at δ 2.13 (equatorial H-6α), the multiplet at δ 2.93 (axial H-14α), the doublet at δ1.06 (axial

H-5α) and the singlet at δ1.24 (axial H-9α). The β configuration of the C-11 acetoxy group was similarly deduced by irradiation of H-11 which produced enhancements in the signals of H-9 (δ 1.24), H-1α (δ 1.80) and both H_2-12 protons (δ 1.46 and 2.35). Finally, irradiation of the aldehyde proton (δ 10.19) resulted in large enhancements in the signals of the angular methyl group (H_3-20, δ 1.03) and H-6β (δ 1.79), demonstrating a 1, 3-diaxial interaction of these three entities and thus assigning the aldehyde group as β.

HREIMS data (432.2153 Δmmu +1.3) yielded a molecular formula of $C_{24}H_{32}O_7$ for spongiane diterpene **222**. Close similarities in NMR data (Table 4.1), and a difference of only two mass units between the molecular ions of **221** and **222**, suggested that the latter compound was an unsaturated analogue of **221**. This assumption was supported by the presence of deshielded olefinic resonances at δ129.6 and 129.9 in the ^{13}C NMR spectrum of **222**. The quaternary character of the signal at δ 129.9 placed this olefinic carbon at a ring junction. Accordingly, the coalesence of the signals at δ 4.46 and 4.04 (H-15α and H-15β) in the ^{1}H NMR spectrum of **221**, to give overlapping triplets (δ 4.23 and 4.29) in the analogous spectrum of **222**, and the downfield shift of H-14 (δ 1.93 to 2.93) located the double bond at the junction of rings C and D. A COSY coupling between the deshielded vinylic proton H-12 (δ 6.65) and the oxymethine proton H-11 (δ 5.86) further confirmed the Δ^{12} position of the olefinic moiety, and although HMBC correlations from H-11 to C-12 were not observed, correlations from H-14 (δ 2.93) and H_2-15 to C-13 were evident. Finally, 1D NOE difference experiments confirmed that the stereochemistry of the aldehyde and the two acetate functionalities in **222** was consistent with that of **221**.

Spongiane diterpenes have been reported from the sponge genera *Aplysilla*,[241,243,244] *Dictyodendrilla*,[242] *Dysidea*,[245] *Igernella*[246] and *Spongia*,[247] and have been shown to be mildly cytotoxic.[243,247] Although the previous examination of *C. hamiltoni* yielded four new unusual homo-diterpenes, hamiltonin A-D (**231-234**), and the sesterterpene hamiltonin E (**235**), no spongiane diterpenes were found.[219] Conversely, no hamiltonin compounds were evident in the extract of the Malangaan Reef *C. hamiltoni* specimens.



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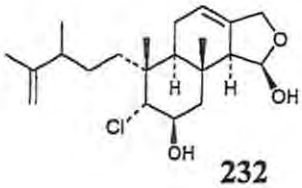

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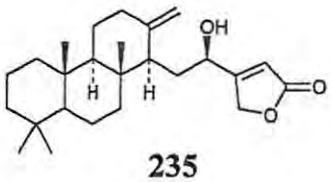

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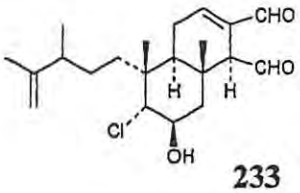

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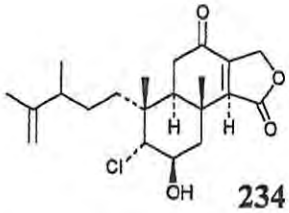

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Table 4.1 ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C ($CDCl_3$, 400 MHz) NMR data for compounds **221** and **222**

	221		**222**	
Atom Number	δ_H (mult., J = Hz)	δ_C (mult.)	δ_H (mult., J = Hz)	δ_C (mult.)
1	0.99 (m)	39.1 (t)	1.09 (t, 12)	39.1 (t)
	1.80 (m)		1.72 (m)	
2	1.58 (br m)	18.3 (t)	1.55 (m)	18.2 (t)
3	1.19 (td, 4, 13)	41.3 (t)	1.19 (td, 4, 13)	41.1 (t)
	1.48 (d, 12)		1.49 (dd, 3, 12)	
4		33.4 (s)		33.3 (s)
5	1.06 (d, 13)	53.9 (d)	1.08 (d, 12)	53.7 (d)
6	1.79 (q, 12)	25.2 (t)	1.91 (m)	25.5 (t)
	2.13 (m)		2.11 (dd, 5, 16)	
7	4.78 (dd, 5, 12)	79.9 (d)	4.87 (dd, 5, 12)	80.5 (d)
8		53.8 (s)		53.2 (s)
9	1.24 (s)	61.3 (d)	1.70 (br s)	58.3 (d)
10		37.7 (s)		37.9 (s)
11	5.59 (br s)	39.1 (t)	5.86 (m)	65.2 (d)
12	1.46 (t, 12)	31.5 (t)	6.65 (t, 4)	129.6 (d)
	2.35 (td, 3, 14)			
13	3.47 (td, 4, 13)	35.3 (d)		129.9 (s)
14	1.93 (br d, 7)	52.3 (d)	2.93 (m)	47.6 (d)
15	4.46 (dd, 3, 10)	68.4 (t)	4.23 (m)	67.9 (t)
	4.04 (t, 8)		4.29 (m)	
16		176.3 (s)		168.3 (s)
17	10.02 (s)	201.0 (d)	10.02 (s)	199.2 (s)
18	0.86 (s)	21.4 (q)	0.89 (s)	21.8 (q)
19	0.92 (s)	33.3 (q)	0.94 (s)	33.5 (q)
20	1.03 (s)	17.9 (q)	1.14 (s)	18.5 (q)
$\underline{CH_3}CO$	2.05 (s)	21.3 (q)	2.07 (s)	21.2 (q)
	1.99 (s)	21.1 (q)	2.02 (s)	21.1 (q)
$CH_3\underline{C}O$		169.6 (s)		169.8 (s)
		169.2 (s)		169.2 (s)

The difference in the chemical composition of *C. hamiltoni* collected on different occasions from two different sites may reflect geographical variation in the chemical composition of specific dietary sponges, or the geographical distribution of the sponges themselves. Whichever may be the case, both sets of nudibranchs sequester highly toxic latrunculins which presumably are critical to the chemical defense of the nudibranchs. Unfortunately, the paucity of material isolated in this study precluded any bioactivity investigations of the spongiane diterpenes. The mild cytotoxicity of similar compounds and the absence of significant activity in antimicrobial and cytotoxicity bioassays for hamiltonins A (**231**) and B (**232**) makes one wonder why these compounds are sequestered along with such potent toxins as the latrunculins. An investigation of the role of compounds **221**, **222** and **231-235** in the chemical ecology of *C. hamiltoni* would be interesting.

4.3 A known spongiane diterpene from *Glossodoris* species 4[80]

Glossodoris sp. 4 (Figure 4.3) is an uncommon nudibranch endemic to southern Africa, and is one of five species of *Glossodoris* found in South Africa, of which only *G. atromarginata* has been fully described.[80] Nine specimens of *Glossodoris* sp. 4 were collected by hand using SCUBA (-10 m) from 'The Gulley' in the Tsitsikamma National Park in March 1998.

Figure 4.3 *Glossodoris* sp. 4 (right) photographed in Algoa Bay with an *Aphelodoris* nudibranch on a *Petrosia* sponge.

The whole animals (length 1-2 cm) were immersed in acetone and stored at –20°C for two months, before being re-extracted with fresh acetone. The acetone extracts were combined, concentrated and partitioned between EtOAc and water. In the ^{1}H NMR spectrum of the crude EtOAc partition fraction (7.1 mg), four prominent peaks of similar height (δ 2.04, 2.06, 2.08 and 2.10) suggested the presence of a tetra-acetate as the single major component of the extract (Figure 4.4). Thus despite the small amount of material in hand, further investigation was undertaken. A single injection of the EtOAc partition fraction (7.1 mg) on normal phase HPLC (3:1 hexane/EtOAc) yielded 6α, 12β, 15α, 16α-tetraacetoxyspongian (**210**, 2.1 mg, 0.23 mg/animal).

Spongiane diterpene **210**, previously isolated from *Chromodoris geminus* collected in Sri Lanka,[248] was identified by comparison of its ^{1}H and ^{13}C NMR data with literature values.[248] The results of a series of 1D NOE difference experiments were also consistent with the reported stereochemistry of the molecule. It is interesting that this uncommon nudibranch is the only known South African chromodorid that is uniformly white.[80] Since this is unlikely to be warning coloration, it is possible that *Glossodoris* sp. 4 is cryptic, like the small white specimens of *G. pallida* from Guam which are camouflaged against the white interior of their externally black prey sponges.[233] Unfortunately, no possible dietary sponges of this nudibranch have been identified since most specimens were collected from the water column in conditions of bad surge. A single specimen of *Glossodoris* sp. 4 was photographed in Algoa Bay (300 km from the Tsitsikamma) on a red *Petrosia* sponge (Figure 4.3), but we consider it improbable that this sponge could be the source of a spongiane diterpene, after preliminary investigation of the brown-spotted *Aphelodoris* nudibranch frequently associated with this sponge yielded polyacetylene compounds.

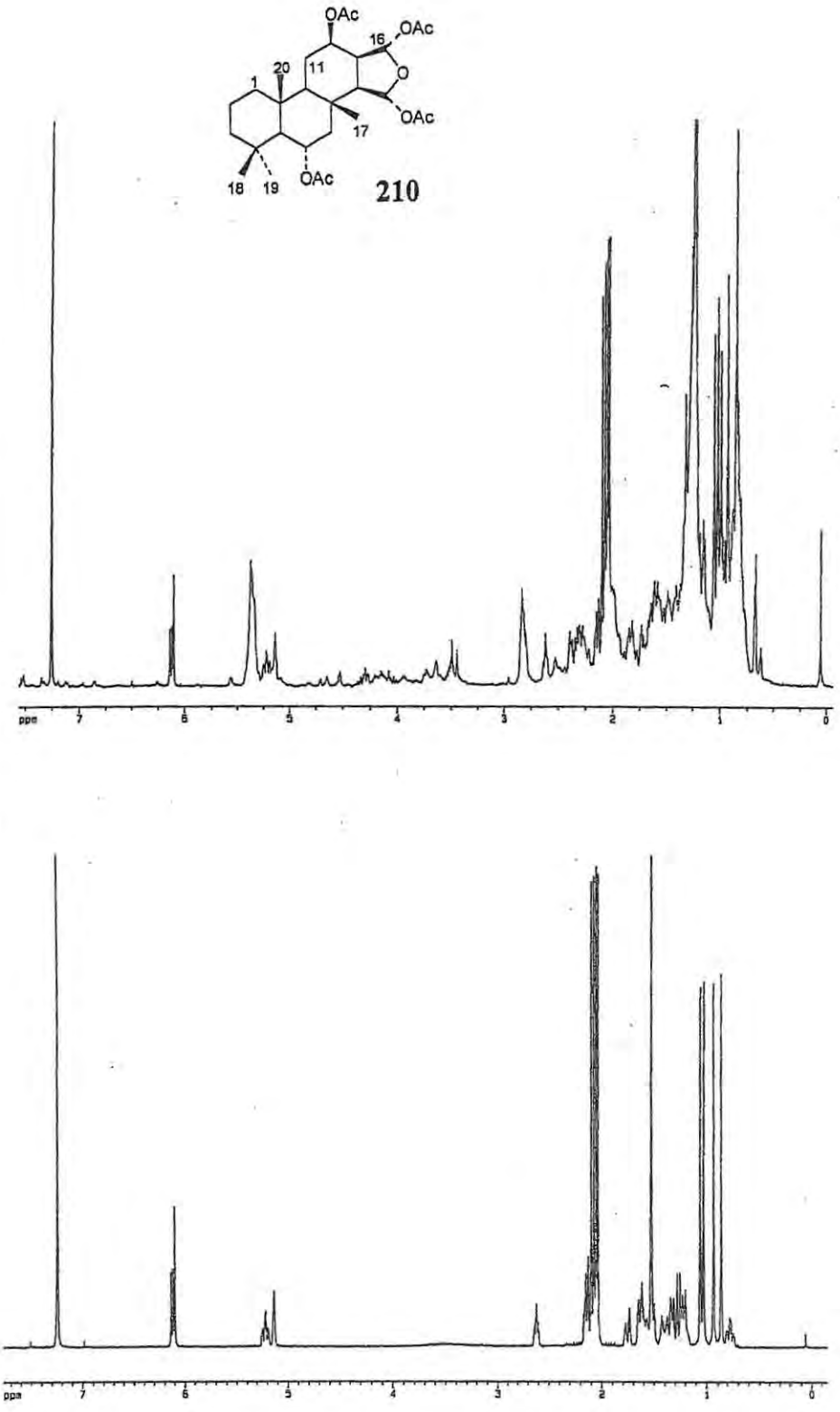


Figure 4.4 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of the crude EtOAc extract of *Glossodoris* sp. 4 (top) and the isolated compound **210** (bottom).

4.4 Furanoterpenes from the chromodorid *Hypselodoris capensis* and two of its dietary sponges, *Fasciospongia* sp. and *Dysidea* sp.

Off the East Cape coast of South Africa, *Hypselodoris capensis* is a common endemic species of chromodorid nudibranch,[80] whose coloration makes it a conspicuous subtidal rocky reef inhabitant. In the Tsitsikamma National Park, *H. capensis* nudibranchs are most frequently observed on a dark grey *Fasciospongia* sponge (Figure 4.5), and may also be seen on a white sponge species of the genus *Dysidea* (Figure 4.5).

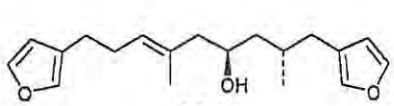
While the association of *Hypselodoris* nudibranchs with *Dysidea* sponges, has been reported from Hawaii,[221] California,[218] Bermuda,[249] and the Mediterranean,[212,213,250,251] the relationships of these nudibranchs with other sponges, unrelated to *Dysidea*, are far less frequently noted.[218] As discussed in earlier, *Hypselodoris* nudibranchs belong to a family of strikingly colored nudibranchs which feed on noxious sponges rarely fed upon by other predators, and sequester bioactive sponge metabolites into specialized storage glands strategically located around the mantle border as part of a chemical defense strategy.

Figure 4.5 Underwater photographs of *H. capensis* on two of its dietary sponges, *Fasciospongia* sp. (left) and *Dysidea* sp. (right)

Furanoid compounds are the preferred defense chemicals of *Hypselodoris* species,[6] apparently attracting the nudibranchs to a variety of suitable prey sponges, as well as

apparently being selected over other metabolites present.[213,250] Indeed, although *Hypselodoris* nudibranchs sequester furanosesquiterpenes from their *Dysidea* prey species, sponges of the genus *Dysidea* have yielded a plethora of other metabolites[252] including polybrominated phenyl ethers, chlorinated amino acid derivatives, cyclic peptides, aromatic sesquiterpenes and sterols which may be judged to be equally characteristic of the sponge genus, even though some of the halogenated metabolites are thought to have a symbiotic algal source. Similarly, furanoterpenes are not the typical metabolites of *Fasciospongia* sponges.[253] However, we have found that the South African *Fasciospongia* species preyed upon by *H. capensis* contained β-substituted linear furanosesterterpene tetronic acids that were sequestered by the nudibranch. Due to their toxicity and their ubiquity in marine dictyoceratid sponges, these compounds have elicited much interest in the last thirty years and are briefly overviewed here.

In the early 1970's, the isolation of C-21 linear difuranoterpenes (oxidised sesterterpenes), *e.g.* furospongin-1 (**236**), from spongiid sponges[254] preceded the discovery of difuranosesterterpene tetronic acids ircinin-1 (**237**) and ircinin-2 (**238**) in *Ircinia oros*,[255] and the furanosesterterpene tetronic acid, fasciculatin (**239**) in *I. fasciculata*.[256] Shortly afterwards, (18*S*)-variabilin (**240**), the logical biosynthetic precursor to fasciculatin and the ircinins with its polyisoprenyl chain, was isolated from the Californian sponge *Ircinia variabilis*.[257] Assignment of the double bond geometry of variabilin as 7*E*, 12*E*, 20*Z* was only completed fifteen years after its initial isolation,[258,259] while the 18*S* absolute stereochemistry was determined by oxidative degradation five years later.[260]



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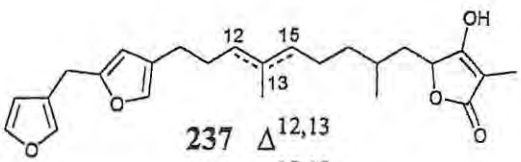
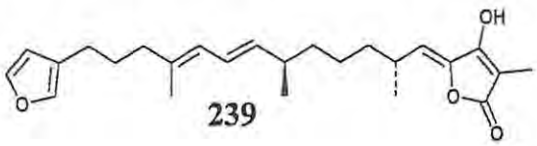


237 $\Delta^{12,13}$

238 $\Delta^{12,15}$



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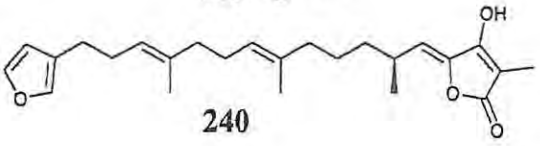
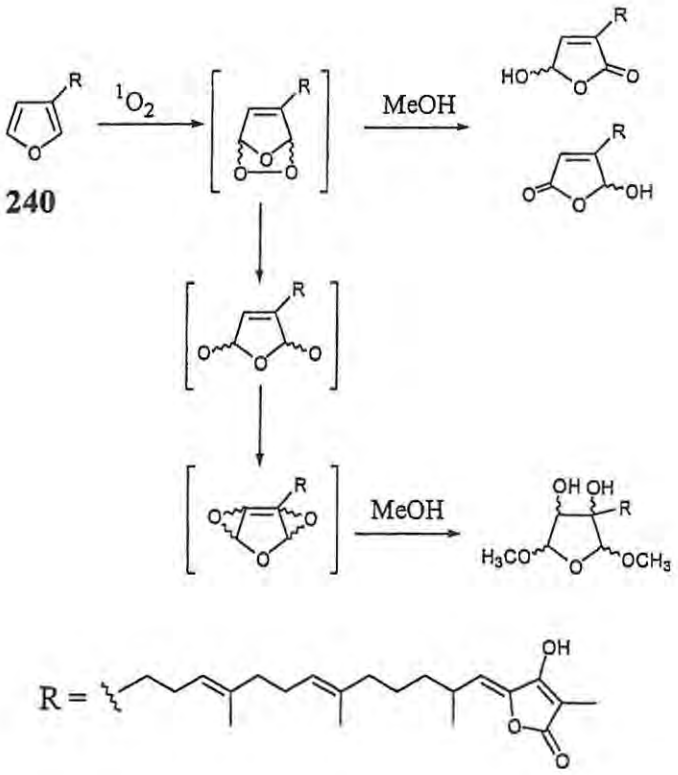


240

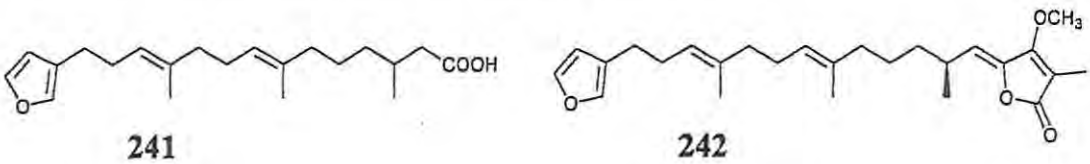
In the mean time, **240** was found to be the major component of many dictyoceratid sponges and has become recognised as the prototype of linear furanosesterterpene tetronic acids, the unsaturation of which lends itself to the occurrence of a plethora of geometric and positional isomers. Much effort has been devoted to differentiating between these different isomers, which are thought to characterise different sponge species, and to assigning their stereochemistry.[260,261] In a study of New Zealand sponges,[262] the occurrence of **240** in all ten of the *Ircinia*, *Psammocinia* and *Sarcotragus* sponges (Family Thorectidae, Order Dictyoceratida) collected could be correlated with the presence in these sponges of fine filaments which permeate the sponge matrix. This morphological feature distinguishes these three genera from other thorectid sponge genera. Interestingly, *Fasciospongia* sponges are morphologically closely related to the *Ircinia* group, although they are transitional to other subgroups within the Family Thorectidae.[262] Thus the furanosesterterpene chemistry of dictyoceratid sponges may serve as a reliable taxonomic indicator of the systematic relationships between these sponges.[262]

In conjunction with their interesting chemistry, the antimicrobial,[257] cytotoxic[262] and possible antiviral[263] activity of linear furanosesterterpene tetronic acids received much attention until the ready decomposition of these compounds in light and air became apparent. Even so, recently there have been reports on the pharmacology[264] of variabilin and its effectiveness as a fish antifeedant.[265] The source of activity in linear furanosesterterpene tetronic acids has been established from detailed autooxidation studies of variabilin.[266] Intriguingly, the stable nonfuranoid oxidation products of variabilin retained the activity observed for variabilin itself. Barrow *et al.*[266] demonstrated that rapid singlet oxygen oxidation of the 3-furan moiety was facilitated by the tetronic acid group which acts as a sensitizer for the production of singlet oxygen. The resulting furan endoperoxides underwent a slow thermal rearrangement to give a range of biologically active oxygenated products (Scheme 4.1).



Scheme 4.1 The singlet oxygen oxidation of variabilin (**240**) to stable diastereomeric pairs of nonfuranoid oxidation products.[266]

In addition, the biological activity of furanoterpene **241** was found to be similar to that of **240**, while 22-*O*-methylvariabilin (**242**) was inactive. Therefore, the biological activity of β-substituted furanosesterterpene tetronic acids was attributed to the presence of terminal polar groups, and the intact 3-furan and tetronic acid moieties, both sites of instability, were shown to be inessential for activity in these compounds.

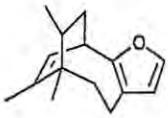


241 **242**

4.4.1 The furanosesquiterpenes isolated from *Hypselodoris capensis* and a *Dysidea* sponge

Sixteen specimens of *H. capensis*, collected in the Tsitsikamma National Park in February 1997, were extracted in acetone and the EtOAc partition fraction of the resulting extract was chromatographed on Si gel to give six crude fractions. Three of these fractions were selected for further purification by normal phase HPLC from their interesting ^{1}H NMR

spectra. The known furanosesquiterpenes nakafuran-8 (**223**) and nakafuran-9 (**224**) were identified as the major constituents of the hexane chromatography fraction.



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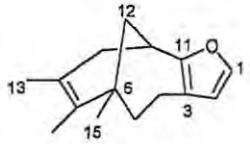


224

Initially, the structure of compound **224** was fully assigned from coherent 1D and 2D NMR spectra and its ^{1}H and ^{13}C NMR data found to be compatible with those reported for nakafuran-9.[221] On closer examination of the ^{13}C NMR data reported by Schulte *et al.*, some minor discrepancies in the assignment of ^{13}C NMR data were evident (Table 4.2). Without the aid of 2D NMR experiments Schulte *et al.*,[221] had indicated that several chemical shift values were interchangeable and we assumed that the resonances at δ 38.5, 38.8 and 41.6 had been erroneously assigned as C-9, C-12 and C-5 respectively.

Table 4.2 Comparative ^{13}C NMR data for nakafuran-9 isolated by Scheuer *et al.*[221] ($CDCl_3$, 25 MHz) and compound **224** isolated from the South African *Dysidea* sponge ($CDCl_3$, 100 MHz)

	Compound*	
Carbon	**nakafuran-9**	**224**
1	138.4 (d)	138.4 (d)
2	113.3 (d)	113.1 (d)
3	118.5 (s)	118.3 (s)
4	23.3 (t)	23.0 (t)
5	41.6 (t)	38.5 (t)
6	37.9 (s)	37.6 (s)
7	129.6 (s)[a]	129.5 (s)
8	126.4 (s)[a]	126.3 (s)
9	38.5 (t)[b]	38.2 (t)
10	32.0 (d)	31.7 (d)
11	156.3 (s)	156.2 (s)
12	38.8 (t)[b]	41.3 (t)
13	30.0 (q)[c]	19.8 (q)
14	31.0 (q)[c]	13.2 (q)
15	20.1 (q)[c]	30.7 (q)

* Values in ppm (mult.); [a, b, c] Values are interchangeable

In the HMBC spectrum of **224** (Figure 4.6), correlations from five ^{1}H signals to the ^{13}C resonance at δ 41.3, and from the corresponding (HMQC correlated) ^{1}H multiplet (δ 1.75) to seven ^{13}C signals, caused us to assign the former ^{13}C resonance to the bridge-head carbon C-12 (Figure 4.6) rather than to C-5 (Table 4.2). Two closely spaced resonances at δ 38.2 and 38.5 in our ^{13}C NMR spectrum of **224** were assigned to C-9 and C-5 respectively after careful consideration of the HMBC correlations from the HMQC correlated ^{1}H signals to these carbon resonances (δ 1.94 and 2.37, and 1.37 and 1.92 respectively, Figure 4.6).

Schulte *et al.*[221] also indicated that their assignments of the vinyl methyls (H_3-13 and H_3-14) as δ 30.0 and 31.0 respectively, and the quartenary methyl (H_3-15) as δ 20.1, were uncertain (Table 4.2). Furthermore, we wondered whether the first two digits of the chemical shift at δ 31.0 (H_3-14) had been accidentally transposed as the value we obtained for this methyl group (δ 13.2) is more consistent with the expected value for an olefinic methyl.[188] This was subsequently confirmed to be the case on inspection of a ^{13}C NMR spectrum of nakafuran-9, kindly provided by Professor Scheuer, which proved identical to that of compound **224**. In the HMQC spectrum of **224**, correlations of the ^{13}C signals at δ 13.2 and 19.8 to two typically deshielded H_3 signals at δ 1.58 and 1.56 respectively in the ^{1}H NMR spectrum of **224** corroborated our assignment of the two vinylic methyls. The third ^{13}C methyl resonance (δ 30.7), showing an HMQC correlation to the methyl singlet at δ 1.06, was assigned as the quartenary methyl group (H_3-15) on the grounds of prominent HMBC correlations from the latter ^{1}H singlet to ^{13}C resonances at δ 37.6 (C-6), 38.5 (C-5), 41.3 (C-12) and 129.5 (C-7, Figure 4.6). While the ^{1}H NMR data reported for nakafuran-9 understandably had not been assigned, the ^{1}H NMR spectrum of compound **224** was also identical to that provided by Professor Scheuer for nakafuran-9.

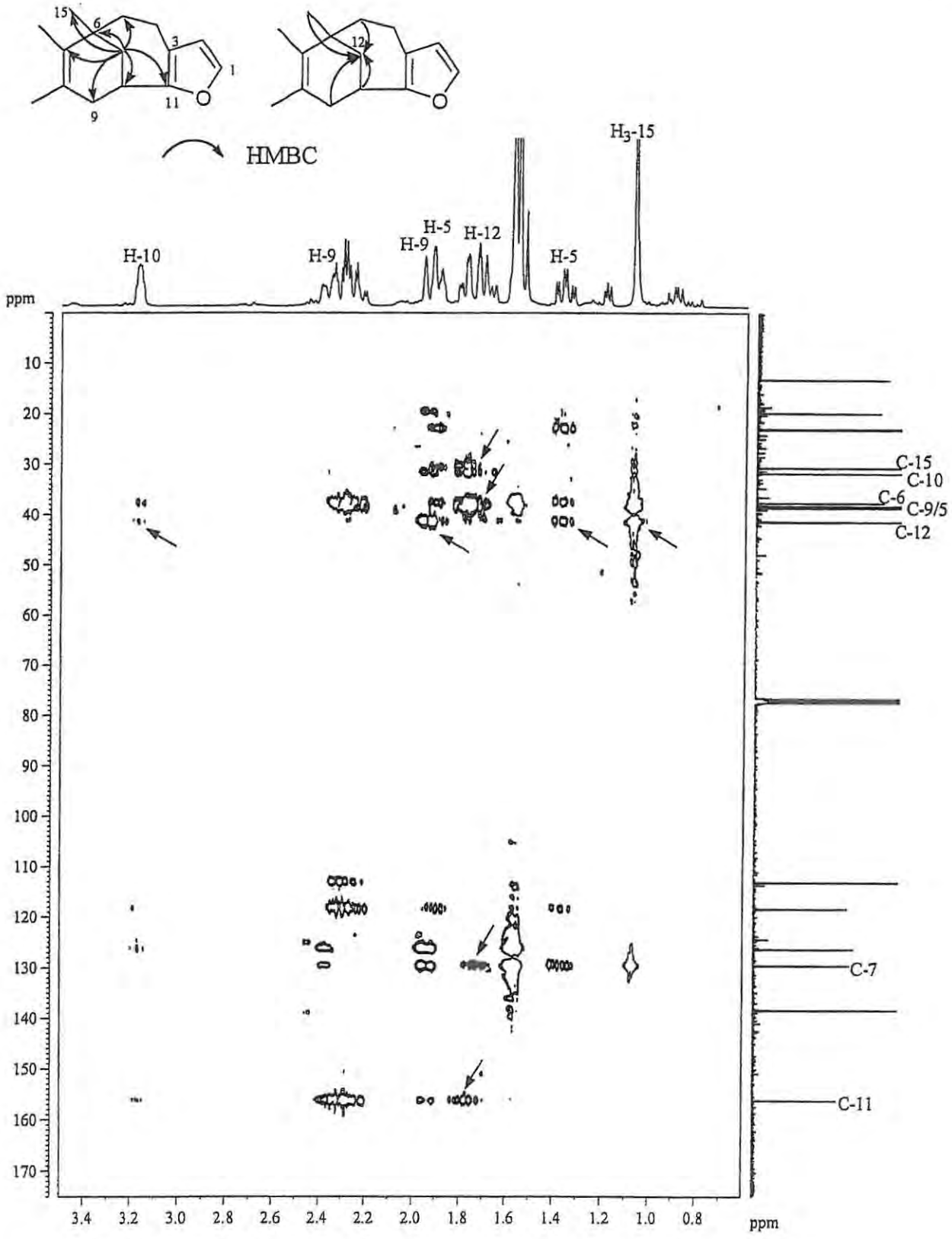


Figure 4.6 A region (F1 = 0 – 175 ppm; F2 = 0.6 – 3.5 ppm) of the HMBC spectrum ($CDCl_3$, 400 MHz, d6 delay time = 60 ms) of nakafuran-9 (**224**) showing the key correlations from H_2-12 and to C-12 which are represented in the accompanying figure.

Unfortunately, an attempt to purify nakafuran-8 (**223**) by reverse phase HPLC resulted in the degradation of this unstable compound, which initially co-eluted in hexane with an unidentified compound from a normal phase HPLC column. The identity of **223**, for which no 2D NMR data was obtained, was confirmed by comparison of its ^{1}H NMR spectrum with that provided for nakafuran-8 by Professor Scheuer.

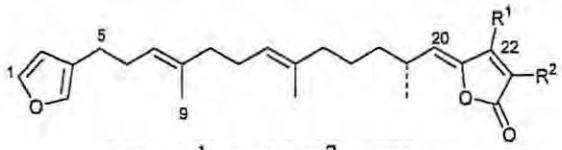
Nakafuran-8 and -9, their isomers or substituted variants, have been isolated from a multitude of *Hypselodoris* nudibranchs,[6] but are well-known *Dysidea* sponge metabolites[252] and have been shown to be sequestered from *Dysidea* sponges by the nudibranchs.[212] For these reasons we thought that a sponge so important in the diet of *H. capensis* would be relatively easy to locate. This proved to be the case when, returning to our collection site in the Tsitsikamma National Park in March 1998, we collected a white *Dysidea* species on which *H. capensis* specimens were observed. ^{1}H NMR spectroscopy of a hexane partition fraction of the crude EtOAc extract from this sponge indicated the presence of furanoid compounds as expected. Subsequent purification of the hexane fraction by open column chromatography and normal phase HPLC (hexane and iso-octane) yielded nakafuran-9 (**224**), and a 2:1 mixture of nakafuran-8 (**223**) and a very similar compound which we were again unable to separate.

4.4.2 The furanosesterterpenes isolated from *Hypselodoris capensis* and a *Fasciospongia* sponge

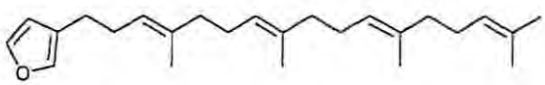
Having established the structures of the main components of the nonpolar fraction of the *H. capensis* extract we turned to the more complex polar fractions. Normal phase HPLC of the two more polar nudibranch chromatography fractions selected gave several fractions containing β-substituted furans, as evidenced by characteristic signals (δ 7.32, 7.19, 6.26) in the ^{1}H NMR spectra of these fractions. Fortunately, the ^{1}H NMR spectrum of an EtOAc partition fraction of the MeOH extract of the *Fasciospongia* sponge indicated the presence of similar compounds. Therefore, mindful of the notorious instability of β-substituted furans,[266] and hampered by a paucity of nudibranch material, we decided to purify the more substantial *Fasciospongia* sponge extract in the hope of

isolating sufficient amounts of pure β-substituted furans for which acceptable spectral data could be obtained in as short a time as possible. Accordingly, the EtOAc partition fraction was applied directly to a normal phase HPLC column to give compounds **195, 225, 226** and **227** in reasonable yield. Comparison of the ^{1}H NMR spectra of these four compounds found in the *Fasciospongia* sponge with those of the unidentified β-substituted furanoterpenes isolated from the nudibranchs showed that all except **227** were present in *H. capensis*. The identity of compound **195** as variabilin was immediately evident on comparison of its ^{1}H and ^{13}C NMR spectra with those of authentic variabilin, which we had in hand.[267]

The positive optical rotation obtained for **195** (+36°) was consistent with that reported for an *R* configuration at C-18 in this compound.[268] Martinez *et al.* assigned this configuration by carrying out comparative ^{1}H NMR studies of the MTPA esters of 2-methylheptane-1,6-diol which was obtained by oxidative degradation of the acetate of **195**.[268] Furospinosulin-1 (**227**) could also be identified by comparison of its NMR and IR spectral data with those reported previously,[195] the tetraprenyl sidechain initially being suggested from integration of the few heavily overlapped signals in the ^{1}H NMR spectrum of **227**.



195 $R^1 = OH, R^2 = CH_3$
225 $R^1 = H, R^2 = CH_3$
226 $R^1 = H, R^2 = CH_2OH$

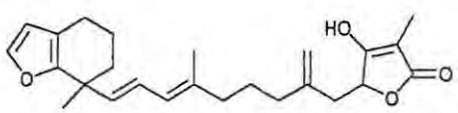


227

Comparison of the spectral data for compounds **225** and **226** with those of **195** showed that these compounds varied only in their substitution pattern around the tetronic acid moiety. Although the spectral data of compound **225** are generally consistent with those of 22-deoxyvariabilin,[269] a significant discrepancy was apparent on comparison of our ^{13}C data with the reported data for this compound (Table 4.3). Kernan *et al.*[269] assigned C-21 as δ 128.9 and C-23 as δ 125.3 for 22-deoxyvariabilin. However, examination of our ^{13}C

NMR spectrum (F1 axis, Figure 4.7) for **225** revealed no signal at δ 125.3, while a prominent resonance at δ 147.5 which showed two bond HMBC correlations to H-20 (4.93) and H-22 (6.94), together with a very weak (4-bond) coupling to H_3-25 (1.97), caused us to assign this signal to C-21 (Figure 4.7). Since only the H-22 and H_3-25 resonances showed correlations to the ^{13}C signal at δ128.9, this signal was assigned to C-23. The analogous C-21 and C-23 chemical shifts reported for variabilin are δ 143.0 and δ 99.1 respectively.[259] Our ^1H NMR spectral data for compound **225** were identical to those reported for 22-deoxyvariabilin, while 1D NOE difference experiments showed reciprocal enhancement of the H-22 and H-20 resonances as expected for the structure given. Infrared spectral data were also in agreement with those reported by Kernan *et al.*,[269] the absence of a broad absorption centred around 3400cm^{-1} being indicative of the deoxy compound.

It is noteworthy that this appears to be the first report of the co-occurrence of variabilin and 22-deoxyvariabilin in the same organism. It was previously thought that these two compounds, which vary only in the oxidation of their tetronic acid moieties, were characteristic of separate groups of sponge genera.[269] While our finding of variabilin in a *Fasciospongia* sponge concurs with the supposition that linear sesterterpenes containing a tetronic acid group are confined to the genera *Ircinia*, *Sarcotragus*, *Psammocinia* and *Fasciospongia*,[269] the presence of 22-deoxyvariabilin in a *Fasciospongia* sponge disproves the hypothesis that linear sesterterpenes containing a terminal unsaturated butenolide are confined to the genera *Taonura* and *Thorecta*.[269] Although the sesterterpene tetronic acid okinonellin A (**243**) has been isolated from an initially misidentified *Fasciospongia* species,[269] there has been no prior report of the occurrence of variabilin itself in this genus.



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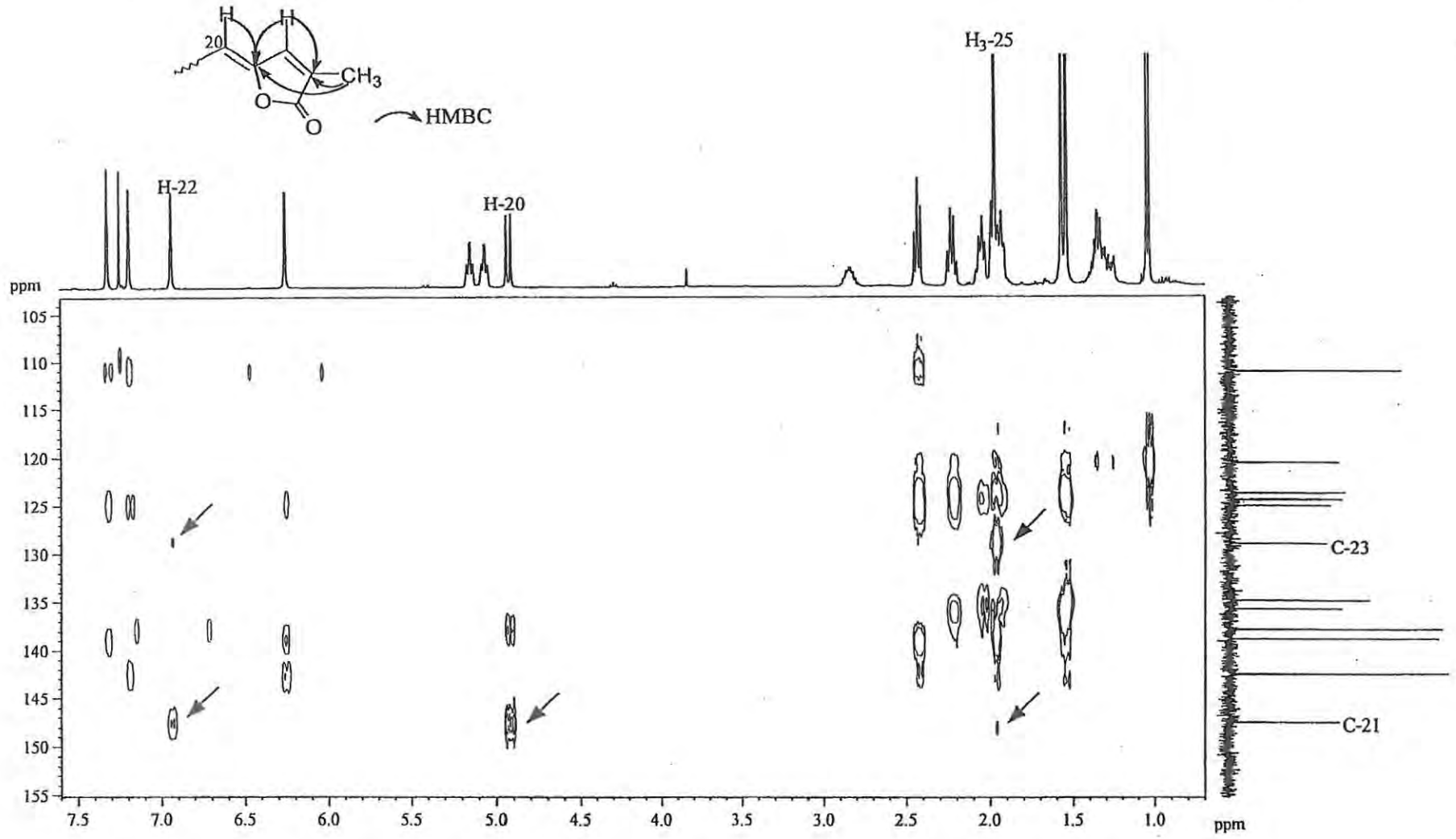


Figure 4.7 A region (F1 = δ 103 – 155 ppm; F2 = δ 0.7 – 7.6 ppm) of the HMBC spectrum ($CDCl_3$, 400 MHz, delay time d6 = 60 msec) of compound **225** used to assign C-21 and C-23 in compound **225** as shown in the accompanying figure.

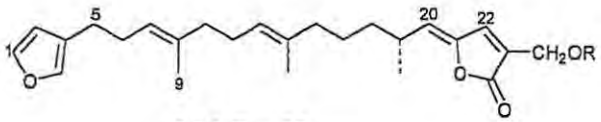
Table 4.3 Comparative ^{13}C NMR data for 22-deoxyvariabilin reported by Kernan *et al.*,[269] ($CDCl_3$, 100 MHz) and compounds **225** and **226** isolated from the South African *Fasciospongia* sponge ($CDCl_3$, 100 MHz)

		Compound*	
Carbon	**22-deoxyvariabilin[269]**	**225**	**226**
1	142.5	142.5 (d)	142.5 (d)
2	111.1	111.1 (d)	111.1 (d)
3	124.9	125.0 (s)	125.0 (s)
4	138.8	138.8 (d)	138.8 (d)
5	25.0	25.0 (t)	25.0 (t)
6	28.4	28.5 (t)	28.5 (t)
7	123.7	123.7 (d)	123.8 (d)
8	135.8	135.8 (s)	135.8 (s)
9	15.8	16.0 (q)	16.0 (q)
10	39.5	39.5 (t)	39.5 (t)
11	26.5	26.6 (t)	26.6 (t)
12	124.3	124.4 (d)	124.5 (d)
13	134.8	134.8 (s)	134.8 (s)
14	16.0	15.8 (q)	15.8 (q)
15	39.7	39.7 (t)	39.7 (t)
16	25.7	25.7 (t)	25.7 (t)
17	36.4	36.7 (t)	36.6 (t)
18	31.3	31.3 (d)	31.6 (d)
19	20.8	20.7 (q)	20.6 (q)
20	120.6	120.5 (d)	123.2 (d)
21	128.9	147.5 (s)	147.5 (s)
22	137.9	137.8 (d)	137.9 (d)
23	125.3	128.9 (s)	131.8 (s)
24	170.1	171.2 (s)	172.8 (s)
25	10.5	10.5 (q)	56.9 (t)

* Values in ppm (mult.)

A molecular formula of $C_{25}H_{34}O_4$ for **226** was confirmed from HREIMS data (398.2468 Δ mmu +1.1), while a broad hydroxyl absorption band from 3660 to 3130cm^{-1} in the IR spectrum of **226** supported the presence of an alcohol functionality in this compound.

The only major differences between the ^{13}C NMR data of **225** and **226** were the absence of a methyl resonance at δ 10.5 and the presence of an additional methylene signal at δ 56.9 in the ^{13}C NMR spectrum of **226**, indicating a different substituent at C-23 in the tetronic acid moiety of this compound. A downfield shift of the C-23 signal from δ 128.9 for **225** to 131.8 for **226** (Table 4.3), and a broad H_3 singlet at δ 4.50 in the ^1H NMR spectrum, suggested a hydroxymethyl substituent at this position, with the hydroxyl proton signal overlapping that of the methylene protons. It should be noted that the downfield position of the ^{13}C signal assigned to C-23 in **226** relative to the signal assigned to C-23 in **225** lends support to our assignment of the tetronic acid chemical shifts of **225**, which differed from those given in the literature for the latter compound. The presence of a hydroxymethyl moiety in **226** was confirmed by its acetylation to give **244**, which resulted in a downfield shift of the methylene proton singlet from δ 4.50 for **226** to δ 4.88 for **244**, and the appearance of an acetate methyl signal (δ 2.10) in the ^1H NMR spectrum of **244**. All other proton chemical shifts remained essentially unchanged. Hydroxymethyl substituents have previously been noted in difuranosesterterpene tetronic acids isolated from Australian sponges of the genera *Taonura*[269] and *Spongia*.[270]



226 R = H
244 R = Ac

The results of our chemical investigations of the endemic South African nudibranchs *Chromodoris hamiltoni*, *Glossodoris* sp. 4 and *Hypselodoris capensis* are consistent with the general trends observed worldwide for nudibranchs of these genera, which tend to sequester bioactive 1,4-dialdehydes often masked as furans.[225] The nature of the secondary metabolites isolated from specimens of *C. hamiltoni*, and an examination of the nudibranchs' gut contents, indicates that the various unknown dietary sponges of these nudibranchs must include a latrunculid sponge, the source of the thiazolidinone macrolide latrunculin B (**220**), and a non-spiculate sponge(s) from which the previously unreported spongiane diterpenes **221** and **222** were sequestered. Few investigations of the chemistry

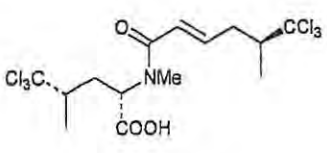
of true *Glossodoris* nudibranchs have been carried out. Although, our finding of a spongiane diterpene (**210**) in *Glossodoris* sp. 4 is only the second report of diterpenes from a *Glossodoris* species, only three other species of *Glossodoris* have been investigated, all of which were found to contain sesterterpenes as discussed in the introduction. In the case of *H. capensis*, both furanosesquiterpenes and furanosesterterpenes are selected from dietary sponges, and there is little doubt as to the classification of this nudibranch as a *Hypselodoris* species. The present study illustrates the ability of *Hypselodoris* nudibranchs to select furanoid compounds, whether they be fifteen, twenty or twenty five carbon compounds, from a diverse range of sponges.

Chapter Five

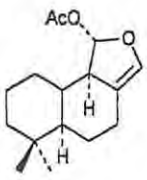
The Structure and Synthesis of a New *Dysidea* Sponge Metabolite

5.1 An overview of *Dysidea* sponge metabolites

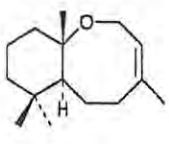
Numerous investigations[252] of more than thirteen *Dysidea* sponge species have yielded a diverse array of metabolites, both within the same species and between different species. Although a few atypical diterpenes,[245,271,272] sesterterpenes[273] and even macrolides[272] have been reported, the metabolites typically isolated from *Dysidea* sponges are chlorinated amino acid derivatives, polybrominated phenyl ethers, cyclic peptides, sterols and sesquiterpenes. In some cases taxonomic assignments of the sponges investigated may be incorrect.[273,274] For instance, several samples of *D. herbacea* which contain polybromo-diphenyl ethers have been reclassified as *Phyllospongia* species.[274] While many polyhalogenated metabolites reported do come from properly identified *Dysidea* sponges,[274] they bear a close resemblance to blue-green algal metabolites and evidence confirming a symbiotic algal source of chlorinated amino acid derivatives, *e.g.* herbacic acid (**245**) from *D. herbacea*,[275] has been presented by two independent research groups.[276] The sesquiterpenes and sterols isolated from *Dysidea* species are considered to be true sponge metabolites and the array of sesquiterpenes routinely reported includes drimanes such as 7-deacetoxy-olepupane (**246**),[31] cyclic ethers such as arenaran (**247**),[277] and aromatic and quinone compounds of mixed biogenesis, *e.g.* avarol (**183**).[199] However, furanosesquiterpenes are undoubtedly the most characteristic secondary metabolites of *Dysidea* sponges.



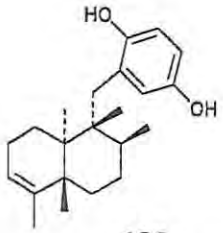
245



246



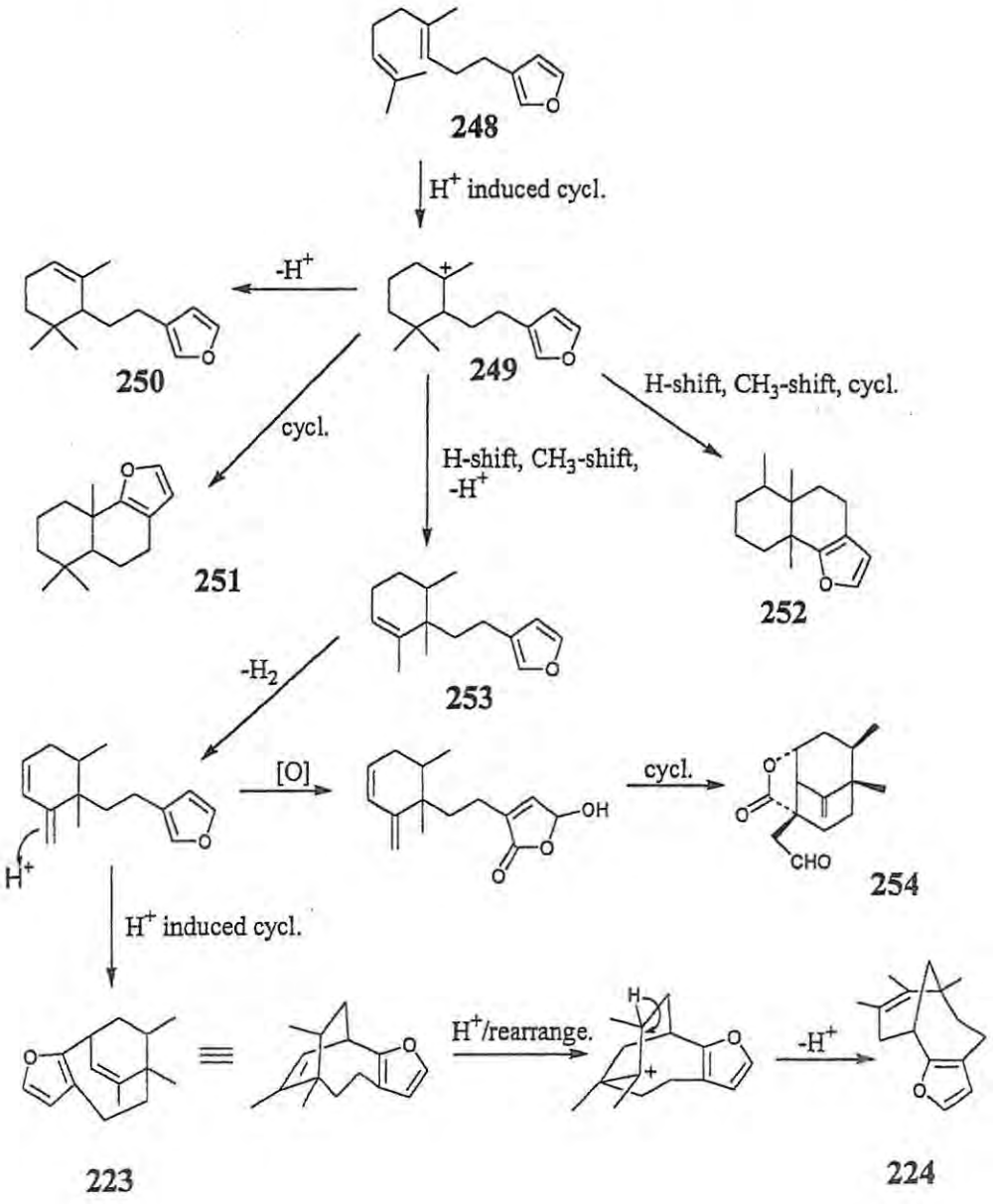
247



183

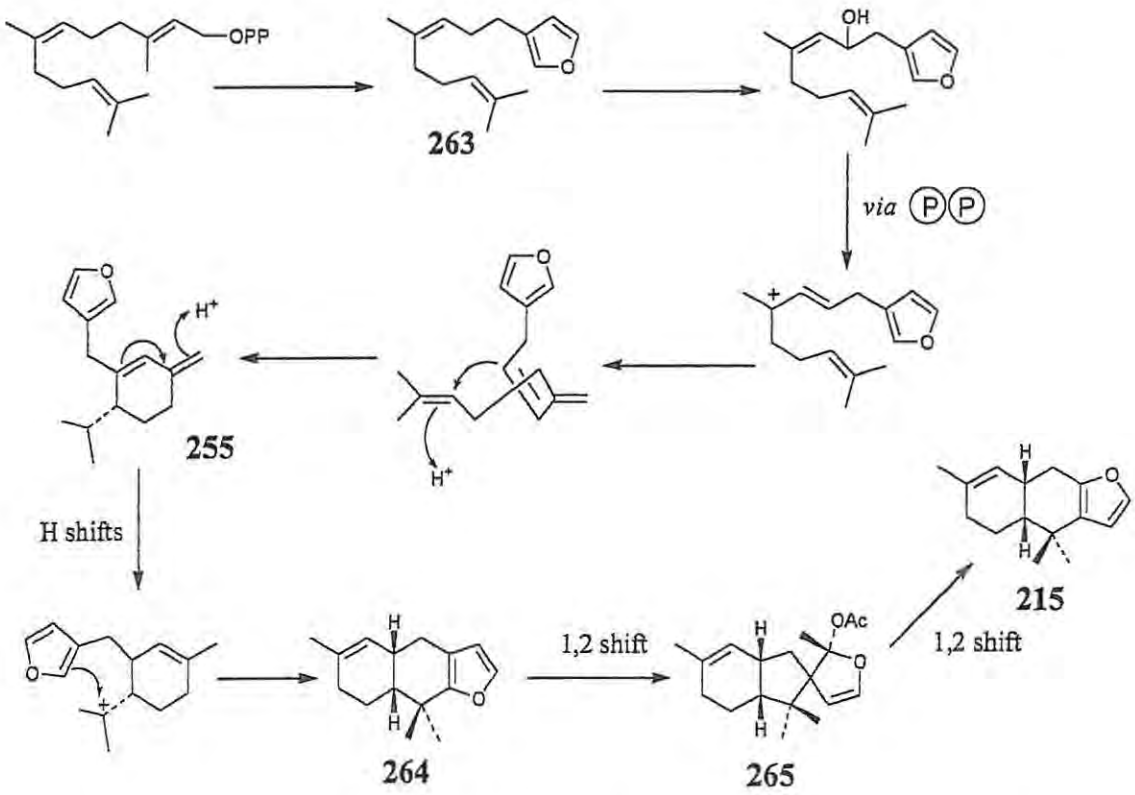
The large complement of *Dysidea* furanosesquiterpenes, and related butenolides, encompasses skeletal types ranging from linear β-substituted furans and difurans, and simple bicyclic derivatives, to fused, spiro-fused and bridged tricyclic systems. Two

distinct biosynthetic pathways from a linear β-substituted furan precursor have been rationalized to account for the structural diversity of *Dysidea* furanosesquiterpenes. In the first pathway (Scheme 5.1),[221] acid-catalysed cyclization of dehydrodendrolasin[278] (**248**) affords (**249**), the protonated precursor of pallescensin 1[279] (**250**), which may cyclize further to produce fused tricycles like pallescensin A[280] (**251**), or undergo 1,2 shifts with or without further cyclization, to afford the various microcionins, *e.g.* microcionin 1 (**252**) and microcionin 2 (**253**).[281] The latter compound is a precursor to the bridged systems of upial[282] (**254**), and also nakafuran-8 (**223**) and –9 (**224**).[221]

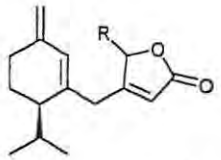


Scheme 5.1 Biogenetic pathway to many *Dysidea* sponge furanosesquiterpenes (adapted from Schulte *et al.*[221])

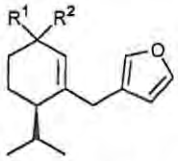
Penlanfuran (**255**),[283] the first reported furanosesquiterpene sponge metabolite of its kind, was isolated from a Brittany sponge, *D. fragilis*, which has subsequently yielded six further similar compounds (**256-261**).[284] The structural skeleton of these compounds is also found in a non-furanoid plant metabolite, humbertiol (**262**)[285] and is not directly related to those of the nakafurans and other cyclic furanosesquiterpenes described above. Guella *et al.*[284] proposed that penlanfuran, as a product of the second biosynthetic pathway to *Dysidea* furanosesquiterpenes, was the result of the cyclization of a hydroxylated hypothetical *Z* isomer of dehydrodendrolasin (Scheme 5.2). The *Z* isomer of dehydrodendrolasin (**263**) has since been reported from *D. herbacea*.[274] Further cyclization of penlanfuran would yield furodysin[286] (**264**), which is related through a 1, 2 alkyl shift to uncommon spirofused sesquiterpenes, *e.g.* spirodysin[287] (**265**). The latter compound was suggested[287] to be the logical precursor to the rearranged furodysinins, *e.g.* furodysinin (**215**).[286]



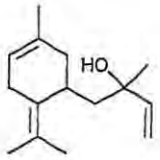
Scheme 5.2 Biogenetic pathway to more unusual *Dysidea* sponge metabolites (adapted from Guella *et al.*[284])



256 R = β-OH
257 R = α-OH



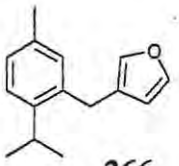
258 R^1 = β-CH_2OAc, R^2 = α- H
259 R^1 = β-CH_2OAc, R^2 = α-OH
260 R = α-CH_2OAc, R^2 = β-OH
261 R^1 + R^2 = O



262

5.2 The isolation and structure determination of tsitsikammafuran (266)

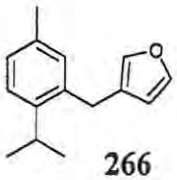
The previous chapter describes the isolation of nakafuran-8 (**223**) and –9 (**224**) from the dorid nudibranch *Hypselodoris capensis*, and the subsequent discovery of these compounds in a dietary *Dysidea* sponge. *H. capensis* specimens were observed on the *Dysidea* sponge in the Tsitsikamma National Park, a marine reserve on the East Cape coast of South Africa from which the nudibranchs and the sponge were collected. In purifying nakafuran-9 from the *Dysidea* sponge, and attempting to isolate nakafuran-8, a further new furanosesquiterpene, tsitsikammafuran (**266**), was isolated.



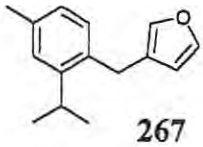
266

Although it was deemed likely from biosynthetic arguments that tsitsikammafuran (**266**, 0.8 mg, 0.0004 % dry wt of sponge) was the aromatic analogue of penlanfuran (**255**), we could not unequivocally assign the structure of the former compound. A molecular mass of 214.1345 (Δmmu –1.2) gained from HREIMS data established a molecular formula of $C_{15}H_{18}O$ for **266**. All fifteen carbon atoms were evident in the ^{13}C NMR spectrum of **266**, and a DEPT experiment indicated the presence of three methyl, one methylene, one shielded methine, six olefinic methines and four olefinic quarternary carbon atoms. Six aromatic proton resonances and one deshielded methylene singlet (δ 3.26) in the ^1H NMR spectrum of **266**, together with the seven degrees of unsaturation implied by the molecular formula, suggested a biaryl structure for **266**, comprising a substituted benzene and a furan ring linked *via* a methylene bridge. Of the six aromatic proton signals present

in the ^{1}H NMR spectrum of **266**, three singlets at δ 6.21 (br s), 7.06 (s) and 7.33 (br s) were consistent with a β-substituted furan ring, while the remaining two mutually coupled doublets and one singlet implied a 1, 3, 4 trisubstituted benzene ring. Besides the [(furan-3-yl)methyl] moiety, an isopropyl and a methyl substituent could be identified from a one proton septet (δ 3.13) mutually coupled to two overlapping methyl doublets (δ 1.17), and a three proton singlet (δ 2.27) respectively. Unfortunately, positioning these three substituents around the benzene ring in the 1,3,4 substitution pattern of either regioisomer **266** or **267** posed a problem.



266



267

Two and three bond HMBC correlations, respectively, from both the isopropyl methine proton and the methylene protons to the same olefinic quartenary carbon (δ 143.8) established an *ortho* relationship between the isopropyl and the [(furan-3-yl)methyl]-substituents. However, a very small, but crucial, HMBC correlation from an aromatic proton doublet (δ 7.17) to a carbon resonance at δ 28.4 (either the isopropyl methine carbon (δ 28.37) or the methylene carbon (δ 28.43)), which could have unequivocally positioned the vicinal benzene protons, could not be assigned with confidence. Although a long-range COSY coupling from the aromatic proton singlet at δ 6.95 to the methylene singlet at δ 3.76 supported structure **266**, there also appeared to be a coupling between the latter signal and the doublet at δ 7.03 (Figure 5.1), making assignment of these long-range COSY couplings ambiguous. Further confusion arose from the results of 1D NOE difference experiments. Irradiation of the isopropyl ^{1}H septet at δ 3.13 produced an enhancement in the methyl singlet at δ 2.27, which is more consistent with structure **267** than structure **266**. Irradiation of the singlet at δ 6.95 and the doublet at δ 7.17 caused enhancements (of similar magnitudes) in the methylene proton signal (δ 3.76) and the methyl signal (δ 2.27). These anomalous results were attributed to the small size of the molecule, and the consequent proximity of the protons of the benzene ring and its

substituents. Unfortunately, a paucity of **266**, and the instability of the furan ring in $CDCl_3$, precluded our further structural investigation of the natural product by NMR using an alternative solvent *e.g.* deuterated benzene.[288]

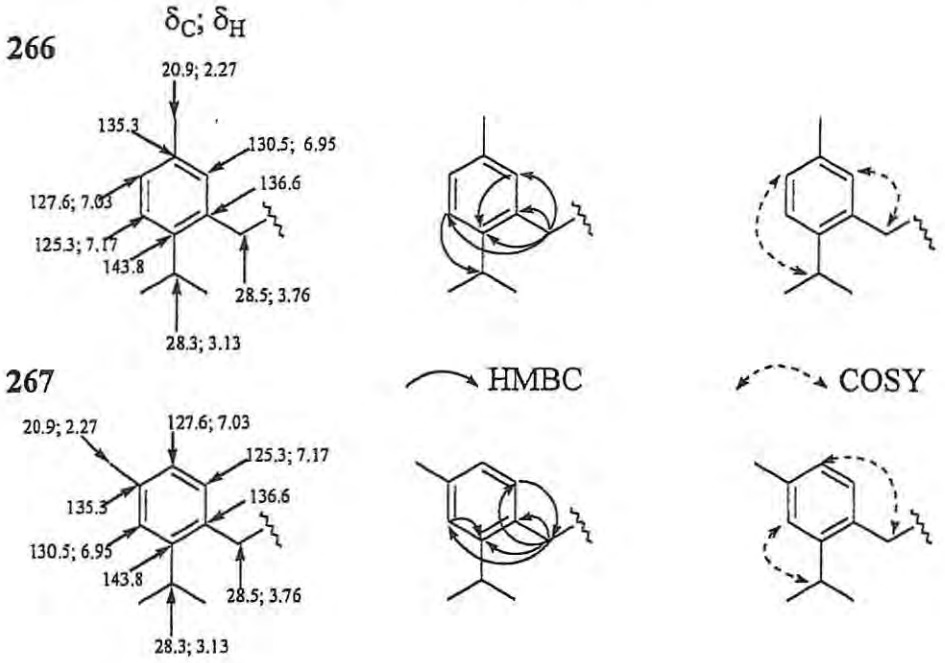


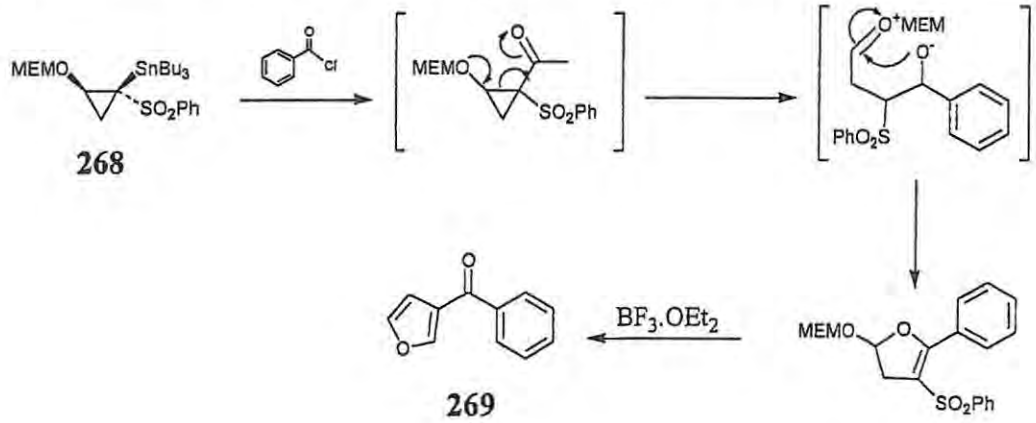
Figure 5.1 HMBC correlations and COSY couplings for tsitsikammafuran as applied to the two alternative regioisomers **266** and **267**.

5.3 Strategies for the synthesis of tsitsikammafuran (266)

Faced with the structural dilemma of two possible regioisomers, conclusive evidence for the proposed structure of tsitsikammafuran was sought *via* synthesis since access to further sponge material in the marine reserve was restricted. In addition, given the very low concentrations of **266** in the *Dysidea* sponge (0.0004 % dry weight), we hoped that a synthetic source of **266** would provide a GC-MS standard for the analysis of *Hypselodoris capensis* extracts which might contain trace quantities of this compound.

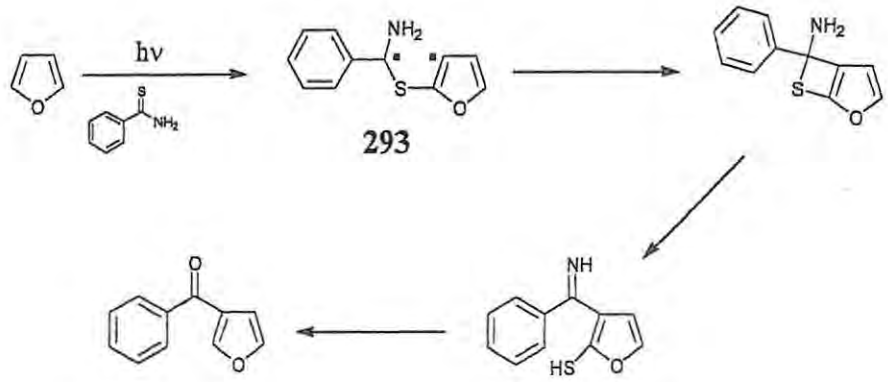
The synthesis of polysubstituted furans has been well documented[289] due to their role as building blocks in synthetic chemistry, as potential pharmaceuticals and their widespread occurrence in natural products. The furan moiety itself is considered to be relatively unreactive in synthetic chemistry and provides a nucleus around which complex cyclic systems may be assembled.[290] Subsequent chemical manipulation of the furan moiety can

provide access to a range of functionalities including 1,4-butendiol, cyclopentenone, cyclic ethers and lactones. 3-Substituted furans, as the most labile furans, are generally unstable to direct light, acid and prolonged heating.[291] Nevertheless, a variety of routes to 3-substituted furans have been developed including less easily rationalised pathways from acyclic precursors, cyclopropane derivatives and rearrangements of 2-substituted furans.[289] Cyclic compounds with a pendant 3-furan moiety like tsitsikammafuran have been prepared in low yield by acylation of stannyl cyclopropane (**268**) and transformation of the resultant dihydrofuran to a 3-acyl furan (**269**) using BF_3 (Scheme 5.3).[292]



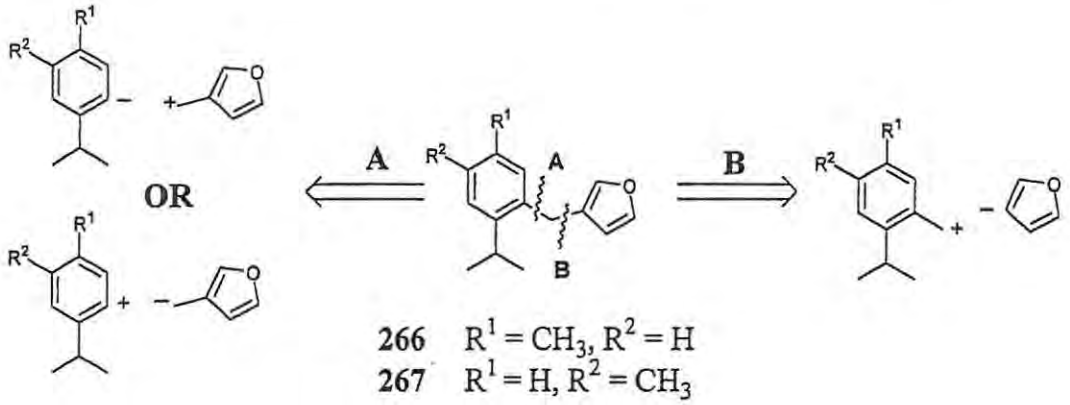
Scheme 5.3 Formation of 3-acyl furans from a stannyl cycopropane derivative (**268**).[292]

3-Acyl furans have also been obtained from the photo-induced reaction of an arenecarbothiamide with an excess of furan (Scheme 5.4),[293] which involves rearrangement of the 2-substituted furan (**270**) initially formed by direct electrophilic attack of the arenecarbothioamide on furan.



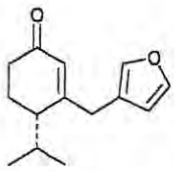
Scheme 5.4 The photo-induced reaction of phenylcarbothioamide with furan to form 3-acyl furans.[293]

Before resorting to any of the above more unusual preparations of 3-substituted furans we considered more familiar routes *via* organometallic reagents. We were interested to know how the NMR data of **266** and **267** would differ. Therefore, at the outset of our synthesis, we envisaged that the direct linkage of two aryl systems would facilitate access to both isomers using the appropriately alkylated benzene precursor in each case. Scheme 5.5 represents three possible approaches to the coupling of the two aromatic rings.

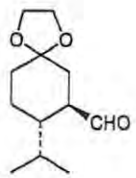


Scheme 5.5 Retrosynthetic possibilities for the concurrent synthesis of **266** and **267**

Formation of the bond **B** would require the generation of 3-lithiofuran to provide the nucleophilic synthon. In defining the absolute configuration of the penlanfurans, Mancini *et al.*[294] prepared the enantiomer of noroxopenlanfuran (**271**) by coupling (**272**) with 3-lithiofuran. However, on further deliberation, we eliminated this synthetic approach on the grounds of the reported instability and poor reactivity of 3-lithiofuran, which readily isomerizes to 2-lithiofuran.[289]



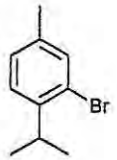
271



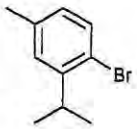
272

Conversely, as is also shown in Scheme 5.5, biaryl coupling to form the bond **A** could be achieved in two ways, depending on the polarity of each aromatic presursor. An initial attempt was made to follow the approach of Tanis *et al.*,[295] using the furyl moiety as the

nucleophile. However, this option entailed preparing the unstable furanyl bromide[296] (from 3-furanmethanol and PPh_3/Br_2) to provide the Grignard reagent which would react with an appropriate aryl halide. In our hands the bromination of 3-furanmethanol proved to be messy and was soon abandoned in favour of 3-furaldehyde, which could serve as the electrophile in the reversed, more usual polarity, bond formation reaction. Thus, it was only necessary to make the aryl bromides (**273**) and (**274**) from which the required benzene nucleophile could be derived by metallation in each case.



273



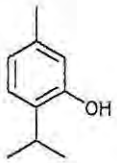
274

5.4 Preparation of 3-bromo-*p*-cymene (273)

Aryl bromination provides access to a wide range of useful synthetic intermediates.[297] Unfortunately, it is often hampered by interfering side reactions, such as bromination of alkyl substituents which takes place in the presence of light and in the absence of a catalyst.[298] Use of a Lewis acid catalyst with molecular bromine generates an electrophilic bromonium species which attacks the electron-rich ring. However, regioselective ring bromination is often difficult to achieve in activated aromatic rings and mixtures of isomers inseparable by fractional distillation are readily obtained.[299]

A survey of the Beilstein literature revealed two syntheses of 3-bromo-*p*-cymene (**273**). The first synthesis, a 1934 preparation from readily available, inexpensive thymol (**275**) in one step using PBr_3/Br_2,[300] and the second, a surface mediated bromination reaction reported by Ranu *et al.*[301] The latter reaction, which combined *p*-cymene and molecular bromine, preadsorbed onto alumina, reportedly gave the 3-bromo isomer together with 10 % of the dibromo isomer (**276**).

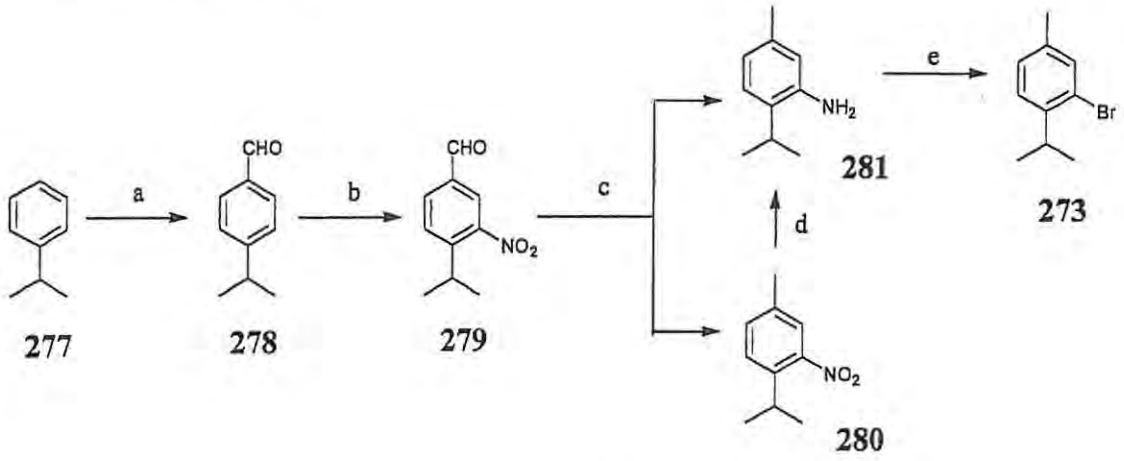
273



275

276

Regrettably, no NMR data for **273** were given in either reference, which was frustrating given our need to ensure that we started our syntheses of **266** and **267** with the correct brominated precursors. We therefore decided to synthesize **273** from cumene (**277**) *via* a much longer, but regiospecific, route (Scheme 5.6). Spectral data for **273** could then be obtained and used for comparison when investigating the products from direct bromination of **275** using the method of Ganguly and Le Fevre[300] which would hopefully yield a single bromo isomer.

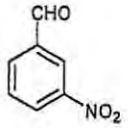


Scheme 5.6 A regiospecific route to 3-bromo-*p*-cymene (**273**)

Reagents and conditions: a. $TiCl_4/Cl_2CH.O.CH_3$, 0-40°C ; b. HNO_3/H_2SO_4, 0°C ; c. $H_2NNH_2.H_2O$, DEG, KOH, Δ; d. $SnCl_2.2H_2O$, conc HCl, Δ; e. i) HBr, $NaNO_2$, 0°C, ii) CuBr/HBr, < 10°C

As shown in Scheme 5.6, freshly distilled cumene (**277**) was formylated, nitrated and reduced to achieve the desired regiospecific substitution pattern on the benzene ring in 3-amino-*p*-cymene (**281**). Formylation[302] and nitration[303] were carried out following standard procedures. Simultaneous reduction of the aldehyde and nitro functionalities in (**279**) was initially attempted using the Huang-Minlon method.[304] Model reactions using *m*-nitrobenzaldehyde (**282**) were used to optimize the reaction conditions. Good yields (83 %) of *m*-toluidine (**283**) were eventually obtained after refluxing the reaction mixture for three hours. However, when these reaction conditions were applied repeatedly to **279**, the fully reduced compound (**281**) was only obtained as the minor product after extending the reflux period from three to six hours.

The major, partially reduced product, 3-nitro-*p*-cymene (**280**), was more easily reduced to **281** by refluxing for 35 min with granulated tin in conc. HCl.[305] Diazotization of the amine group, followed by bromination in a Sandmeyer reaction[306] gave **273** in low yield. Although the Sandmeyer reaction was not optimized, it is felt that the low yield was due partly to the apparent volatility of **273** during the concentration *in vacuo* of the relatively large amounts of solvent resulting from chromatography of the reaction mixture.



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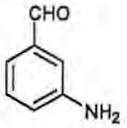

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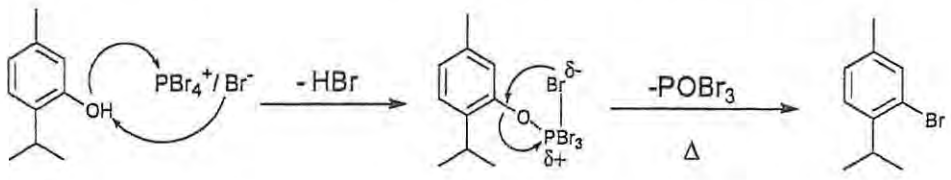
With spectral data for **273** in hand the bromination of thymol (**275**) was then investigated for a more convenient, larger scale production of **273** *via* the method of Ganguly and Le Fevre.[300] The addition of Br_2 to PBr_3 (both liquids) under rigorously anhydrous conditions resulted in the immediate formation of a yellow crystalline mass. Confirmation that this yellow solid was PBr_5 was provided by the successful substitution of PBr_5 purchased from Sigma® for PBr_3/Br_2 in the reaction. The bromination of thymol is an unusual reaction and the reaction mechanism is tentatively rationalised below in Scheme 5.7. In our proposed mechanism, PBr_3 and Br_2 react to form PBr_5, a crystalline solid which is known to exist as PBr_4^+/Br^-.[307] The electrophilic PBr_4^+ attacks the hydroxyl moiety of thymol to form the phosphonium ether (**284**) with concomitant release of HBr gas. The release of HBr gas was observed immediately on addition of the thymol to PBr_5 when a vigorous reaction accompanied by fuming occurred. Subsequent refluxing of the reaction mixture at high temperatures promotes simultaneous elimination of phosphorous oxybromide and attack of bromide at the deactivated 3 position on the ring.



287 **284** **273**

Scheme 5.7 A suggested mechanism for the bromination of thymol (**275**)

Our first attempts at this reaction were surprisingly successful. Steam distillation of the reaction mixture after a two hour reflux period, yielded a clean mixture of starting material and the target bromoisomer (**273**). Removal of the unreacted phenol by washing with KOH gave pure **273** in 53 % yield. It was felt that chromatography of the crude reaction mixture may be more efficient than steam distillation in smaller scale reactions. By-passing the steam-distillation also allowed the composition of the crude reaction mixture to be examined by ^{1}H NMR prior to chromatography. On all four occasions that the crude reaction mixture obtained from the bromination reaction was purified by chromatography, a preliminary ^{1}H NMR spectrum of the crude reaction mixture showed the presence, in varying proportions, of thymol (**275**), 3-bromo-*p*-cymene (**273**) and a third tri-substituted aromatic compound (Figure 5.2). Unfortunately, the latter compound could not be recovered from the Si gel, and was assumed to be the phosphonium-thyml ether (**284**) since it was more prevalent in reaction mixtures which had been refluxed in an oil bath on cold days when only 'gentle' refluxing (210°C as opposed to ≥ 240°C) was achieved.

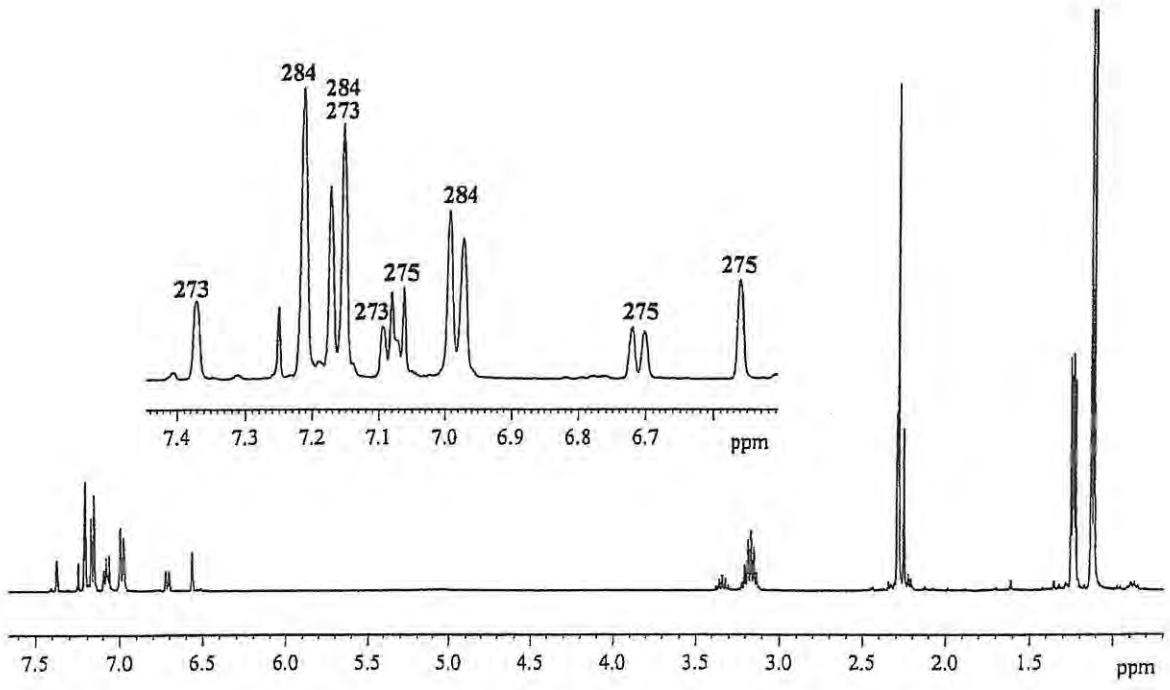
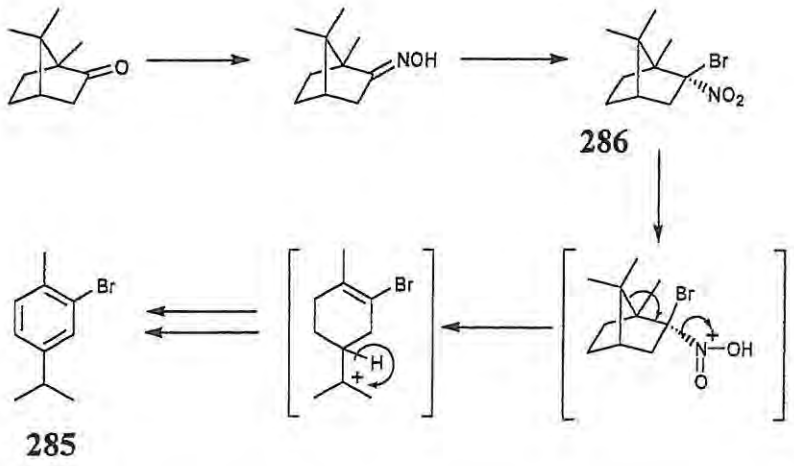


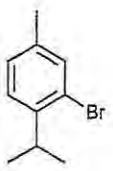
Figure 5.2 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of a crude bromination reaction mixture which was only 'gently' refluxed. Inset shows aromatic peaks assigned to 3-bromo-*p*-cymene (**273**), thymol (**275**) and the phosphonium ether (**284**)

Confirmation that the unknown compound was not 2-bromo-*p*-cymene (**285**), which should have eluted off the Si gel column in hexane along with **273**, was provided by a comparison of the ^{1}H NMR data for the former two compounds. An authentic sample of **285** had been prepared concurrently in our laboratory[308] *via* a regiospecific method involving the intriguing acid catalysed rearrangement of bromonitrocamphane (**286**, Scheme 5.8).[309] Therefore, it is clear that the bromination of **275** via the method of Ganguly and Le Fevre[300] is a selective route for the production of a single monobromo isomer.



Scheme 5.8 The regiospecific synthesis of 2-bromo-*p*-cymene (**285**) from camphor[309]
Reagents and conditions: a. EtOH, $NH_2OH.HCl/H_2O$, NaOH (s); b. KOH, KOBr, 0°C; c. conc H_2SO_4, hexane, >25°C

Interestingly, when only two, instead of four, equivalents of thymol were used, a mixture of **273** and the dibromo isomer **276** were obtained in a 2:1 ratio. Although the two isomers could be separated by reverse phase chromatography, low yields of both **273** and **276** were obtained from the large volumes of mobile phase (9:1 MeOH/water) necessary for reverse phase HPLC.



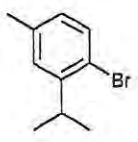
273

276

The presence of two bromine atoms in **276** was suggested from characteristic 1:2:1 abundance patterns of mass fragments (294, 292, 290 and 279, 277, 275) in the EIMS spectrum for this compound, and HRFABMS data confirmed a molecular formula of $C_9H_{10}Br_2$ for **276**. Six clearly resolved aromatic resonances in the ^{13}C NMR spectrum of **276** indicated an asymmetric substitution of the two bromine atoms in this compound. Placement of the two bromine atoms at C-2 and C-5, rather than C-3 and C-5, followed from the presence of two aromatic proton singlets in the ^{1}H NMR spectrum of **276**. With no spectral data in the recent chemical literature at hand, conclusive evidence for the 1,2,4,5 tetrasubstitution pattern around the benzene ring was provided by three bond HMBC correlations from the protons of the methyl substituent (δ 2.32) to the brominated quartenary carbon C-2 (δ 124.1) and from the isopropyl methine proton (δ 3.26) to the brominated C-5 carbon (δ 122.6).

5.5 Preparation of 4-bromo-*m*-cymene (274)

The most obvious approach to the preparation of 4-bromo-*m*-cymene (**274**) was to use the by now familiar method of Ganguly and Le Fevre[300] to brominate the *meta*-substituted equivalent (**287**) of thymol (**275**). Unfortunately the lack of a ready supply of **287** necessitated its preparation from our limited store of potential starting materials. An alternative route which would allow rapid access to **274** was the direct propylation of *p*-bromotoluene.[309] However, the required propylene precursor for this reaction was not immediately available, and instead we attempted to prepare **287** by direct propylation of *p*-cresol (**288**) using di-isopropyl ether in the presence of a clay catalyst.



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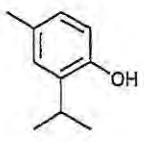

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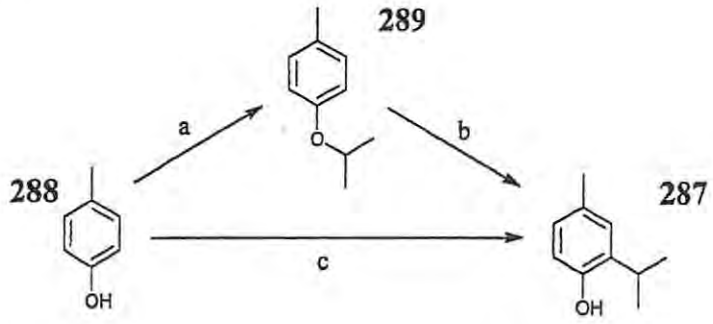
288

Our preliminary attempt at the latter reaction was unsuccessful. The method followed[311] somewhat surprisingly involved the addition of peroxide free di-isopropyl ether (boiling point 67°C) to a mixture of **288** and acid activated Fuller's Earth clay catalyst refluxing at

180°C. With hindsight we realized that the low yield reported by Carpenter and Easter[311] could be due in part to an inability to react all of the volatile ether with **288** because of the high temperature of the refluxing reaction mixture. Accordingly, the reaction was carried out by stirring the precombined catalyst, di-isopropyl ether and *p*-cresol (**288**) in a small autoclave (180°C) for two hours. However, an incalcitrant product mixture was again obtained, and it was felt that the problem lay either in the degree of activation of the Fuller's Earth or in the quality of the Fuller's Earth itself.

At this point we sought an alternative route to **287** and attempted to rearrange isopropyl-*p*-cresyl ether (**289**) (Scheme 5.9), in what was initially anticipated to be a simple, high yielding and much cleaner procedure[312] than the direct propylation reactions. A Williamson ether reaction using *p*-cresol and freshly prepared isopropyl bromide afforded **289** in 80 % yield.



Scheme 5.9 The two methods investigated for the propylation of *p*-cresol (**288**) to obtain **287**
Reagents and conditions: a. i) Na (s)/EtOH, ii) isopropyl bromide, Δ; b. $AlCl_3$, 0-50°C; c. diisopropyl ether, montmorillonite/50 % conc H_2SO_4, Δ

Rearrangement of the ether has been shown to be an intermolecular process involving scission of the isopropyl group by the $AlCl_3$, since the use of benzene as a solvent results in the formation of cumene and *p*-cresol.[312] Although we avoided the use of solvent, we found that treatment of the ether with $AlCl_3$ yielded only 7 % of the rearranged *ortho*-substituted *p*-cresol (**287**), the major alkali-soluble product being *p*-cresol (**288**). Surprisingly, the reaction mixture was described as a viscous solution in the literature.[312] In our hands, the very viscous mixture readily solidified to form a glassy gum, hampering

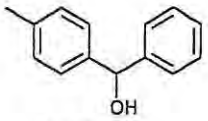
efficient stirring even when a large mechanical stirrer was used. Therefore, we thought it probable that our high yield of **288** in this rearrangement reaction resulted from the cleavage of unreacted ether by the concentrated hydrochloric acid added during the work-up to complete the hydrolysis of the $AlCl_3$ complex.

In the mean time, given the awkwardness of the $AlCl_3$-catalyzed rearrangement procedure, we turned our attention once more to the direct propylation of *p*-cresol (**288**),[311] substituting the Fuller's Earth catalyst with light kaolin, which was similarly activated with a few drops of conc. H_2SO_4. A ^1H NMR spectrum of the crude kaolin reaction mixture showed that **287** was present in about 11% yield. This suggested that the problem lay with the clay catalyst, and prompted us to try yet another catalyst, montmorillonite clay. Montmorillonite was a much more active catalyst, but mixtures of products were still obtained and vacuum distillation of these mixtures always resulted in impure fractions, with the desired product appearing in two distillate fractions. Reverting to a more efficient distillation technique, pure **287** (bp 114°C, 112 mmHg, lit.[311] 82°C, 3 mmHg) was the final fraction obtained from a spinning band distillation column using a Cartesian diver for precise control of the applied vacuum. This rather laborious distillation method was very successful, being repeated twice with perfect reproducibility of distilling temperatures and pressures. NMR spectra showed the three distillates collected to be pure. The first and second distillate fractions obtained were cumene (**277**) and *p*-cresol (**288**) respectively. Undoubtedly, improved yields of **287** could be obtained for the propylation reaction by optimizing the activation of the montmorillonite clay with H_2SO_4. However, since enough **287** had been obtained for our purposes, we decided to move on and obtained **274** in 54 % yield using the bromination procedure of Ganguly and Le Fevre[300] as described ealier (5.3) for the bromination of thymol (**275**).

5.6 Biaryl coupling of 3-bromo-*p*-cymene (273) with 3-furaldehyde

At the start of the synthesis, model coupling reactions had been performed on *p*-bromotoluene with firstly benzaldehyde and secondly 3-furaldehyde. High yields were obtained using a mole equivalent of *n*-BuLi to provide the nucleophilic lithiated toluene

which then attacked the aldehyde to give the biaryl secondary alcohols (**290**) and (**291**) respectively.[313] In contrast, coupling of 3-furaldehyde with *p*-tolyl magnesium bromide in a Grignard reaction[314] yielded a complex mixture of products. Consequently we focussed our attention on the use of organolithium reagents as a means of generating the nucleophilic aryl system, even though **273** proved far too sterically hindered for successful lithiation to take place under the same conditions as were used for *p*-bromotoluene.



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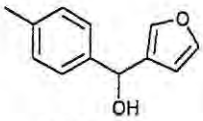

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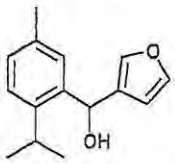
The reactivity of organolithium reagents, as a function of the covalent character of the C-Li bond, is related to the structure of the alkyl group and the co-ordinating ability of the solvent.[315] THF is usually the solvent of choice because *n*-BuLi is less stable and therefore more reactive in THF than in diethyl ether.[315] Even so, aggregation of the organolithium molecules in THF due to the covalency of the C-Li bond may be considerable with a concomittant reduction in reactivity of *n*-BuLi. That this was a problem in our system was confirmed when addition of the Lewis base DABCO to the reaction mixture resulted in a 24 % yield of (**292**).



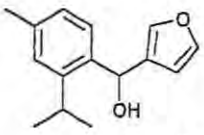
292

It is assumed that additives such as DABCO complex with the lithium reagent, increasing the polarity and therefore the carbanionic character of the C-Li bond, as well as reducing polymeric associations of the organolithium molecules.[315] Although the reaction was repeated using other Lewis bases such as hexamethyl phosphoramide (HMPA) and 1,3-dimethyltetrahydro-2(1H)-pyrimidinon as additives, it was only with tetramethyl ethylene diamine (TMEDA, 1 equivalent) that a similar yield of **292** (27 %) was obtained. Fortuitously, when the amount of TMEDA added was doubled, a doubling of the yield of

coupled product (54 %) was observed, and very little of the brominated starting material was recovered. However, the use of four and then six equivalents of TMEDA produced no further increase in the yield of **292**. Branched chain alkyllithium compounds are the reagents of choice for several similar coupling reactions reported in the literature.[316,317] and should be more reactive than *n*-BuLi due to less efficient aggregation of the branched chain molecules. However, the replacement of *n*-BuLi with *t*-BuLi, in the presence of two equivalents of TMEDA, on a single occasion resulted in a slightly reduced yield of **292** (22 %), estimated from the ^{1}H NMR spectrum of the reaction mixture. From these results we could conclude that the yield of **292** was more significantly influenced by the amount of TMEDA present than the type of organolithium reagent used.

5.7 Biaryl coupling of 4-bromo-*m*-cymene (274) with 3-furaldehyde

As soon as we had achieved some success in preparing **292** *via* lithiation of **273** in the presence of TMEDA, we undertook the preparation of (**293**) from 4-bromo-*m*-cymene (**274**). The latter bromo compound was reacted with *n*-BuLi, in the presence of TMEDA (1 equivalent) followed by 3-furaldehyde. Considering the steric bulk of the isopropyl group, it was expected that the yield of the coupling reaction using 4-bromo-*m*-cymene (**274**) would be the same as that obtained when 3-bromo-*p*-cymene (**273**) was used (27 %). However, a slightly higher isolated yield (32%) of **293** was obtained from **274**. This yield was of interest to us after a coupling of 2-bromo-*p*-cymene[308] (**285**) with 3-furaldehyde gave an unexpectedly low yield (15 %) of (**294**).



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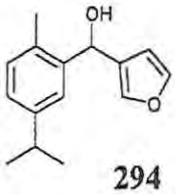


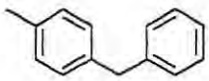
294

In the latter reaction lithiation takes place *meta* to the isopropyl group and *ortho* to the methyl substituent of **285**. In contrast, the structure of 4-bromo-*m*-cymene (**274**) requires lithiation to take place *ortho* to the isopropyl group and *para* to the methyl group. The relatively low yield obtained using **285** would suggest that the combined *ortho/para-*

inductive effect of the isopropyl and methyl groups is important in enhancing lithiation and the steric bulk of the isopropyl group may not hinder halogen-lithium exchange as much as was initially expected. Needless to say, the coupling reaction would have to be repeated several times with each of the bromo-cymene compounds to confirm this initial observation before conclusive deductions concerning the mechanism of lithiation could be made.

5.8 Reductive dehydroxylations of 292 and 293

Reductive dehydroxylation of **292** and **293** to afford **266** and **267** respectively, initially presented a problem in that the instability of the furan ring precluded the use of the usual acid-catalysed or refluxed reactions.[317,318] Thus, although dehydroxylation of the model compound **290** using triethylsilane and tri-fluoroacetic acid afforded (**295**),[317] an attempt to repeat the reaction using model compound **291**, to yield (**296**), resulted in the degradation of this compound. Similarly, attempted removal of the hydroxyl group *via* tosylation, was unsuccessful.



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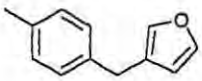

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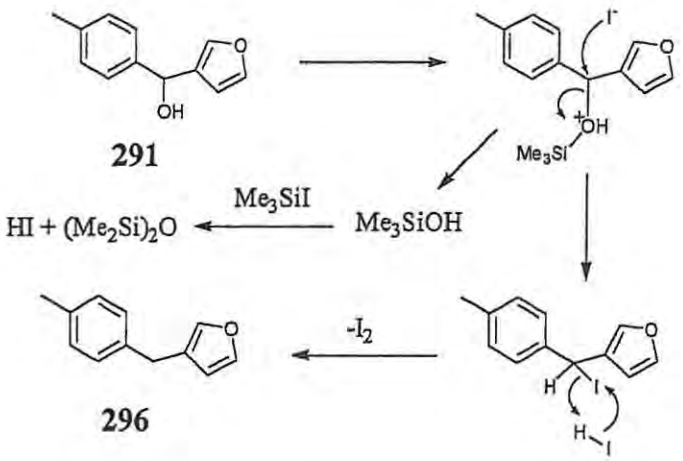
Success was finally achieved by following the benzylic reductive dehydroxylation method of Perry *et al.*[319] who reported near quantitative yields of dehydroxylated 2-furyl biaryl compounds obtained under mild, neutral conditions using iodotrimethylsilane. Iodotrimethylsilane is generated *in situ* from chlorotrimethylsilane and sodium iodide. The mechanism, as described by Perry *et al.* (Scheme 5.10), involves removal of the hydroxyl through formation and cleavage of the silyl ether. The resulting silyl alcohol reacts with further iodotrimethylsilane releasing HI which sequentially iodates and deiodates the biaryl moiety to afford the reduced product with the concomitant release of molecular iodine.



Scheme 5.10 Mechanism for the reductive benzylic dehydroxylation of **291** with iodotrimethylsilane (adapted from Perry *et al.*[319]).

The model compound **296** was obtained in a 90 % yield from **291**, and subsequent application of Perry *et al.*'s protocol to **292** gave **266** in quantitative yield. An exact match of the ^{1}H (Figure 5.3) and ^{13}C NMR data for the synthetic compound **266** with those of the isolated tsitsikammafuran established the identity of the natural product, not surprisingly, as the aromatic analogue of penlanfuran.

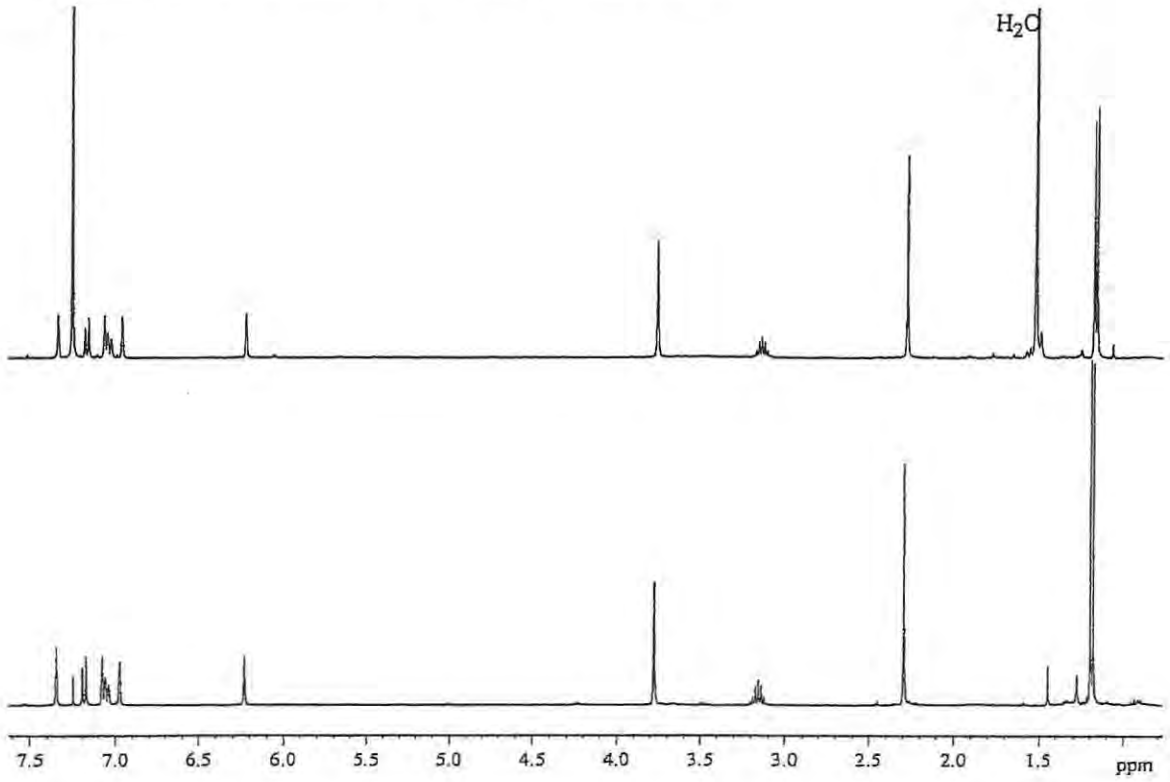


Figure 5.3 ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of isolated (upper) and synthesized (lower) tsitsikammafuran (**266**)

The NMR data of the alternative isomer **267** proved similar to that of **266**. The ^{13}C NMR data for **266** and **267** showed only slight differences (≤ 4 ppm, Table 6.2) in the chemical shifts of corresponding benzene ring carbons (C-1 to C-6) of each compound. However, the two compounds may be distinguished from their ^{1}H NMR spectra (Figure 5.4) due to the shielding effect that the furan ring has on the benzene ring protons. In compound **266** the benzene ring H-2 proton (δ 6.95, s) is adjacent to the [(furan-3-yl)methyl] substituent and consequently resonates upfield of aromatic protons H-5 and H-6 (δ 7.17 and 7.03 respectively). In the ^{1}H NMR spectrum of the 4-[(furan-3-yl)methyl] substituted compound **267**, the proton singlet of H-2 (δ 7.09), which is positioned between the methyl and isopropyl substituents, is downfield of the relatively shielded doublets of H-5 (δ 7.03) and H-6 (δ 6.93). The ^{1}H and ^{13}C NMR data for a third regioisomer (**297**) are also presented for comparison (Figure 5.4 and Table 6.2 respectively) and the intermediate chemical shifts of the benzene ring protons of **297** further illustrate the relative shielding effects of the [(furan-3-yl)methyl], methyl and isopropyl substituents in these compounds. In compound **297**, the isolated proton is shielded by the adjacent furan moiety as in **266**. However, the isopropyl group provides less shielding for H-3 in **297** than is provided by the methyl group for H-2 in **266**. Compound **297** was prepared in the same manner as **266** and **267**, by the reaction of 2-bromo-*p*-cymene (**285**) with 3-furaldehyde and subsequent dehydroxylation of the coupled product **294**, as part of a project to compare the feeding deterrent properties of **266** and its regioisomers.[308] The potential agrochemical and biomedical properties of **266**, **267**, **292-294** and **297** are currently under investigation.

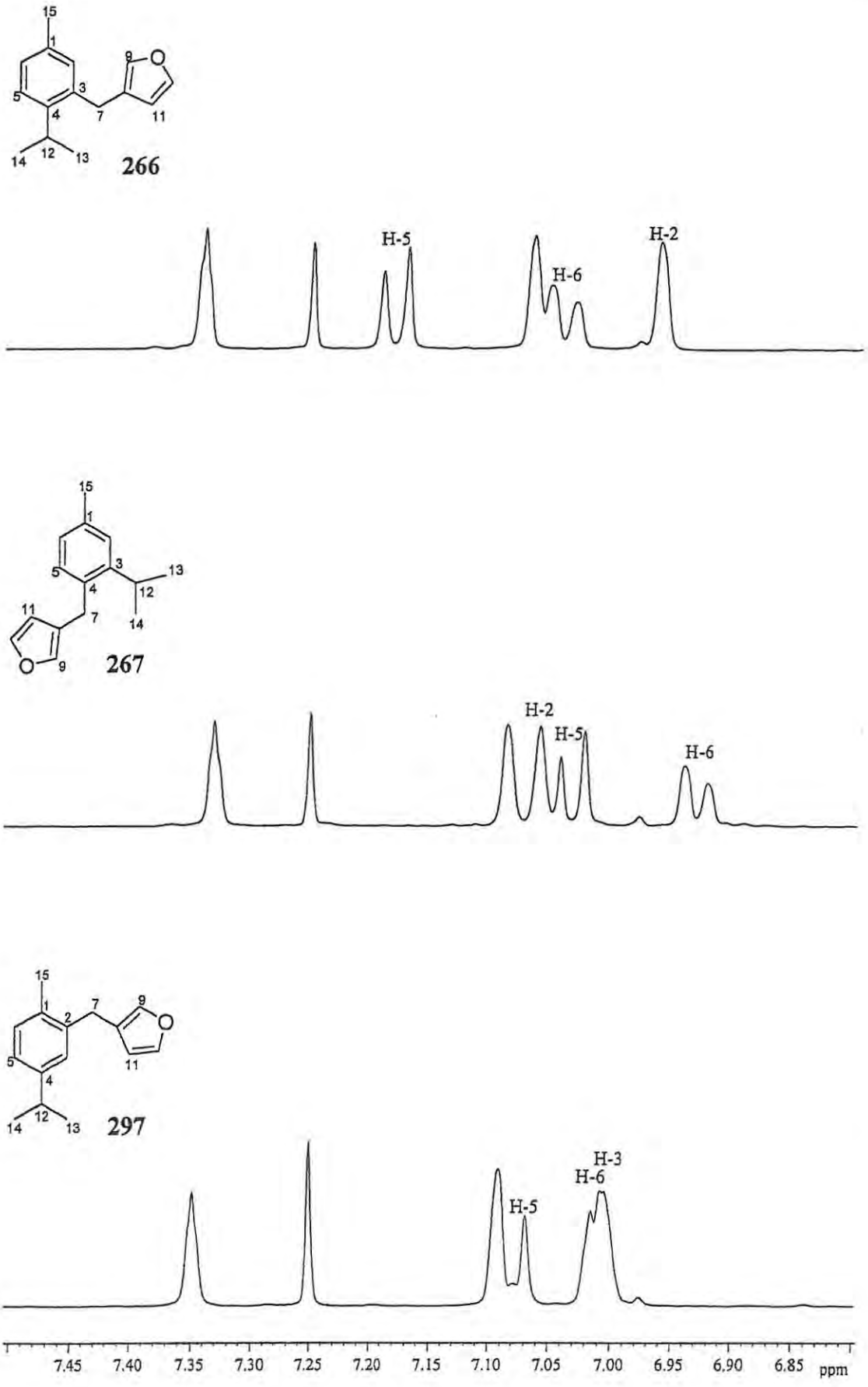


Figure 5.4 Aromatic regions of the ^{1}H NMR spectra ($CDCl_3$, 400 MHz) for compounds **266**, **267** and **297** showing the relative chemical shifts of the benzene ring protons.

Table 5.1 ^{13}C ($CDCl_3$, 100 MHz) and ^{1}H ($CDCl_3$, 400 MHz) NMR data for compounds **266**, **267** and **297**

	266		267		297	
	δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)	δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)	δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)
1	135.1 (s)		133.7 (s)		133.5 (s)	
2	130.5 (d)	6.95 (s)	126.1 (d)	7.09 (s)	138.1 (s)	
3	136.6 (s)		146.5 (s)		127.4 (d)	7.01 (s)
4	143.8 (s)		136.2 (s)		146.6 (s)	
5	125.3 (d)	7.17 (d, 8)	129.7 (d)	7.03 (d, 8)	130.2 (d)	7.08 (d, 8)
6	127.6 (d)	7.03 (d, 8)	126.4 (d)	6.93 (d, 8)	124.3 (d)	7.01 (br d)
7	28.4 (t)	3.76 (s)	28.0 (t)	3.76 (s)	29.0 (t)	3.72 (s)
8	124.8 (s)		124.9 (s)		123.8 (s)	
9	139.6 (d)	7.06 (s)	139.5 (d)	7.06 (s)	139.6 (d)	7.09 (s)
10	142.9 (d)	7.33 (s)	142.8 (d)	7.33 (s)	142.9 (d)	7.35 (s)
11	111.2 (d)	6.21 (s)	111.2 (d)	6.21 (s)	111.3 (d)	6.23 (s)
12	28.4 (d)	3.13 (sept, 7)	28.7 (d)	3.14 (sept, 7)	33.7 (d)	2.84 (sept, 7)
13	23.9 (q)	1.17 (d, 7)	23.8 (q)	1.18 (d, 7)	24.1 (q)	1.21 (d, 7)
14	23.9 (q)	1.17 (d, 7)	23.8 (q)	1.18 (d, 7)	24.1 (q)	1.21 (d, 7)
15	20.9 (q)	2.27 (s)	21.2 (q)	2.32 (s)	18.9 (q)	2.25 (s)

We were able to establish appropriate GC-MS conditions for tsitsikammafuran (**266**) in preparation to screen individual *H. capensis* specimens for the presence of this *Dysidea* sponge metabolite. However, unfortunately time constraints and the distance of our collection site in the Tsitsikamma National Park, which is often made inaccessible by highly erratic sea surface conditions, prevented the collection of further *H. capensis* specimens. Therefore, we were unable to establish whether *H. capensis* sequesters tsitsikammafuran from its prey *Dysidea* sponges together with nakafuran-8 (**223**) and –9 (**224**).

In conclusion, it is interesting that the South African *Dysidea* species contained both the bridged bicyclic nakafurans and the tethered bicyclic penlanfuran analogue. While *Dysidea fragilis* from Hawaii yielded only the nakafurans, the same sponge species from Brittany contained only penlanfuran. The latter mutually exclusive occurrence of the two structural classes caused the classification of the Hawaii and Brittany sponges as the same species to be questioned.[284] Here, it is evident that these different furanosesquiterpenes should not be used as chemotaxonomic markers, since it is possible for a single species to possess the enzymes required for both biogenetic pathways.

Chapter Six

Experimental

6.1 General Experimental Procedures

The ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded using standard pulse sequences on a Bruker AMX400 spectrometer from 1997 to September 1999, and on a Bruker Avance 400 spectrometer from September 1999 to May 2000. Chemical shifts are reported in ppm and referenced to residual undeuterated solvent resonances. Coupling constants have been recorded directly from the NMR spectra and corresponding coupling constants have not been matched. Optical rotations were measured on a Perkin-Elmer 141 polarimeter at the sodium-D line (589 nm). Following standard protocol, the concentration is expressed in g/100 mL. Infra-red data were obtained on a Perkin Elmer Spectrum 2000 FT-IR spectrometer with compounds as films (neat) on NaCl discs. LRMS were recorded on a Hewlett-Packard 5988A or a Finnigan GCQ spectrometer at 70 eV. HREIMS and HRFABMS were obtained by Dr. P. Boshoff and Prof. L. Fourie of the Mass Spectrometry Units at the Cape Technikon, Cape Town, and the University of Potchefstroom, Potchefstroom respectively. The X-ray crystallographic analysis was carried out by Drs D. Eggleston and R. Copley of Smithkline Beecham U.K. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected.

All solvents were distilled before use. Normal phase semipreparative HPLC separations were performed on a Whatman Magnum 9 Partisil 10 column using a Spectra-Series P100 isocratic pump and a Waters 410 Differential Refractometer. Reverse phase semipreparative HPLC separations were performed on a Phenomenex Luna 10 μ C18 column using a Spectra-Physics IsoChrom LC pump and a Waters R401 Differential Refractometer. Analytical normal phase thin layer chromatography (TLC) was performed on DC-Alufolien Kieselgel $60F_{254}$ plates and analytical reverse phase TLC was performed on DC-Ferigplatten $RP18F_{254}s$ plates. The plates were viewed under UV light (254 nm) and were developed by spraying with 10 % H_2SO_4 in MeOH followed by heating. Column chromatography was performed using Kieselgel 60 (230-400 mesh) silica.

Where a reaction temperature is not stated, the reaction was carried out at room temperature. All reactions requiring anhydrous conditions were conducted in flame-dried apparatus under an atmosphere of dry nitrogen or using an anhydrous calcium chloride guard tube. Dry solvents were stored over the appropriate drying agent under an atmosphere of dry nitrogen. Prior to their use in dry reactions, diethyl ether and tetrahydrofuran were distilled from sodium metal/benzophenone ketyl and dichloromethane was distilled from calcium hydride. Organic extracts were dried over anhydrous sodium or magnesium sulfate. All reactions were magnetically stirred unless otherwise stated.

6.2 Chapter Two Experimental

6.2.1 Collection and extraction of *Aplysia parvula*.

In February and March 1997, two collections of the sea hare *Aplysia parvula* (length 2-3 cm) were made, by hand using SCUBA (-6 m), in the Tsitsikamma National Park (34°01' S, 23°54' E), on the South East Cape coast of South Africa. A voucher specimen of *A. parvula* is retained in the Rhodes University marine invertebrate collection (APLY97.004). In each case, the specimens collected (numbering 19 and 30 respectively), were stored in acetone for one week before the concentrated acetone extract was partitioned between EtOAc and water. The dried and concentrated EtOAc phases of the two acetone extracts (91.6 mg and 144.2 mg respectively) gave identical ^{1}H NMR spectra and therefore were combined (235.8 mg) and applied to an LH20 column (10 g sephadex, 1:1 $CHCl_3$/MeOH). The column fractions were combined according to their TLC profiles to afford three main fractions. Fraction 3 was filtered in hexane and the hexane soluble portion was subjected to normal phase HPLC (hexane) to yield (3*Z*)-bromofucin (**120**, 3.6 mg, < 0.1 mg/animal).

(3*Z*)-bromofucin (129): white solid; $[\alpha]^{24}_{D}$ –0.5° (*c* 0.25, $CHCl_3$); IR ν_{max} 3291, 2126, 1490, 1174, 1139, 1009, 963 and 881 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2.1; EIMS *m/z* (rel. int.) 319 (26), 277 (26), 194 (35), 121 (22), 109 (47), 105 (22), 71 (33); 69 (28), 53 (43), 43 (100); HREIMS *m/z* 389.9838 (calcd for $C_{15}H_{20}{}^{79}Br_2O_2$, 389.9830).

6.2.2 Collection and extraction of *Aplysia dactylomela*.

Four large specimens of *A. dactylomela* (16-20 cm) were collected by hand from the intertidal zone at Cape Recife Nature Reserve, Algoa Bay on the Eastern Cape coast of South Africa in March 1998 and identified by Ms S. Kuiters of the Zoology Department, University of Port Elizabeth. Of this collection, one was a green color variant in which the predominant background color was pale green. The other three specimens had a background color that was predominantly red brown. A voucher specimen of *A.*

dactylomela is retained in the Rhodes University marine invertebrate collection (APLY98.001).

The pale green *A. dactylomela* specimen was dissected and the digestive gland removed and placed in acetone for one week, while the digestive glands of the three red specimens were separately dissected out and placed together in acetone, also for one week. The two acetone extracts were worked up separately and initially partitioned between EtOAc and water. The EtOAc partition fraction of the green color variant was concentrated (927 mg) and subjected to Si gel column chromatography (hexane, hex/EtOAc 9:1, 8:2, 7:3, 1:1, EtOAc). The crude chromatography fractions were combined according to their TLC profiles to give eleven main fractions which were monitored by ^{1}H NMR spectroscopy and the standard brine shrimp assay.[139] Algoane (**121**, 59.0 mg, 6.6 % calculated from mass of concentrated EtOAc partition layer) crystallized slowly as large needles from main fraction 4 (8:2 hex/EtOAc). A further 15 mg of algoane was obtained from normal phase HPLC (8:2 hex/EtOAc) of the remainder of main fraction 4. Normal phase HPLC (19:1 hex/EtOAc) of the nonpolar, bioactive main fraction 2 yielded 1-deacetoxy-8-deoxyalgoane (**123**, 19 mg, 2.1 %), and two impure fractions, which, after further HPLC, afforded nidificene (**125**, 6 mg, 0.7 %, hexane) and prepacifenol epoxide (**101**, 11 mg, 1.2 %, 9:1 hex/EtOAc).

The concentrated EtOAc partition fraction (3.05 g) from the three red color variants was similarly chromatographed on Si gel and the test tube fractions collected were monitored by TLC and combined accordingly to afford nine main fractions which were subjected to the standard brine shrimp assay. Once again, algoane (**121**, 158.3 mg) crystallized spontaneously from a bioactive crude fraction (main fraction 3). The remainder of main fraction 3 (333.4 mg) was reapplied to Si gel to afford six further crude fractions. The second of these fractions yielded 1-deacetoxyalgoane (**122**, 16.7 mg, 5.6 mg/animal) after normal phase HPLC (9:1 hex/EtOAc), while normal phase HPLC (7:3 hex/EtOAc) of the third crude fraction yielded further algoane (**121**, 23.8 mg), pacif-7-enediol (**104**, 15.1 mg, 5.0 mg/animal) and ibhayinol (**124**, 4.9 mg, 1.6 mg/animal).

Algoane (121): colorless prisms (hex/EtOAc, mp 188-192°C) $[\alpha]^{22}_{D}$ +51° (*c* 0.60, $CHCl_3$); IR ν_{max} 3368, 1721, 1373, 1232, 1074, 1034, cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2.2; EIMS *m/z* (rel. int.) 315 (10), 253 (20), 188 (35), 173 (34), 161 (26), 109 (92), 108 (69), 69 (45), 43 (100); HRFABMS *m/z* 489.0043 (calcd for $C_{17}H_{27}{}^{79}Br_2ClO_4$, 489.0042).

1-Deacetoxyalgoane (122): colorless prisms (hex/EtOAc, mp 152-155°C); $[\alpha]^{22}_{D}$ +18° (*c* 0.40, $CHCl_3$); IR ν_{max} 3220, 2973, 1460, 1387, 1075, 994, 785 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2.2; HRFABMS *m/z* 430.9987 (calcd for $C_{15}H_{25}{}^{79}Br_2ClO_2$, 430.9988).

1-Deacetoxy-8-deoxyalgoane (123): colorless prisms (hex/EtOAc, mp 138-142°C); $[\alpha]^{22}_{D}$ +23° (*c* 1.15, $CHCl_3$); IR ν_{max} 3618, 2976, 1463, 1386, 1008, 781 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2.2; HRFABMS *m/z* 415.0034 (calcd for $C_{15}H_{25}{}^{79}Br_2ClO$, 415.0039).

Ibhayinol (124): white amorphous powder; $[\alpha]^{22}_{D}$ +9° (*c* 0.57, $CHCl_3$); IR ν_{max} 3437, 2956, 1460, 1387, 1093, 1060 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2.3; HRFABMS *m/z* 351.0725 (calcd for $C_{15}H_{24}{}^{79}BrClO_2$, 351.0726).

Prepacifenol epoxide (101): colorless prisms; mp 95-97°C; $[\alpha]^{22}_{D}$ +110° (*c* 0.73, $CHCl_3$); IR ν_{max} 3568, 2978, 1447, 1387, 1089, 805 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2.4; EIMS *m/z* (rel. int.) 319 (26), 277 (26), 194 (35), 121 (22), 109 (47), 105 (22), 71 (33); 69 (28), 53 (43), 43 (100); HRFABMS *m/z* 441.9552 (calcd for $C_{15}H_{21}{}^{79}Br_2ClO_3$, 441.9541).

Nidificene (125): white amorphous powder; $[\alpha]^{22}_{D}$ +27° (*c* 1.18, $CHCl_3$); IR ν_{max} 2971, 1638, 1451, 1092, 905, 866 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 2.4; EIMS *m/z* (rel. int.) 396 (12), 319 (32), 283 (43); 281 (44), 201 (56), 109 (100), 95 (70), 93 (41), 69 (83); HRFABMS *m/z* 395.9858 (calcd for $C_{15}H_{23}{}^{79}Br_2Cl$, 395.9856).

Pacif-7-enediol (104): white crystals; mp 168-170°C; $[\alpha]^{22}_{D}$ -34° (*c* 0.34, $CHCl_3$); IR ν_{max} 3437, 2925, 1644, 1472, 1091, 1033, 911 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.92 (3H, s, H_3-12), 1.32 (3H, s, H_3-13), 1.75 (3H, s, H_3-15), 2.10 (1H, t, *J* = 14 Hz, H-5), 2.25 (1H, dd, *J* = 3, 14 Hz, H-5), 2.27 (1H, t, *J* = 14 Hz, H-2), 2.41 (1H, br s, O<u>H</u>), 2.62 (1H, dd, *J* = 5, 15 Hz, H-2), 4.10 (1H, s, H-9), 4.34 (1H, dd, *J* = 2, 13 Hz, H-4), 4.60 (1H, s, H-8), 4.86 (1H, dd, *J* = 5, 13 Hz, H-1), 5.16 (1H, s, H-14), 5.35 (1H, s, H-14) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.4 (q, C-12), 25.5 (q, C-13), 31.4 (q, C-15), 32.6 (t, C-5), 45.9 (t, C-2), 49.0 (s, C-11), 53.7 (s, C-6), 58.7 (d, C-4), 68.9 (s, C-3), 76.3 (d, C-8), 77.2 (d, C-1), 83.2 (d, C-9), 112.4 (s, C-10), 114.7 (t, C-14), 148.8 (s, C-7) ppm.

8-Acetoxyalgoane (132): compound **121** (5.8 mg), Ac_2O (1.0 mL) and dry pyridine (0.5 mL) were left to stand for 16 hours and the solvents removed under reduced pressure to yield **132** as a pale yellow solid: $[\alpha]^{22}_{D}$ +6° (*c* 0.58, $CHCl_3$); IR ν_{max} 3544, 1743, 1372, 1219 1035, 756 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.94 (3H, s, H_3-12), 1.08 (3H, s, H_3-14), 1.40 (3H, s, H_3-13), 1.74 (3H, s, H_3-15), 1.97 (1H, m, H-9), 2.01 (1H, m, H-5), 2.07 (3H, s, H_3-17), 2.15 (3H, s, H_3-19), 2.57 (1H, m, H-2), 2.64 (1H, t, *J* = 13 Hz, H-5), 2.80 (1H, dd, *J* = 3, 15 Hz, H-2), 3.06 (1H, p, *J* = 8 Hz, H-9), 4.11 (1H, t, *J* = 9 Hz, H-10), 4.32 (1H, br s, O<u>H</u>), 4.89 (1H, dd, *J* = 4, 9 Hz, H-4), 4.89 (1H, br s, H-8), 5.09 (1H, d, *J* = 2 Hz, H-1) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.5 (q, C-14), 21.5 (q, C-17), 21.5 (q, C-19), 21.9 (q, C-12), 23.3 (q, C-13), 27.4 (q, C-15), 41.2 (t, C-5), 41.4 (t, C-9), 41.5 (t, C-2), 47.5 (s, C-11), 53.3 (s, C-7), 59.3 (d, C-4), 59.9 (d, C-10), 69.5 (s, C-3), 73.9 (d, C-1), 78.6 (s, C-6), 83.0 (d, C-8), 168.0 (s, C-18), 168.6 (s, C-16) ppm; HRFABMS *m/z* 531.0148 (calcd for $C_{19}H_{29}{}^{79}Br_2ClO_5$, 531.0149).

Pacif-7-ene diacetate (136): compound **104** (2.0 mg), Ac_2O (0.2 mL) and dry pyridine (0.5 mL) were left to stand for 16 hours and the solvents removed under reduced pressure to yield **136** as a yellow solid: IR ν_{max} 2969, 1745, 1355, 1227, 1048, 1029, 1010 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.95 (3H, s, H_3-12), 1.33 (3H, s, H_3-13), 2.09 (3H, s, CH_3CO), 2.11 (3H, s, CH_3CO), 2.13 (1H, m, H-5), 2.24 (1H, dd, *J* = 2, 14 Hz, H-5), 2.28

(1H, m, H-2), 2.64 (1H, dd, J = 5, 14 Hz, H-2), 4.26 (1H, dd, J = 2, 13 Hz, H-4), 4.70 (1H, dd, J = 5, 13 Hz, H-1), 5.21 (1H, s, H-14), 5.26 (1H, s, H-9), 5.51 (1H, s, H-14), 5.59 (1H, s, H-8) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.3 (q, C-12), 21.0 (q, $\underline{C}H_3CO$), 21.3 (q, $\underline{C}H_3CO$), 25.1 (q, C-13), 31.5 (q, C-15), 32.5 (t, C-5), 45.8 (t, C-2), 49.5 (s, C-11), 53.9 (s, C-6), 58.3 (d, C-4), 69.0 (s, C-3), 74.7 (d, C-8), 77.4 (d, C-1), 79.5 (d, C-9), 105.5 (s, C-10), 117.5 (t, C-14), 143.9 (s, C-7), 168.4 (s, $CH_3\underline{C}O$), 169.3 (s, $CH_3\underline{C}O$) ppm; HRFABMS *m/z* 526.982998 (calcd for $C_{19}H_{26}{}^{79}Br_2ClO_5$, 526.983549).

X-ray diffraction of 121: Large crystals of **121** were grown via the slow evaporation of an EtOAc/hex solution. A single crystal was cleaved several times to obtain appropriate dimensions for X-ray analysis and flash cooled in a stream of N_2 gas to 150(2) K. Lattice parameters were determined from the setting angles of 25 reflections well distributed in reciprocal space and measured using a Nonius MACH3 diffractometer. Intensity data were collected using graphite monochromated copper radiation and an ω-2θ variable scan speed technique. Three check reflections were monitored to assess any crystal movement during the experiment. The intensities of these three reflections were measured every hour of exposure time and showed a variation of 10.5%. Data were corrected for this variation and for Lorentz and polarization effects. An absorption correction was applied using psi-scan data. The structure was solved and refined using the SHELXTL package.[320] Atomic co-ordinates and anisotropic displacement parameters were refined for the nonhydrogen atoms. Co-ordinates were refined for the hydroxyl hydrogen atoms, with O-H distances being restrained to 0.84(2)Å. The remaining hydrogen atoms were included in idealized positions, either riding on the carbon atoms to which they were attached or, for the methyl hydrogen atoms, being treated as rigid, rotating groups. For all hydrogen atoms, isotropic displacement parameters were assigned as an appropriate multiple (either 1.2 or 1.5) of U(eq) for the atom to which they were bonded. The full-matrix least-squares refinement (on F^2) of 229 variables converged ($\Delta/\sigma_{max} < 0.001$) to values of the conventional crystallographic residuals R1 = 0.0365 (*wR2* = 0.0965) for 3733 observed data with $I \geq 2\sigma(I)$ and R1 = 0.0372 (*wR2* = 0.1008) for all data. The

goodness-of-fit was 1.078. The function minimized was $\Sigma w(F_o^2 - F_c^2)^2$. Weights, w, were assigned to the data as $w = 1/[\sigma^2 (F_o^2) + (0.0579P)^2 + 2.2595P]$ where $P = [\max(F_o^2,0) + 2F_c^2]/3$. A final difference Fourier map showed residual density between 0.603 e $Å^{-3}$ and –0.609 e $Å^{-3}$. The absolute configuration was assigned on the basis of the absolute structure parameter,[57] which refined to a value of 0.02(2). Values of the neutral atom scattering factors and real and imaginary dispersion corrections were taken from the *International Tables for X-ray Crystallography.*[321]

Crystal data:[322] colorless block; 0.50 x 0.43 x 0.37 mm; orthorhombic; space group $P2_12_12_1$ (no. 19); unit cell dimensions a = 8.2536(5) Å, b = 11.4458(7) Å, c = 21.063(2) Å, V = 1989.8(2) $Å^3$; Z = 4; d_{calc} = 1.638 Mg m^{-3}; μ (Cu K_α, λ = 1.54178Å) = 6.554 mm^{-1}.

6.3 Chapter Three Experimental

6.3.1 Invertebrate material

Nineteen specimens of the arminacean nudibranch *Leminda millecra* were collected in October 1998 from Algoa Bay by hand using SCUBA equipment (-20 to 40m). A further thirteen specimens were similarly collected from Algoa Bay in February 1999. Both of these collections were made in collaboration with the CRRF who were making large scale marine invertebrate collections in Algoa Bay at these times. GC, GC-MS and LC analyses were performed on Rhodes University octocoral voucher specimens from both CRRF collections and crude octocoral extracts provided by the National Cancer Institute (USA) from the 1998 CRRF collection. In March 2000, eight *L. millecra* specimens, and three species of gorgonians (*Leptogorgia* sp., *L. palma* and an *Acabaria* species) upon which they were found, were collected from two sites (-18m and -23m) at White Sands in Algoa Bay for GC analysis. A voucher specimen of *L. millecra* is retained in the Rhodes University marine invertebrate collection (KUPE98.012).

6.3.2 Extraction and Isolation of *Leminda millecra* metabolites

The acetone extracts of the nudibranch specimens from the 1998 and 1999 collections were worked up separately and initially partitioned between EtOAc and water. The two concentrated EtOAc partition fractions obtained gave identical ^{1}H NMR spectra, and therefore were combined (1.78 g) and subjected to chromatography on a Si gel column using gradient elution (hexane, hex/EtOAc 8:2, 1:1, and EtOAc). Fractions collected were combined according to their TLC profiles to give seven main fractions, of which fractions 1, 3 and 4 were selected for further purification due to their interesting ^{1}H NMR spectra. As shown in Scheme 3.1, further chromatography of fraction 1 afforded millecrone A (**142**, 93 mg, 2.9 mg/animal) and isofuranodiene (**149**, 11.0 mg, 0.3 mg/animal). Fraction 3 afforded millecrone B (**143**, 19 mg, 0.6 mg/animal), algoafuran (**150**, 1.5 mg, 0.05 mg/animal), cubebenone (**151**, 129 mg, 4.0 mg/animal), 8-hydroxycalamenene (**152**, 16 mg, 0.5 mg/animal), **153** (9.2 mg), **154** (8.2 mg), **157** (3.1 mg, 0.1 mg/animal) and **158** (8.2 mg, 0.3 mg/animal). Fraction 4 afforded more of

compounds **153** (19.3 mg, 0.9 mg/animal in total) and **154** (6.4 mg, 0.5 mg/animal in total), together with the hydroquinones **155** (154.7 mg, 4.8 mg/animal), **156** (9.8 mg, 0.3 mg/animal), **158** (8.3 mg, 0.3 mg/animal) and **159** (3.6 mg, 0.1 mg/animal).

Millecrone A (142): colorless oil; $[\alpha]^{21}_{D}$ +41° (*c* 0.60, $CHCl_3$); IR ν_{max} 2939, 1694, 1615, 1463, 1190, 1088, 942, cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.68 (3H, d, *J* = 7 Hz, H_3-12), 0.82 (3H, d, *J* = 7 Hz, H_3-13), 1.08 (3H, s, H_3-15), 1.29 (5H, m, H_2-2, H-3, H-10, H-11), 1.42 (1H, m, H-10), 1.54 (1H, m, H-9), 1.69 (1H, m, H-3), 1.83 (1H, m, H-9), 2.64 (2H, m, H-8, H-4), 2.44 (1H, d, *J* = 6 Hz, H-5), 2.42 (1H, m, H-8), 5.13 (1H, t, *J* = 2 Hz, H-14), 5.88 (1H, d, *J* = 2 Hz, H-14) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7 (q, C-13), 21.6 (q, C-12), 23.8 (t, C-9), 25.2 (q, C-15), 27.9 (t, C-3), 29.6 (t, C-8), 34.0 (d, C-11), 35.3 (t, C-10), 41.9 (t, C-2), 45.7 (d, C-4), 46.0 (s, C-1), 62.4 (d, C-5), 121.4 (t, C-14), 146.0 (s, C-7), 201.8 (s, C-6) ppm; EIMS *m/z* (rel. int.) 220 (3), 178 (14), 177 (100), 149 (15), 121 (11), 109 (13), 93 (15), 81 (13), 79 (13); HRFABMS *m/z* 221.190521 (calcd for $C_{15}H_{25}O$, 221.190541).

Millecrone B (143): colorless oil; $[\alpha]^{20}_{D}$ +155° (*c* 0.65, $CHCl_3$); IR ν_{max} 2954, 1651, 1455, 1376, 1316, 1286, 990 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.69 (1H, m, H-7), 0.81 (1H, br t, *J* = 11 Hz, H-6), 1.02 (3H, d, *J* = 7 Hz, H_3-14), 1.05 (3H, s, H_3-12), 1.16 (3H, s, H_3-13), 1.40 (1H, m, H-3), 1.76 (3H, s, H_3-15), 2.20 (1H, m, H-4), 2.33 (2H, m, H-2, H-8), 1.86 (1H, m, H-3), 2.63 (2H, m, H-2, H-5), 2.78 (1H, dd, *J* = 5, 15 Hz, H-8) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.9 (q, C-15), 15.4 (q, C-14), 15.9 (q, C-13), 22.9 (d, C-7), 25.6 (s, C-11), 28.2 (q, C-12), 31.3 (d, C-6), 32.3 (t, C-3), 34.0 (t, C-2), 37.2 (d, C-4), 41.9 (t, C-8), 45.2 (d, C-5), 130.3 (s, C-10), 166.1 (s, C-1), 200.8 (s, C-9) ppm; EIMS *m/z* (rel. int.) 218 (21), 203 (100), 176 (34), 175 (50), 161 (51), 147 (31), 121 (59), 119 (31), 105 (43), 91 (54); HREIMS *m/z* 218.1677 (calcd for $C_{15}H_{22}O$, 218.1669).

Isofuranodiene (149): colorless crystals; IR ν_{max} 2927, 1767, 1439, 1385, 1137, 1080, 851, 755 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.27 (3H, s, H_3-15), 1.59 (3H, s, H_3-14),

1.78 (1H, td, J = 5, 12 Hz, H-7), 1.92 (3H, d, J = 1 Hz, H_3-13), 2.12 (2H, m, H_2-8), 2.23 (1H, dt, J = 3, 12 Hz, H-7), 3.07 (2H, d, J = 7 Hz, H_2-4), 3.43 (1H, d, J = 16 Hz, H-11), 3.53 (1H, d, J = 16 Hz, H-11), 4.74 (1H, t, J = 7 Hz, H-5), 4.93 (1H, dd, J = 6, 11 Hz, H-9), 7.06 (1H, s, H-1) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.9 (q, C-13), 16.2 (q, C-15), 16.5 (q, C-14), 24.3 (t, C-4), 26.8 (t, C-8), 39.5 (t, C-7), 40.9 (t, C-11), 118.9 (s, C-3), 121.9 (s, C-2), 127.6 (d, C-5), 128.9 (s, C-6), 129.0 (d, C-9), 134.3 (s, C-10), 136.0 (d, C-1), 149.7 (s, C-12) ppm; HRFABMS *m/z* 216.151437 (calcd for $C_{15}H_{20}O$, 216.151415).

Algoafuran (150): colorless oil; IR ν_{max} 2930, 1739, 1371, 1234, 1023, 894 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.71 (2H, m, H_2-4'), 2.00 (3H, s, H_3-6), 2.07 (3H, s, H_3-12'), 2.24 (4H, t, J = 8 Hz, H_2-3', H_2-5'), 4.98 (2H, s, H_2-10'), 5.01 (2H, d, J = 18 Hz, H_2-9'), 5.04 (H, d, J = 17 Hz, H-8'), 5.22 (1H, d, J = 18 Hz, H-8'), 6.10 (1H, s, H-3), 6.16 (1H, s, H-1'), 6.36 (1H, dd, J = 18 Hz, H-7'), 7.13 (1H, s, H-5) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 9.6 (q, C-6), 21.0 (q, C-12'), 26.7 (t, C-4'), 31.0 (t, C-5'), 35.6 (t, C-3'), 63.3 (t, C-10'), 112.5 (d, C-3), 113.3 (t, C-8'), 115.9 (t, C-9'), 118.0 (d, C-1'), 121.5 (s, C-4), 134.6 (s, C-2'), 138.8 (d, C-5), 138.8 (d, C-7'), 146.0 (s, C-6'), 151.8 (s, C-2), 171.1 (s, C-11') ppm; HRFABMS *m/z* 274.156705 (calcd for $C_{17}H_{22}O_3$, 274.156895).

Cubebenone (151): yellow oil; $[\alpha]^{23}_D$ +126° (*c* 0.67, $CHCl_3$); IR ν_{max} 1694, 1607, 1447, 1377, 1323, 1247, 1028, 872, 831, 610 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 3.1; EIMS *m/z* (rel. int.) 218 (26), 203 (33), 175 (31), 161 (25), 147 (32), 136 (100), 121 (58), 105 (35), 91 (32); HRFABMS *m/z* 219.174802 (calcd for $C_{15}H_{23}O$, 219.174891).

8-Hydroxycalamenene (152): yellow oil; $[\alpha]^{22}_D$ +36° (*c* 0.53, $CHCl_3$); IR ν_{max} 3437, 1619, 1579, 1464, 1287, 1240, 1165, 1030, 974, 904, 842 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 3.1; EIMS *m/z* (rel. int.) 218 (79), 176 (73), 175 (100), 160 (33), 159 (28), 147 (34), 121 (20); HREIMS *m/z* 218.1679 (calcd for $C_{15}H_{22}O$, 218.1671).

2-(9-Keto-3, 7, 11-trimethyl-2, 6-dodecadienyl)-5-methyl-*p*-quinone (153): bright yellow oil; λ_{max} (MeOH) nm (log ε) 252 (3.76); IR ν_{max} 1712, 1656, 1614, 1445, 1366, 1284, 1238, 1133, 909 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.89 (6H, d, J = 7 Hz, H_3-12, H_3-13), 1.60 (3H, s, H_3-14), 1.61 (3H, s, H_3-15), 2.02 (3H, s, H_3-7'), 2.09 (1H, t, J = 7 Hz, H-11), 2.07 (2H, m, H_2-4), 2.16 (2H, m, H_2-5), 2.28 (2H, d, J = 7 Hz, H_2-10), 3.02 (2H, s, H_2-8), 3.10 (2H, d, J = 7 Hz, H_2-1), 5.15 (1H, t, J = 7 Hz, H-2), 5.21 (1H, t, J = 7 Hz, H-6), 6.48 (1H, s, H-3'), 6.58 (1H, d, J = 1 Hz, H-6') ppm; ^{13}C NMR data see Table 3.2; EIMS *m/z* (rel. int.) 342 (8), 215 (27), 190 (36), 189 (21), 175 (69), 151 (18), 137 (20), 121 (22), 85 (50), 57 (100), 41 (33); HREIMS *m/z* 342.2198 (calcd for $C_{22}H_{30}O_3$, 342.2193).

2-(9-Keto-3, 7, 11-trimethyl-2, 7-dodecadienyl)-5-methyl-*p*-quinone (154): bright yellow oil; λ_{max} (MeOH) nm (log ε) 250 (3.45); IR ν_{max} 1682, 1657, 1615, 1445, 1384, 1238, 1134, 909 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.90 (6H, d, J = 7 Hz, H_3-12, H_3-13), 1.56 (2H, m, H_2-5), 1.62 (3H, s, H_3-15), 1.85 (3H, d, J = 1 Hz, H_3-14), 2.01 (3H, d, J = 2 Hz, H_3-7'), 2.08 (2H, t, J = 7 Hz, H_2-4), 2.11 (1H, m, J = 7 Hz, H-11), 2.25 (2H, d, J = 7 Hz, H_2-10), 2.51 (2H, t, J = 8 Hz, H_2-6), 3.10 (2H, d, J = 7 Hz, H_2-1), 5.15 (1H, t, J = 7 Hz, H-2), 6.02 (1H, s, H-8), 6.49 (1H, d, J = 2 Hz, H-3'), 6.57 (1H, d, J = 2 Hz, H-6') ppm; ^{13}C NMR data see Table 3.2; EIMS *m/z* (rel. int.) 342 (12), 205 (18), 187 (13), 176 (16), 175 (100), 149 (20), 109 (11), 95 (13), 81 (10); HRFABMS *m/z* 343.227298 (calcd for $C_{22}H_{31}O_3$, 343.227321).

2-(9-Keto-3, 7, 11-trimethyl-2, 6-dodecadienyl)-5-methyl-*p*-quinol (155): orange oil; λ_{max} (MeOH) nm (log ε) 295 (3.27); IR ν_{max} 3396, 1698, 1420, 1368, 1192, 1003, 872 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.89 (6H, d, J = 7 Hz, H_3-12, H_3-13), 1.63 (3H, s, H_3-14), 1.69 (3H, s, H_3-15), 2.11 (3H, m, H_2-4, H-11), 2.16 (3H, s, H_3-7'), 2.19 (2H, m, H_2-5), 2.31 (2H, d, J = 6 Hz, H_2-10), 3.03 (2H, s, H_2-8), 3.26 (2H, d, J = 7 Hz, H_2-1), 4.83 (1H, br s, O<u>H</u>-1'), 5.19 (1H, t, J = 6 Hz, H-6), 5.28 (1H, t, J = 7 Hz, H-2), 5.72 (1H, br s, O<u>H</u>-4'), 6.54 (1H, s, H-3'), 6.56 (1H, s, H-6') ppm; ^{13}C NMR data see Table 3.2; EIMS

m/z (rel. int.) 344 (59), 244 (27), 215 (21), 189 (41), 177 (29), 176 (23), 175 (100), 161 (13), 137 (30); HRFABMS *m/z* 344.235138 (calcd for $C_{22}H_{32}O_3$, 344.235145).

2-(9-Keto-3, 7, 11-trimethyl-2, 7-dodecadienyl)-5-methyl-*p*-quinol (156): orange oil; λ_{max} (MeOH) nm (log ε) 294 (3.19), 232 (4.31); IR ν_{max} 3369, 1667, 1603, 1420, 1380, 1192, 873 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.92 (6H, d, *J* = 7 Hz, H_3-12, H_3-13); 1.61 (2H, m, H_2-5); 1.66 (3H, s, H_3-15); 1.90 (3H, s, H_3-14); 2.14 (1H, m, H-11); 2.16 (2H, m, H_2-4); 2.17 (3H, s, H_3-7'); 2.29 (2H, d, *J* = 7Hz, H_2-10); 2.68 (2H, t, *J* = 8 Hz, H_2-6); 3.27 (2H, d, *J* = 8 Hz, H_2-1); 4.54 (1H, s, O<u>H</u>-4'); 5.35 (1H, t, *J* = 7 Hz, H-2); 6.08 (1H, s, H-8); 6.55 (1H, s, H-6'); 6.83 (1H, s, H-3'); 6.96 (1H, s, O<u>H</u>-1') ppm; EIMS *m/z* (rel. int.) 344 (54), 205 (30), 189 (86), 177 (33), 176 (35), 175 (100), 137 (31), 133 (29), 91 (27), 81 (38); ^{13}C NMR data see Table 3.2; HRFABMS *m/z* 344.235132 (calcd for $C_{22}H_{32}O_3$, 344.235145).

5-(9-Keto-3, 7, 11-trimethyl-2, 7-dodecadienyl)-2-methyl-4-acetophenol (157): yellow oil; λ_{max} (MeOH) nm (log ε) 281 (3.52), 242 (4.11); IR ν_{max} 3369, 1760, 1667, 1607, 1445, 1368, 1214, 1180, 1010, 915 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.92 (6H, d, *J* = 7Hz, H_3-12, H_3-13), 1.61 (3H, s, H_3-15), 1.63 (2H, m, H_2-5), 1.64 (1H, m, H-11), 1.92 (3H, s, H_3-14), 2.17 (2H, m, H_2-4), 2.19 (3H, s, H_3-7'), 2.27 (3H, s, H_3-9'), 2.30 (2H, d, *J* = 7 Hz, H_2-10), 2.72 (2H, br t, *J* = 9 Hz, H_2-6), 3.13 (2H, d, *J* = 8 Hz, H_2-1), 5.32 (1H, t, *J* = 7 Hz, H-2), 6.10 (1H, s, H-8), 6.73 (1H, s, H-6'), 7.01 (1H, s, H-3'), 8.13 (1H, s, O<u>H</u>-4') ppm; ^{13}C NMR data see Table 3.2; EIMS *m/z* (rel. int.) 386 (18), 344 (30), 227 (100), 189 (51), 175 (44), 149 (45), 111 (44), 95 (65), 81 (56), 67 (43); HRFABMS *m/z* 386.245669 (calcd for $C_{24}H_{34}O_4$, 386.245711).

Chromenol (158): orange oil; λ_{max} (MeOH) nm (log ε) 331 (3.11), 267 (3.68), 222 (4.43); IR ν_{max} 3404, 1702, 1459, 1368, 1178, 1005, 919, 873 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.88 (6H, d, *J* = 7 Hz, H_3-12, H_3-13), 1.34 (3H, s, H_3-15), 1.57 (3H, s, H_3-14), 1.68 (2H, dm, H_2-4), 2.10 (2H, sept, H_2-5), 2.17 (3H, s, H_3-7'), 2.26 (2H,d, *J* = 7 Hz, H_2-

10), 2.98 (2H, s, H_2-8), 4.41 (1H, br s, O<u>H</u>-4'), 5.23 (1H, t, *J* = 7 Hz, H-6), 5.51 (1H, d, *J* = 10 Hz, H-2), 6.24 (1H, d, *J* = 10 Hz, H-1), 6.41 (1H, s, H-3'), 6.54 (1H, s, H-6') ppm; ^{13}C NMR data see Table 3.2; EIMS *m/z* (rel. int.) 342 (12), 325 (12), 300 (16), 299 (48), 283 (18), 281 (34), 227 (25), 225 (25), 211 (25), 209 (27); HRFABMS *m/z* 342.219402 (calcd for $C_{22}H_{30}O_3$, 342.219495).

2-(9-Hydroxy-3, 7, 11-trimethyl-2, 6-dodecadienyl)-5-methyl-*p*-quinol (159): orange oil; λ_{max} (MeOH) nm (log ε) 294 (3.24); IR ν_{max} 3369, 1421, 1381, 1191, 872 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.93 (6H, d, *J* = 7 Hz, H_3-12, H_3-13), 1.22 (1H, m, H-10), 1.25 (1H, s, O<u>H</u>-9), 1.49 (1H, m, H-10), 1.64 (3H, s, H_3-14), 1.67 (3H, s, H_3-15), 1.82 (1H, sept, *J* = 7 Hz, H-11), 1.96 (1H, dd, *J* = 3, 10 Hz, H-8), 2.16 (3H, s, H_3-7'), 2.18 (3H, m, H_2-4, H-5), 2.21 (1H, dm, *J* = 16 Hz, H-8), 2.29 (1H, pent, *J* = 8 Hz, H-5), 3.26 (2H, d, *J* = 7 Hz, H_2-1), 3.76 (1H, bm, H-9), 4.86 (1H, bs, O<u>H</u>-1'), 5.24 (1H, m, H-6), 5.27 (1H, m, H-2), 5.29 (1H, br s, O<u>H</u>-4'), 6.45 (1H, s, H-3'), 6.57 (1H, s, H-6') ppm; ^{13}C NMR data see Table 3.2; EIMS *m/z* (rel. int.) 344 (10), 326 (13), 278 (8), 241 (8), 213 (9), 189 (8), 176 (16), 175 (100), 105 (10), 91 (11); HRFABMS *m/z* 346.250742 (calcd for $C_{22}H_{34}O_3$, 346.250795).

6.3.3 Hydrogenation of cubebenone (151)

Hydrogenation of **151** (9.0 mg) over Pd-C catalyst (20 mg) in the usual manner gave **162** as a yellow oil; $[\alpha]^{20}_D$ +65° (*c* 0.35, $CHCl_3$); IR ν_{max} 1719, 1456, 1370, 1256, 1023, 870 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.49 (1H, qd, *J* = 2, 12 Hz, H-2), 0.82 (1H, qd, *J* = 2, 13 Hz, H-3), 0.87 (3H, d, *J* = 6 Hz, H_3-14), 0.92 (3H, d, *J* = 7 Hz, H_3-13), 0.97 (3H, d, *J* = 7 Hz, H_3-12), 1.04 (1H, m, H-4), 1.07 (1H, t, *J* = 4 Hz, H-10), 1.12 (3H, d, *J* = 7 Hz, H_3-15), 1.43 (1H, dd *J* = 2, 13 Hz, H-3), 1.61 (1H, m, *J* = 7 Hz, H-11), 1.67 (1H, t, *J* = 4 Hz, H-5), 1.68 (1H, d, *J* = 12 Hz, H-2), 1.73 (1H, q, *J* = 9 Hz, H-7), 2.13 (1H, q, *J* = 9 Hz, H-7), 2.43 (1H, m, H-6) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.2 (q, C-15), 19.9 (q, C-12/13), 19.9 (q, C-14), 20.2 (q, C-13/12), 25.3 (d, C-1), 26.7 (t, C-3), 27.1 (d, C-10), 29.6 (d, C-6), 30.6 (t, C-2), 33.6 (d, C-11), 37.6 (d, C-5), 40.9 (t, C-7), 44.7 (d, C-4), 45.0 (s,

C-9), 215.9 (s, C-8) ppm; EIMS *m/z* (rel. int.) 220 (39), 177 (34), 149 (59), 135 (44), 109 (30), 97 (41), 57 (52), 55 (69), 43 (94), 41 (100); HREIMS *m/z* 220.1818 (calcd for $C_{15}H_{24}O$, 220.1826).

6.3.4 Attempted degradation of cubebenone (151)

GC analyses were performed on a Hewlett Packard 6890C FID gas chromatograph using a J&W Scientific DB-1 capillary column (0.25 x 30 m) and front inlet and detector temperatures of 250°C. The split ratio used was 30:1 and the injection volume was 1 μL. GC analysis of a sample of cubebenone (**151**, 0.1 $mgmL^{-1}$, retention time 21.50 min) which had been stored at –20°C for 10 months revealed the presence of a trace amount (2 %) of 8-hydroxycalamenene (**152**, retention time 24.92 min) in the sample. A portion of the same sample (2.5 mg) was sealed in a 1 mL glass ampoule and heated in a hot block for a total of six hours. After being heated for two hours at 135°C, GC analysis of the sample showed no relative increase in the amount of **152**. The temperature was raised to 150°C and two further GC injections of the sample were made after four and six hours. The GC oven temperature profile used was as follows: 5 min at 40°C, 10°C/min increment to 150°C, 5 min at 150°C, 5°C/min increment to 180°C.

6.3.5 Derivatization of cubebenone (151)

Reduction of 151: A large excess of sodium borohydride (200 mg, 5.3 mmol) in absolute EtOH (5 mL) was added to a solution of **151** (85 mg, 0.4 mmol) in absolute EtOH (5 mL). The reaction mixture was stirred for seven hours, until no starting material could be detected by TLC. The reaction was quenched with water (2 mL) and the EtOH was removed *in vacuo*. The aqueous residue was then extracted with EtOAc (3 x 5 mL), dried and concentrated. The crude product (72 mg) obtained was subjected to normal phase HPLC (8:2 hex/EtOAc) to afford the two hydroxy diastereomers **164** (31 mg, 36 %) and **165** (10 mg, 11 %).

Cubeb-1-α-ol (164): colorless oil; $[\alpha]^{23}_D$ +28° (*c* 0.19, $CHCl_3$); IR ν_{max} 3339, 2953, 1459, 1372, 1055, 1019 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.52 (1H, qd, *J* = 2, 13 Hz), 0.78 (3H, m), 0.95 (12H, m), 1.12 (1H, br s), 1.39 (1H, m), 1.60 (4H, m), 1.93 (2H, m), 2.09 (1H, m), 4.35 (1H, br t, *J* = 8 Hz) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.5 (q), 18.1 (q), 18.8 (q), 20.0 (d), 20.2 (q), 27.2 (t), 27.6 (d), 31.6 (t), 32.7 (d), 33.8 (d), 33.9 (d), 38.4 (t), 39.2 (s), 44.1 (d), 75.0 (d) ppm; HRFABMS *m/z* 222.198328 (calcd for $C_{15}H_{26}O$, 222.198366).

Cubeb-1-β-ol (165): white crystals; $[\alpha]^{22}_D$ +19° (*c* 0.74, $CHCl_3$); IR ν_{max} 3339, 2947, 1450, 1369, 1032, 1008 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.32 (1H, s), 0.78 (1H, m), 0.88 (3H, d, *J* = 6 Hz), 0.93 (3H, d, *J* = 7 Hz), 1.00 (3H, d, *J* = 6 Hz), 1.09 (3H, d, *J* = 6 Hz), 1.15 (1H, m), 1.68 (1H, m), 2.03 (1H, m), 2.48 (1H, m), 4.06 (1H, d, *J* = 6 Hz) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.0 (q), 20.1 (q), 20.3 (q), 22.3 (d), 23.0 (q), 26.8 (t), 28.1 (d), 33.0 (d), 33.2 (t), 33.8 (d), 34.2 (d), 37.2 (s), 40.8 (t), 44.7 (d), 81.5 (d) ppm; HRFABMS *m/z* 222.125613 (calcd for $C_{15}H_{26}O$, 222.198366).

***p*-Bromobenzoylation of 164:** *p*-Bromobenzoyl bromide (137 mg, 0.52 mmol), dry benzene (1.5 mL), and dry pyridine (0.5 mL) were added to compound **164** (29 mg, 0.13 mmol) under anhydrous conditions and the mixture was stirred overnight. Water (3 mL) was added and the reaction mixture refluxed (75 min) in a water bath. After removal of the benzene *in vacuo*, the aqueous residue was extracted with $CHCl_3$ (3 x 5 mL) and the combined $CHCl_3$ layers were washed with HCl (5 %, 2 x 10 mL), $NaHCO_3$ (5 %, 2 x 10 mL) and water (10 mL) before being dried and concentrated. The crude product was filtered and purified by normal phase HPLC (hexane) to afford **163** (37 mg, 70 %) as a white solid; $[\alpha]^{23}_D$ +14° (*c* 0.87, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.55 (1H, q, *J* = 12 Hz), 0.92 (5H, m), 1.00 (12H, m), 1.07 (1H, m), 1.39 (1H, m), 1.60 (1H, m), 1.69 (1H, oct, *J* = 6 Hz), 1.82 (1H, *J* = 6 Hz, sept), 2.26 (2H, m), 5.57 (1H, t, *J* = 8 Hz), 7.56 (2H, dd, *J* = 1, 8 Hz), 7.87 (2H, dd, *J* = 1, 8 Hz) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.0 (d), 18.8 (q), 18.9 (q), 19.5 (q), 19.9 (q), 26.8 (t), 28.1 (d), 31.3 (t), 33.1 (d), 33.1 (d), 33.8 (d),

35.3 (t), 37.0 (s), 43.6 (d), 78.9 (d), 127.0 (s), 127.7 (s), 131.0 (d), 131.0 (d), 131.6 (d), 131.6 (d), 166.2 (s) ppm; HRFABMS *m/z* 404.135146 (calcd for $C_{22}H_{29}{}^{79}BrO_2$, 404.135091).

6.3.6 Extraction of samples for *Leminda millecra* and octocoral GC and LC analyses

The supernatants (70 % EtOH) of twenty eight octocoral voucher specimens from the October 1998 and February 1999 collections were concentrated *in vacuo*, and the aqueous residues were extracted with EtOAc (3 x 20 mL) and dried to yield between 2 and 25 mg of concentrated crude extract from each specimen. Eight specimens of *Leminda millecra* and three pieces of different gorgonians (*Leptogorgia* sp., *L. palma* and an *Acabaria* species) were extracted separately in acetone, and the resulting crude acetone extracts were partitioned between water and EtOAc (3 x 20 mL) and dried to afford between 2 and 30 mg of concentrated crude extract from each specimen.

6.3.7 GC analyses of pure compounds, octocoral and nudibranch extracts

GC analyses were performed on a Hewlett Packard 6890C FID gas chromatograph using a J&W Scientific DB-1 capillary column (0.25 x 30 m) and front inlet and detector temperatures of 250°C. The split ratio used was 15:1 and the injection volume was 1 μL.

Solutions (0.1 mg mL^{-1}) of each of the pure compounds **142** (20.17 min), **143** (25.25 min), **151** (21.50 min), **152** (24.92 min) and **158** (41.11 min), and a mixture of all five compounds, were analysed by GC to provide standards for the analysis of crude octocoral and nudibranch extracts. The oven temperature profile used for the analysis of all pure samples and crude extracts was as follows: 5 min hold at 40°C, 10°C/min increment to 150°C, 5 min hold at 150°C, 5°C/min increment to 280°C, 10 min hold at 280°C.

EtOAc solutions (0.5 mg mL^{-1}) of the twenty-eight octocoral voucher specimen extracts, eighteen organic octocoral extracts provided by the NCI, and extracts of the individual nudibranchs (x 8) and octocoral pieces collected (x 3) were filtered and qualitatively

analysed by GC for the presence of the standard compounds **142**, **143**, **151**, **152** and **158**. Voucher specimen sample OCDN 6385 contained millecrone A (**142**), as did NCI crude extracts of OCDN 6174 and OCDN 6176. Each of the eight *L. millecra* specimens collected individually, and the octocoral *Leptogorgia palma*, contained cubebenone (**151**), 8-hydroxycalamenene (**152**) and a third compound (retention time 18.33 min) which was later tentatively identified from a ^{1}H NMR spectrum of the *L. palma* extract as isofuranodiene (**149**).

6.3.8 GC-MS analyses of pure compounds, octocoral and nudibranch extracts

The crude extracts OCDN 6385, OCDN 6165 and OCDN 6169 thought to contain **142**, and the individual nudibranchs and *Leptogorgia palma* thought to contain **151** and **152** were subjected to GC-MS using a J&W Scientific DB-1 capillary column (0.25 x 30 m) to confirm the results of the GC analyses. To accommodate the effect of the MS vacuum on peak retention times, the oven temperature program used was altered as follows: 5 min at 40°C, 10°C/min increment to 150°C, hold 5 min at 150°C, 8°C/min increment to 280°C, hold 15 min at 280°C. A mass range of 50 to 440 Da was recorded every 1.00 s and the source and transfer line temperatures were maintained at 200°C and 275°C respectively. The mass fragment patterns of the peaks selected at 23.27, 24.28 and 27.58 min in the chromatograms of the crude extracts were consistent with those obtained for pure **142**, **151** and **152** respectively.

6.3.9 LC analyses of pure compounds, octocoral and nudibranch extracts

Farnesyl quinols **155** and **156** (0.1 mg mL^{-1}) were separately applied to a Wakosil II 5C18RS microbore column (1 x 150 mm) 7:3 MeOH/H_2O, flow rate 0.1 mL min^{-1}) in tandem with a SpectraSERIES UV100 detector to provide standards for the screening of the eighteen organic octocoral extracts from the NCI. Analysis of the eighteen extracts (0.2 mg mL^{-1}) under the same conditions led to further investigation of two extracts (OCDN 6004 and OCDN 6167). Coinjection of OCDN 6004 with compound **155** and of OCDN 6167 with **155** and **156** showed that neither of the samples contained the respective standards.

6.4 Chapter Four Experimental

6.4.1 Collection and extraction of *Chromodoris hamiltoni.*

Fifteen specimens of *Chromodoris hamiltoni* (length 2-4 cm) were collected by hand using SCUBA (-27 m) from Malangaan Reef off Ponto do Oura on the border of Mozambique and South Africa in September 1995 (voucher specimen MOZ95.005). The whole nudibranchs were stored in acetone for four months at ambient temperature before the supernatant was decanted and the specimens were re-extracted with further acetone. The acetone extracts were combined, concentrated and partitioned between EtOAc and water. Latrunculin B (**220**, 4.2 mg, 0.28 mg/animal) was obtained directly from normal phase HPLC (3:2 hex/EtOAc) of the crude EtOAc partition fraction. Further purification of a less polar HPLC fraction (7:3 hex/EtOAc) afforded 7β, 11β-diacetoxy-16-oxospongian-17-al (**221**, 2.1 mg, 0.14 mg/animal) and 7β, 11β-diacetoxy-16-oxospongi-12-en-17-al (**222**, 1.9mg, 0.13 mg/animal).

Latrunculin B (220): colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.94 (3H, d, J = 7 Hz, H_3-20), 1.90 (3H, s, H_3-19), 2.31 (2H, m, H_2-5), 2.66 (2H, m, H-4, H-8), 3.38 (1H, dd, J = 6, 12 Hz, H-17), 3.45 (1H, dd, J = 9, 12 Hz, H-17), 3.82 (2H, br t, J = 8 Hz, H-16, O<u>H</u>), 4.22 (1H, br t, J = 12 Hz, H-11), 5.04 (1H, t, J = 11 Hz, H-7), 5.24 (1H, td, J = 3, 11 Hz, H-6), 5.44 (1H, br s, H-13), 5.67 (1H, s, H-2), 5.74 (1H, br s, N<u>H</u>) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 22.2 (q, C-20), 23.9 (q, C-19), 26.9 (t, C-4/5), 28.7 (t, C-17), 28.9 (d, C-8), 31.0 (t, C-9/10), 31.2 (t, C-9/10), 31.5 (t, C-14), 35.3 (t, C-12), 35.8 (t, C-4/5), 61.4 (d, C-16), 62.5 (d, C-11), 68.7 (d, C-13), 97.9 (s, C-15), 117.8 (d, C-2), 127.4 (d, C-6), 135.8 (d, C-7), 154.5 (s, C-3), 165.3 (s, C-1), 174.8 (s, C-18) ppm; HRFABMS *m/z* 395.176584 (calcd for $C_{20}H_{29}NO_5S$, 395.176645).

7β, 11β-Diacetoxy-16-oxospongian-17-al (221): colorless oil; $[\alpha]^{21}_{D}$ = +38° (*c*, 0.19, $CHCl_3$); ^{1}H and ^{13}C NMR data see Table 4.1; EIMS *m/z* (rel int) 314 (6), 201 (5), 123 (9), 109 (19), 91(12), 69(15), 55 (12), 43 (100); HREIMS *m/z* 434.2287 (calcd for $C_{24}H_{34}O_7$, 434.2296).

7β, 11β-Diacetoxy-16-oxospongi-12-en-17-al (222): colorless oil; $[\alpha]^{21}_D$ = +97° (*c*, 0.18, $CHCl_3$); ^1H and ^{13}C NMR data see Table 4.1; EIMS *m/z* (rel int) 205 (5), 161(9), 133(9), 123(13), 109(25), 105(12), 95(11), 91(17), 81(13), 69(18), 43(100); HREIMS *m/z* 432.2153 (calcd for $C_{24}H_{32}O_7$, 432.2140).

6.4.2 Collection and extraction of *Glossodoris* sp. 4.

Nine specimens of *Glossodoris* sp. 4 were collected by hand using SCUBA (-10 m) from the Tsitsikamma National Park (34°01' S, 23°54' E) on the South East coast of South Africa in March 1998 (voucher specimen TSI98.006). The whole animals (length 1-2 cm) were immediately immersed in acetone and stored at –20°C for two months. After decanting the supernatant acetone, the nudibranchs were re-extracted with further acetone. The acetone extracts were combined, concentrated and partitioned between EtOAc and water. A single injection of the EtOAc partition fraction (7.1 mg) was made on normal phase HPLC (3:1 hex/EtOAc) and yielded **210** (2.1 mg, 0.23 mg/animal).

6α, 12β, 15α, 16α-Tetraacetoxyspongian (210): colorless oil; $[\alpha]^{23}_D$ = +38° (*c*, 0.17, $CHCl_3$), lit.[248] $[\alpha]_D$ +40° (*c*, 0. 7, $CHCl_3$); IR ν_{max} 3401, 2927, 1767, 1439, 1385, 1137, 1080, 1023, 851, 755 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.78 (1H, td, *J* = 2, 12 Hz, H-1), 0.86 (3H, s, H_3-18), 0.94 (3H, s, H_3-20), 1.03 (3H, s, H_3-19), 1.06 (3H, s, H_3-17), 1.21 (1H, br t, *J* = 11 Hz, H-3), 1.22 (1H, d, *J* = 12 Hz, H-9), 1.27 (1H, d, *J* = 11 Hz, H-5), 1.32 (1H, t, *J* = 11 Hz, H-7), 1.33 (1H, br d, *J* = 11 Hz, H-3), 1.39 (1H, br d, J = 13 Hz, H-2), 1.60 (1H, br t, J = 13 Hz, H-2), 1.62 (1H, t, J = 13 Hz, H-11), 1.63 (1H, br d, *J* = 12 Hz, H-1), 1.78 (1H, br d, *J* = 12 Hz, H-11), 2.04 (3H, s, $C\underline{H}_3CO$), 2.06 (3H, s, $C\underline{H}_3CO$), 2.08 (3H, s, $C\underline{H}_3CO$), 2.10 (3H, s, $C\underline{H}_3CO$), 2.14 (1H, d, *J* = 7 Hz, H-14), 2.15 (1H, d, *J* = Hz, H-7), 2.62 (1H, br t, *J* = 7 Hz, H-13), 5.14 (1H, d, *J* = 1 Hz, H-12), 5.22 (1H, td, *J* = 3, 11 Hz, H-6), 6.11 (1H, s, H-16), 6.13 (1H, d, *J* = 7 Hz, H-15) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.4 (q, C-20), 17.7 (q, C-17), 18.1 (t, C-2), 21.2 (q, $\underline{C}H_3CO$), 21.3 (q, $\underline{C}H_3CO$), 21.3 (q, $\underline{C}H_3CO$), 21.9 (q, $\underline{C}H_3CO$), 22.1 (q, C-18), 23.6 (t, C-11), 33.3 (s, C-4), 35.4 (s, C-8), 36.1 (q, C-19), 39.1 (s, C-10), 39.6 (t, C-1), 43.2 (t, C-3), 45.9 (d, C-13),

48.9 (t, C-7), 49.6 (d, C-9), 57.0 (d, C-14), 58.9 (d, C-5), 67.6 (d, C-12), 69.7 (d, C-6), 99.5 (d, C-16), 99.7 (d, C-15), 169.7 (s, $CH_3\underline{C}O$), 170.0 (s, $CH_3\underline{C}O$), 170.1 (s, $CH_3\underline{C}O$), 170.2 (s, $CH_3\underline{C}O$); HRFABMS *m/z* 403.248398 ($[M\text{-}2OAc]^+$, calcd for $C_{24}H_{35}O_5$, 403.248449).

6.4.3 Collection and extraction of *Hypselodoris capensis* and two dietary sponges, *Fasciospongia* sp. and *Dysidea* sp.

6.4.3.1 Invertebrate material

In February 1997, sixteen specimens of the nudibranch *Hypselodoris capensis* (length 1-3 cm) were collected using SCUBA (-10 m) in the Tsitsikamma National Park (34°01' S, 23°54' E) on the South East coast of South Africa (voucher specimen TSI97.001), together with a portion of the *Fasciospongia* sponge on which they were found. The sponge is massive, horizontally extended and lobate, with the larger oscules (3 mm diameter) along the raised ridges. Live coloration is a black-brown exterior with a lighter brown interior. It is compressible and spongy. The surface appears hispid when the fasciculated primary fibres penetrate the dermis. Primary fibres (65 μm in diameter) contain foreign inclusions (spicules) when near the surface, whereas the secondary fibres (30 μm in diameter) are most often clear. Specimens exude copious amounts of mucus soon after collection, and when dried, the skeleton is a reddish brown network of fibres. The sponge is an undescribed species of the genus *Fasciospongia* (Order Dictyoceratida, Family Thorectidae). A voucher specimen has been deposited at the South African Museum, Cape Town, South Africa (SAMA 24697).

In March 1998, we returned to the site of our initial collection of *Hypselodoris capensis* and its dietary *Fasciospongia* sponge in the Tsitsikamma National Park. Underwater observation of *H. capensis* specimens on a different sponge led us to collect a portion of this undulating white sponge. The sponge is typically horizontally extended, standing only 3-5 centimetres high. It has large oscula (2mm diameter) along raised ridges and is fairly hard but breakable. The bumpy conulose exterior is a dirty white colour in life and is covered with a layer of large sand grains. Internally, it's skeleton is a reticulation of

large fibres, heavily cored with sand grains. The sponge most likely represents a new species of the genus Dysidea (Family Dysididae, Order). A voucher specimen is held at Rhodes University (DYS98.005).

6.4.3.2 Extraction and Isolation

The nudibranchs were stored in acetone for one week, while the *Fasciospongia* sponge (302 g wet mass) was steeped in methanol over the same period. These initial extracts were partitioned between EtOAc and water to yield 309 mg and 953 mg of crude organic extract respectively. The nudibranch extract was chromatographed on a Si gel column eluted with a solvent gradient system of pure hexane, hex/EtOAc 9:1, 8:2, 6:4, 1:1 and pure EtOAc. Six crude fractions were obtained by combining column fractions with similar TLC profiles. Normal phase HPLC (hexane) was used to purify the least polar of these fractions which yielded nakafuran-8 (**223**) and -9 (**224**, 32 mg, 2 mg/animal). In attempting to further purify **223** using reverse phase HPLC (MeOH/H_2O 9:1), this labile compound degraded, preventing any further analysis. When subjected to normal phase HPLC, two of the more polar crude fractions yielded (18*R*)-variabilin (**195**, 12 mg, 0.75 mg/animal, 6:4 hex/EtOAc,), 22-deoxyvariabilin (**225**, 2.0 mg, 0.13 mg/animal, 9:1 hex/EtOAc) and 22-deoxy-23-hydroxymethylvariabilin (**226**, 1.7 mg, 0.11 mg/animal, 6:4 hex/EtOAc,). 380mg of the crude *Fasciospongia* sponge extract was subjected to normal phase HPLC (7:3 hex/EtOAc), from which furospinosulin-1 (**227**, 136 mg), **195** (105 mg) and **226** (5.6 mg) were obtained. Further HPLC purification (5:1 hex/EtOAc) of the third fraction obtained from the initial HPLC run yielded **225** (14.7 mg).

The freeze-dried *Dysidea* sponge (180 g) was stored in EtOAc for 3 days and the concentrated extract was partitioned between hexane and 10 % aqueous MeOH. The resulting hexane partition fraction (358 mg) was applied to 10 g Si gel in hexane. Repeated normal phase HPLC of the hexane column fraction (49 mg) using hexane (3.5 ml/min) followed by iso-octane (3 ml/min) yielded nakafuran-9 (**224**, 19 mg) and a 1:1 mixture (10 mg) of nakafuran-8 (**223**) and an unidentified, very similar, compound presumed to be an isomer of the latter compound.

Nakafuran-9 (224): colorless oil; λ_{max} (hexane) 219 nm (ε 4752); IR ν_{max} 2910, 1508, 1450, 1161, 1073, 1050 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.07 (3H, s, H_3-15), 1.37 (1H, td, J = 4, 13 Hz, H-5), 1.56 (3H, s, H_3-13), 1.58 (3H, s, H_3-14), 1.72 (1H, br d, J = 13 Hz, H-12), 1.79 (1H, dd, J = 4, 13 Hz, H-12), 1.92 (1H, br t, J = 13 Hz, H-5), 1.94 (1H, br d, J = 17 Hz, H-9), 2.23 (1H, td, J = 4, 16 Hz, H-4), 2.34 (1H, td, J = 4, 16 Hz, H-4), 2.37 (1H, dd, J = 5, 17 Hz, H-9), 3.16 (1H, br s, H-10), 6.05 (1H, d, J = 1 Hz, H-2), 7.11 (1H, d, J = 1 Hz, H-1) ppm; ^{13}C NMR data see Table 4.2; EIMS *m/z* (rel. int.) 216 (100), 201 (37), 174 (35), 173 (24), 161 (19), 145 (19), 131 (18), 122 (21), 120 (30), 109 (28), 91 (34), 77 (26), HREIMS *m/z* 216.1503 (calcd for $C_{15}H_{20}O$, 216.1514).

(18*R*)-Variabilin (195): colorless oil; $[\alpha]^{23}_D$ +36° (*c* 0.96, $CHCl_3$); IR ν_{max} 3660-2380, 2940, 1730, 1632, 1450, 1305, 1065 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.04 (3H, d, J = 7 Hz, H_3-19), 1.34 (2H, br m, H_2-17), 1.36 (2H, br m, H_2-16), 1.54 (3H, s, H_3-14), 1.57 (3H, s, H_3-9), 1.81 (3H, s, H_3-25), 1.93 (2H, m, H_2-15), 1.98 (2H, br d, J = 7 Hz, H_2-10), 2.05 (2H, br d, J = 7 Hz, H_2-11), 2.23 (2H, q, J = 7 Hz, H_2-6), 2.43 (2H, t, J = 7 Hz, H_2-5), 2.80 (1H, br m, H-18), 5.07 (1H, t, J = 6 Hz, H-12), 5.15 (1H, t, J = 6 Hz, H-7), 5.29 (1H, d, J = 10 Hz, H-20), 6.26 (1H, s, H-2), 7.19 (1H, s, H-4), 7.32 (1H, s, H-1) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 6.1 (q, C-25), 15.8 (q, C-14), 16.03 (q, C-9), 20.6 (q, C-19), 25.0 (t, C-5), 25.7 (t, C-16), 26.6 (t, C-11), 28.4 (t, C-6), 30.9 (d, C-18), 36.6 (t, C-17), 39.5 (t, C-15), 39.7 (t, C-10), 99.6 (s, C-23), 111.1 (d, C-2), 116.1 (d, C-20), 123.7 (d, C-7), 124.4 (d, C-12), 125.0 (s, C-3), 134.8 (s, C-13), 135.8 (s, C-8), 138.8 (d, C-4), 142.5 (d, C-1), 142.7 (s, C-22), 161.7 (s, C-21), 171.7 (s, C-24) ppm; EIMS *m/z* (rel. int.) 203 (11), 193 (5), 175 (21), 163 (9), 153 (24), 135 (48), 123 (21), 101 (22), 95 (31), 81 (100), 69 (45); HREIMS *m/z* 398.2458 (calcd for $C_{25}H_{34}O_4$, 398.2457).

22-Deoxyvariabilin (225): colorless oil; $[\alpha]^{23}_D$ -73° (*c* 0.20, $CHCl_3$); IR ν_{max} 2920, 1765, 1666, 1620, 850, 740 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.04 (1H, d, J = 7 Hz, H-19), 1.32 (1H, br m, H-17), 1.36 (2H, br m, H_2-16), 1.54 (3H, br s, H_3-14), 1.57 (3H, s, H_3-9), 1.94 (2H, br t, J = 7 Hz, H_2-15), 1.97 (3H, br s, H_3-25), 1.98 (2H, br t, J = 7 Hz,

H_2-10), 2.05 (2H, q, J = 7 Hz, H_2-11), 2.23 (2H, q, J = 8 Hz, H_2-6), 2.43 (2H, t, J = 8 Hz, H_2-5), 2.85 (1H, br m, H-18), 4.93 (1H, d, J = 10 Hz, H-20), 5.07 (1H, td, J = 1, 7 Hz, H-12), 5.15 (1H, td, J = 1, 7 Hz, H-7), 6.26 (1H, d, J = 1 Hz, H-2), 6.94 (1H, s, H-22), 7.19 (1H, s, H-4), 7.32 (1H, s, H-1) ppm; ^{13}C NMR data see Table 4.3; EIMS *m/z* (rel. int.) 179 (13), 175 (17), 149 (17), 137 (60), 123 (27), 95 (20), 81 (100), 69 (25); HREIMS *m/z* 382.2517 (calcd for $C_{25}H_{34}O_3$, 382.2508).

22-Deoxy-23-hydroxymethylvariabilin (226): yellow oil; $[\alpha]^{23}_D$ +18° (*c* 0.31, $CHCl_3$); IR ν_{max} 3660-3130, 1760, 1665, 1620, 825, 772, 720 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.06 (1H, d, J = 7 Hz, H-19), 1.33 (2H, br m, H_2-17), 1.36 (2H, br m, H_2-16), 1.55 (3H, br s, H_3-14), 1.58 (3H, s, H_3-9), 1.95 (2H, br t, J = 7 Hz, H_2-15), 1.98 (2H, br t, J = 7 Hz, H_2-10), 2.05 (2H, q, J = 7 Hz, H_2-11), 2.23 (2H, q, J = 8 Hz, H_2-6), 2.43 (2H, t, J = 8 Hz, H_2-5), 2.86 (1H, br m, H-18), 4.50 (2H, br s, H_2-25), 5.08 (1H, m, H-12), 5.09 (1H, d, J = 10 Hz, H-20), 5.15 (1H, td, J = 1, 7 Hz, H-7), 6.26 (1H, d, J = 1 Hz, H-2), 7.17 (1H, s, H-22), 7.19 (1H, s, H-4), 7.32 (1H, s, H-1) ppm; ^{13}C NMR data see Table 4.3; EIMS *m/z* (rel. int.) 175 (15), 153 (14), 149 (23), 135 (38), 123 (20), 109 (27), 95 (28), 81 (100), 69 (62), 55 (43), 41 (25); HREIMS *m/z* 398.2468 (calcd for $C_{25}H_{34}O_4$, 398.2457).

Acetylation of 226: Acetylation of **226** (1.5mg) with pyridine (0.4ml) and acetic anhydride (0.2ml) in the usual manner gave **244** as a yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.06 (1H, d, J = 7 Hz, H-19), 1.33 (2H, br m, H_2-17), 1.36 (2H, br m, H_2-16), 1.55 (3H, br s, H_3-14), 1.58 (3H, s, H_3-9), 1.95 (2H, br t, J = 7 Hz, H_2-15), 1.98 (2H, br t, J = 7 Hz, H_2-10), 2.05 (2H, q, J = 7 Hz, H_2-11), 2.10 (3H, br s, OOCC$\underline{H}_3$), 2.23 (2H, q, J = 8 Hz, H_2-6), 2.43 (2H, t, J = 8 Hz, H_2-5), 2.86 (1H, br m, H-18), 4.88 (2H, br s, H_2-25), 5.08 (1H, m, H-12), 5.09 (1H, d, J = 10 Hz, H-20), 5.15 (1H, td, J = 1, 7 Hz, H-7), 6.26 (1H, d, J = 1 Hz, H-2), 7.19 (1H, s, H-22), 7.19 (1H, s, H-4), 7.32 (1H, s, H-1) ppm; HREIMS *m/z* 440.2550 (calcd for $C_{27}H_{36}O_5$, 440.2561).

Furospinosulin-1 (227): colorless oil; IR ν_{max} 2920, 1740, 1660, 1505, 1400, 1380, 1025 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.60 (3H, s, H_3-9), 1.60 (3H, s, H_3-14), 1.60 (3H, s, H_3-19), 1.60 (3H, s, H_3-25), 1.68 (3H, s, H_3-24), 2.00 (2H, br m, H_2-10), 2.00 (2H, br m, H_2-15), 2.00 (2H, br m, H_2-20), 2.07 (2H, br m, H_2-11), 2.07 (2H, br m, H_2-16), 2.07 (2H, br m, H_2-21), 2.24 (2H, q, J = 7 Hz, H_2-6), 2.45 (2H, t, J = 7 Hz, H_2-5), 5.11 (1H, m, H-12), 5.11 (1H, m, H-17), 5.11 (1H, m, H-22), 5.18 (1H, t, J = 7 Hz, H-7), 6.27 (1H, s, H-2), 7.20 (1H, s, H-4), 7.33 (1H, s, H-1) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.9 (q, C-14), 16.0 (q, C-9), 17.7 (q, C-24) 25.1 (t, C-5), 25.7 (q, C-19), 25.7 (q, C-25), 26.6 (t, C-11), 26.7 (t, C-16), 26.8 (t, C-21), 28.5 (t, C-6), 39.7 (t, C-10), 39.7 (t, C-15), 39.7 (t, C-20), 111.1 (d, C-2), 123.8 (d, C-7), 124.2 (d, C-22), 124.3 (d, C-17), 124.4 (d, C-12), 125.0 (s, C-3), 131.2 (s, C-23), 134.9 (s, C-13), 135.0 (s, C-18), 135.8 (s, C-8), 138.8 (d, C-4), 142.5 (d, C-1) ppm; EIMS *m/z* (rel. int.) 285 (3), 204 (10), 189 (8), 175 (7), 161 (11), 136 (24), 135 (25), 121 (24), 95 (30), 81 (100), 69 (90); HREIMS *m/z* 354.2933 (calcd for $C_{25}H_{38}O$, 354.2922).

6.5 Chapter Five Experimental

6.5.1 Isolation of tsitsikammafuran (266) from a *Dysidea* sponge

The collection (March 1998), description and extraction of the new *Dysidea* sponge from the Tsitsikamma National Park, is given in Chapter Four. A hexane partition fraction (358 mg) of the EtOAc extract of the sponge was applied to 10 g Si gel. Normal phase HPLC (hexane, 3.5 ml/min) of the first (hexane) column fraction (49 mg) yielded tsitsikammafuran (**266**) as a colorless oil (0.8 mg, 0.0004 % dry wt of sponge); IR ν_{max} 2962, 1501, 1455, 1382, 1024, 873, 767, 599 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 5.1; EIMS *m/z* (rel. int.) 214 (10), 178 (34), 149 (25), 133 (100), 121 (34), 69 (39), 57 (55), 55 (44), 43 (45), 41 (42); HREIMS *m/z* 214.1345 (calcd for $C_{15}H_{18}O$, 214.1357).

6.5.2 Preparation of 3-bromo-*p*-cymene (273) from cumene (277)

6.5.2.1 Preparation of 4-isopropylbenzaldehyde (278)

A solution of freshly distilled cumene (1.25 g, 10.4 mmol) in dry CH_2Cl_2 (15 mL) under a N_2 atmosphere was cooled to 0°C in an ice-salt bath before titanium tetrachloride (2.42 mL, 17.4 mmol) was added in one portion. Dichloromethyl methyl ether (787 µL, 8.7 mmol) was added dropwise, with stirring, to the reaction mixture and stirring at 0°C was continued for 10 min after addition of the ether was complete. The reaction mixture was then allowed to warm to ambient temperature before being heated to 40°C for 15 min. The resulting solution was poured onto 4 g of crushed ice, extracted with CH_2Cl_2 (3 x 30 ml), and the combined extracts were washed with brine (20 mL), filtered to remove titanium oxides, dried and concentrated. 4-Isopropylbenzaldehyde (**278**) was isolated as a yellow oil (880 mg, 68 %) by chromatography of the concentrate on Si gel in 19:1 hex/EtOAc after filtration to remove titanium oxides; IR ν_{max} 2964, 1702, 1608, 1213, 840, 828, 764 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.27 (6H, d, J = 8 Hz), 2.98 (1H, sept, J = 8 Hz), 7.37 (2H, d, J = 8 Hz), 7.80 (2H, d, J = 8 Hz), 9.96 (1H, s) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.5 (q), 23.5 (q), 34.4 (d), 127.1 (d), 129.9 (d), 134.5 (s), 156.1 (s), 191.9 (s) ppm; EIMS *m/z* (rel. int.) 151 (74), 148 (60), 147 (63), 130 (65), 129 (92), 119

(100), 115 (71), 105 (53), 79 (44), 77 (50); HRFABMS *m/z* 149.096712 (calcd for $C_{10}H_{13}O$, 149.096640).

6.5.2.2 Preparation of 3-nitro-4-isopropylbenzaldehyde (279)

The aldehyde **278** (2.04 g, 13.8 mmol) was dissolved in technical grade conc. H_2SO_4 (12 mL) and cooled in an ice-salt bath. Fuming HNO_3 (1 mL) in conc. H_2SO_4 (2 mL) was added dropwise to the vigorously stirred mixture so that the temperature of the ice bath remained below 0°C. The mixture was stirred for 15 min before the ice bath was removed and stirring was continued at ambient temperature for 1.5 h. The reaction mixture was poured onto 200 ml crushed ice and extracted with EtOAc (3 x 50 mL). The combined EtOAc layers were washed with brine (2 x 70 mL) followed by 10 % $NaHCO_3$ solution (2 x 70 mL) before being dried. Si gel chromatography (8:2 hex/EtOAc) yielded 3-nitro-4-isopropylbenzaldehyde (**279**) as an orange oil (1.99 g, 75 %); IR ν_{max} 1703, 1614, 1531, 1356, 1194, 1054 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.28 (6H, d, J = 7 Hz), 3.41 (1H, sept, J = 7 Hz), 7.64 (1H, d, J = 8 Hz), 8.01 (1H, dd, J = 2, 8 Hz), 8.13 (1H, d, J = 2 Hz), 9.97 (1H, s) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.2 (q), 23.2 (q), 29.0 (d), 124.8 (d), 128.6 (d), 132.3 (d), 134.8 (s), 148.7 (s), 150.1 (s), 189.4 (s) ppm; EIMS *m/z* (rel. int.) 193 (68), 175 (76), 164 (100), 146 (49), 145 (62), 132 (49), 128 (66), 115 (45), 91 (43); HREIMS *m/z* 193.073776 (calcd for $C_{10}H_{13}NO_3$, 193.073893).

6.5.2.3 Model reaction: Huang-Minlon reduction of m-nitrobenzaldehyde (282) to m-toluidine (283)

m-Nitrobenzaldehyde (**282**, 1.06 g, 7.01 mmol) and hydrazine hydrate (1.70 mL, 33.9 mmol) were refluxed in diethylene glycol (13 mL) for 30 min, after which time a concentrated aqueous solution of KOH (1.55 g in 1.4 mL water) was added slowly. The dark brown solution was refluxed for 3 h until it had become pale brown. Dilution with water was followed by steam distillation of the mixture and extraction of the distillate with $CHCl_3$ (3 x 30 mL). The combined $CHCl_3$ layers were back-washed with water, dried and concentrated to give *m*-toluidine (**283**, 589 mg, 83 %); ^1H NMR ($CDCl_3$, 400

MHz) δ 2.33 (3H, s), 3.62 (2H, br s), 6.54 (1H, d, J = 8 Hz); 6.55 (1H, s), 6.65 (1H, d, J = 8 Hz), 7.11 (1H, t, J = 8 Hz) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.3 (q), 112.1 (d), 115.8 (d), 119.3 (d), 129.0 (d), 138.9 (s), 146.3 (s) ppm.

6.5.2.4 Attempted Huang-Minlon reduction of 3-nitro-4-isopropylbenzaldehyde (279)

Compound **279** (1.93 g, 10.0 mmol) and hydrazine hydrate (2.5 mL, 51.5 mmol) were refluxed in diethylene glycol (25 mL) for 30 min, after which time a concentrated aqueous solution of KOH (2.3 g in 8 mL water) was added slowly. The reaction mixture was refluxed for 6 hours until the dark brown solution had changed to a pale brown color. Dilution with water was followed by steam distillation of the mixture and extraction of the distillate with $CHCl_3$ (3 x 30 mL). The combined $CHCl_3$ layers were back-washed with water, dried and concentrated. Partially reduced product, 3-nitro-*p*-cymene (**280**, 1.27 g, 64 %), was separated from the fully reduced product (**281**, 440 mg, 36 %) on Si gel (hexane, 8:2 hex/EtOAc). Compound **280** was obtained as a yellow oil; IR ν_{max} 2970, 1528, 1356, 828, 806 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.25 (6H, d, J = 8 Hz), 2.35 (3H, s), 3.35 (1H, sept, J = 7 Hz), 7.32 (2H, t, J = 9 Hz), 7.47 (1H, s) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.4 (q), 23.5 (q), 23.5 (q), 28.1 (d), 123.8 (d), 127.2 (d), 133.2 (d), 136.6 (s), 139.3 (s), 149.5 (s) ppm; EIMS *m/z* (rel. int.) 162 (100), 144 (59), 134 (82), 119 (28), 118 (44), 115 (66), 106 (24), 91 (93), 77 (29); HRFABMS *m/z* 180.102503 (calcd for $C_{10}H_{14}NO_2$, 180.102454).

*6.5.2.5 Reduction of 3-nitro-*p*-cymene (280)*

The partially reduced compound **280** (1.03 g, 5.8 mmol) was added in portions to a gently warmed and stirred slurry of stannous chloride (5.40 g, 23.9 mmol) in conc. HCl (10 mL). A small amount of EtOH (~5 mL) was required to complete the dissolution of the two reactants before the mixture was finally refluxed for 35 min. The resulting yellow solution was made alkaline with 30 % NaOH and extracted with ether (3 x 30 mL). After drying and evaporation of the ether, the crude product was chromatographed on Si gel

(hexane, 8:2 hex/EtOAc) to yield a small amount of undecomposed $SnCl_2$-amino-*p*-cymene complex (56 mg) and pure **281** (744 mg, 87 %) as a yellow oil; IR ν_{max} 3465, 3372, 2961, 1623, 1510, 1296, 801 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.30 (6H, d, J = 8 Hz), 2.30 (3H, s), 2.90 (1H, sept, J = 8 Hz), 3.61 (2H, br s), 6.55 (1H, s), 6.66 (1H, d, J = 8 Hz), 7.08 (1H, d, J = 8 Hz) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.8 (q), 22.3 (q), 22.3 (q), 27.3 (d), 116.5 (d), 119.7 (d), 125.2 (d), 129.7 (s), 136.0 (s), 143.1 (s) ppm; EIMS *m/z* (rel. int.) 149 (42), 134 (100), 119 (33), 117 (16), 115 (15), 106 (7), 91 (11), 77 (6); HRFABMS *m/z* 149.120334 (calcd for $C_{10}H_{15}O$, 149.120450).

*6.5.2.6 Sandmeyer bromination of 3-amino-*p*-cymene (**281**)*

The amine **281** (300 mg, 2.0 mmol) was slurried in conc. HBr (5 mL) and heated to reflux before being cooled to 0°C in an ice-salt bath. An aqueous solution of $NaNO_2$ (152 mg, 2.21 mmol, in 3 mL water) was added dropwise to the stirred reaction mixture so as to maintain the temperature below 10°C. After addition of the $NaNO_2$ was complete, the reaction mixture was stirred for 15 min at 0°C. The cold, crude reaction mixture containing the diazonium salt was added dropwise *via* a Pasteur pipette to an ice-cooled solution of freshly prepared CuBr in conc. HBr (5 mL). Again, the temperature of the CuBr/HBr solution was monitored so that it remained below 10°C during this addition. An orange precipitate of a Cu complex soon formed on the surface of the reaction mixture which was left to stand overnight at ambient temperature. $CHCl_3$ (3 x 10 mL) was used to extract the aqueous reaction mixture. The combined extracts were washed with brine (20 mL), dried, filtered, and the $CHCl_3$ evaporated to yield a crude mixture that was fractionated on Si gel (hexane, 9:1 hex/EtOAc). The volatile product (**273**) was obtained as a colorless oil (68 mg, 26 %); IR ν_{max} 2960, 2927, 1501, 1463, 1379, 816 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.24 (6H, d, J = 7 Hz), 2.30 (3H, s), 3.34 (1H, sept, J = 7 Hz), 7.09 (1H, d, J = 8 Hz), 7.17 (1H, d, J = 8 Hz), 7.38 (1H, s) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.5 (q), 22.9 (q), 22.9 (q), 32 .4 (d), 124.0 (s), 126.3 (d), 128.4 (d), 133.2 (d,), 137.1 (s), 144.2 (s) ppm; EIMS *m/z* (rel. int.) 214 (33), 212 (32), 199 (91), 197 (87), 118 (100), 117 (51); HRFABMS *m/z* 213.027795 (M+1, calcd for $C_{10}H_{14}{}^{79}Br$, 213.027887).

6.5.3 Preparations of 3-bromo-*p*-cymene (273) from thymol (275)

6.5.3.1 PBr_3/Br_2 bromination of thymol (275)

Bromine liquid (0.95 mL, 18.4 mmol) was cautiously added dropwise to cooled PBr_3 (1.75 mL, 18.4 mmol) under anhydrous conditions to form bright yellow crystalline PBr_5. Thymol (**275**, 10.0 g, 66.6 mmol) was then added in small portions to the PBr_5 in the reaction flask with warming to 50°C in a water bath. When the vigorous reaction had ceased, after all of **275** had been added, the light orange solution was refluxed for 2.5 h in a sandbath over a bunsen burner. At the end of this period, water was added and the mixture was steam distilled. The distillate was taken up in ether, separated from the aqueous layer, and washed with 10 % KOH (3 x 30 mL), water (30 mL), and dried. Some remaining **275** was removed by chromatography of the mixture (3.02 g) on 10 g Si gel (hexane). Pure **273** was obtained as a colorless oil (2.08 g, 53 %). The spectral data of **273** prepared by this method were identical with those of **273** prepared in *6.5.2.6*.

6.5.3.2 PBr_5 (Sigma®) bromination of thymol (275)

Thymol (**275**, 8.80 g, 58.6 mmol), which had been pre-dried under high vacuum in a dessicator, was weighed into a reaction flask under anhydrous conditions in a glove box. Similarly, PBr_5 (Sigma®, 3.99 g, 9.3 mmol) was weighed into a solids addition flask that was then connected, in the glove box, to the reaction flask containing the thymol. The apparatus containing the PBr_5 and thymol was removed from the glove box and connected to a pre-dried reflux condenser held under N_2. The PBr_5 was transferred to the reaction flask in small portions, by twisting the solids addition flask, while warming the reaction mixture to 50°C in an oil bath. After this addition was complete, and the vigorous effervescence had ceased, the N_2 source was replaced by a calcium chloride guard tube and the reaction mixture was heated at 150°C for 2 h. Finally, the temperature was raised to 230°C and the reaction mixture refluxed for a further 2 h. The reaction was continually monitored by TLC to determine the temperature and heating time required for formation of **273**. The dark brown reaction mixture was taken up in ether, dried and concentrated (6.54 g) before being chromatographed on 60 g Si gel. A 1:1 mixture (1.7 g) of 3-bromo-*p*-cymene (**273**) and 2, 5-dibromo-*p*-cymene (**276**) was eluted in hexane.

A small amount (24 mg) of this mixture of isomers was subjected to reverse phase HPLC (9:1 MeOH/H_2O). 2, 5-Dibromo-*p*-cymene (**276**) was obtained as a colorless oil; IR ν_{max} 2964, 1471, 1383, 1070, 1046, 880 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.21 (6H, d, J = 7 Hz, H_3-9, H_3-10), 2.32 (3H, s, H_3-7), 3.26 (1H, sept, J = 7 Hz, H-8), 7.38 (1H, s, H-3), 7.39 (1H, s, H-6) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 22.1 (q, C-7), 22.7 (q, C-9), 22.7 (q, C-10), 32.6 (d, C-8), 122.6 (s, C-5), 124.1 (s, C-2), 130.3 (d, C-3), 134.4 (d, C-6), 137.0 (s, C-1), 146.7 (s, C-4) ppm; EIMS *m/z* (rel. int.) 294 (19), 292 (37), 290 (18), 279 (46), 277 (100), 275 (49), 198 (36), 196 (34), 117 (15), 115 (19); HRFABMS *m/z* 289.930921 (calcd for $C_{10}H_{12}{}^{79}Br_2$, 289.930573).

6.5.4 Preparations of 4-hydroxy-m-cymene (287) from p-cresol (288)

6.5.4.1 Preparation and rearrangement of isopropyl-p-cresyl ether (289)

NaOEt was prepared *in situ* by adding Na metal (9.0 g, 0.38 mmol) to absolute EtOH (140 mL, excess) and refluxing gently. Once all of the Na had reacted, the flask was cooled and *p*-cresol (288, 38.0 g, 0.35 mmol) was added. After a few minutes to allow the flask to cool down again, isopropyl bromide (47 g, 0.38 mmol) was added in small portions *via* a Pasteur pipette. The mixture was then refluxed for 6 h. Excess EtOH and isopropyl bromide were removed *in vacuo* and water was added to dissolve the precipitate of NaBr. The reaction mixture was taken up in EtOAc and washed with KOH (15 %, 2 x 150 mL), to remove any unreacted phenol, and water (2 x 50 mL). After drying, the concentrated crude product was vacuum distilled (104-105°C, 38 mmHg, lit.[312] 194°C, 760 mmHg) to yield **289** as a colorless liquid (42.0 g, 80 %); IR ν_{max} 1509, 1384, 1372, 1241, 1123, 957, 820 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.43 (6H, d, J = 6 Hz), 2.40 (3H, s), 4.59 (1H, sept, J = 6 Hz), 6.91 (2H, d, J = 8 Hz), 7.17 (2H, d, J = 8 Hz) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.3 (q), 22.0 (q), 22.0 (q), 69.8 (d), 115.9 (d), 115.9 (d), 129.6 (d), 129.6 (d), 129.8 (s), 155.7 (s) ppm; EIMS *m/z* (rel. int.) 150 (27), 108 (100), 107 (74), 80 (17), 79 (33), 77 (19); HRFABMS *m/z* 151.112317 (calcd for $C_{10}H_{15}O$, 151.11290).

Compound **289** (20 g, 133 mmol) was added dropwise to ice-cooled $AlCl_3$ (20 g, 150 mmol) under anhydrous conditions. Although the viscous reaction mixture was mechanically stirred, warming to 50°C was necessary to free the stirring rod once addition of the ether was complete. After vigorous stirring for 3 h, the reaction mixture was left to stand for a further 3 h. The reaction was quenched by the addition of ice followed by conc. HCl (20 mL). The organic layer was taken up in ether (50 mL), separated, washed with water (2 x 25 mL) and extracted with 15 % NaOH (3 x 100 mL). This NaOH solution containing the product as the sodium phenoxide was then neutralised with conc. HCl. The organic layer which separated out was taken up in ether, separated, washed with water (30 mL) and dried. Vacuum distillation using an oil pump gave crude *p*-cresol (68-84°C, 5 mmHg) as the initial, major fraction. The *ortho* substituted product 4-hydroxy-m-cymene (**287**) was obtained in 7 % yield as the second fraction (90-93°C, 5 mmHg, lit.[323] 93-94°C, 5 mmHg); white crystalline solid (mp 35°C, lit.[311] 36-37°C); IR ν_{max} 3401, 2957, 1506, 1257, 1081, 808, 773 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.26 (6H, d, J = 7 Hz), 2.29 (3H, s), 3.20 (1H, sept, J = 7 Hz), 4.72 (1H, s), 6.65 (1H, d, J = 8 Hz), 6.87 (1H, d, J = 8 Hz), 7.01 (1H, s) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.4 (q), 22.6 (q), 22.6 (q), 26.6 (d), 115.1 (d), 126.9 (d), 130.0 (d), 134.3 (s), 150.5 (s) ppm; EIMS *m/z* (rel. int.) 150 (36), 135 (100), 115 (26), 107 (29), 91 (33); HRFABMS *m/z* 151.112198 (calcd for $C_{10}H_{15}O$, 151.112290).

6.5.4.2 Direct propylation of p-*cresol (288)*

In a small autoclave equipped with a magnetic stirrer and heating element, *p*-cresol (**288**, 50.0 g, 0.462 mol) and diisopropyl ether (47.0 g, 0.462 mol) were combined with montmorillonite (6.0 g) which had been activated by stirring with 50% conc. H_2SO_4 (0.2 g) beforehand. The mixture was heated to 180°C and left to stir for 3 h in the autoclave. After being left to stand overnight, the effervescent mixture was filtered into a pear flask and vacuum distilled using a short fractionating column. The initial fraction, *p*-cresol (26-49°C, 2 mmHg vacuum pump), was followed by two further fractions that were collected while the temperature rose constantly to 68°C. These latter two fractions were

combined (36.5 g) and redistilled using a spinning band distillation column equipped with a Cartesian diver to moderate the vacuum obtained with an oil pump. The first two fractions consisted of cumene (**277**, 84.5-88.4°C, 12 mmHg; lit.[324] 152-153°C, 760 mmHg) and *p*-cresol (**288**, 91-95°C, 12 mmHg; lit.[324] 88.6°C, 10mmHg) respectively. 4-Hydroxy-*m*-cymene (**287**, 12.0 g, 17 % yield) was collected as the final fraction at 113-114°C (12 mmHg). The spectral data of this compound were consistent with those for **287** prepared in *6.5.4.1.*

6.5.5 Preparation of 4-bromo-*m*-cymene (274) from 4-hydroxy-*m*-cymene (287)

Bromine liquid (665 μL, 12.9 mmol) was cautiously added dropwise to cooled PBr_3 (1.23 mL, 12.9 mmol) under dry conditions to form bright yellow crystalline PBr_5. The reaction flask was then warmed to 50°C before phenol **287** (7.76 g, 51.7 mmol) was added in small portions causing vigorous effervescence. When addition was complete and effervescence had ceased, the mixture was 'gently' refluxed in an oil bath (240°C) for 2 h. The reaction mixture (7.98 g) was taken up in EtOAc, dried and concentrated. After chromatography on 150 g Si gel using hexane, the crude brominated product obtained was further purified in hexane on 10 g Si gel. 4-Bromo-*m*-cymene (**287**) was obtained as a colorless oil (1.48 g, 54 %); IR ν_{max} 2961, 2929, 1502, 1463, 1382, 815, 720 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.25 (6H, d, J = 7 Hz, H_3-9, H_3-10), 2.31 (3H, s, H_3-7), 3.35 (1H, sept, J = 7 Hz, H-8), 6.86 (1H, dd, J = 2, 8 Hz, H-6), 7.09 (1H, d, J = 1 Hz, H-2), 7.40 (1H, d, J = 8 Hz, H-5) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.1 (q, C-7), 22.8 (q, C-9), 22.8 (q, C-10), 32.7 (d, C-8), 120.9 (s, C-4), 127.4 (d, C-2), 128.1 (d, C-6), 132.4 (d, C-5), 137.3 (s, C-1), 146.9 (s, C-3) ppm; EIMS *m/z* (rel. int.) 215 (82), 213 (85), 134 (100), 119 (8), 105 (7), 91 (8); HRFABMS *m/z* 213.027802 (M+1, calcd for $C_{10}H_{14}{}^{79}Br$, 213.027887).

6.5.6 Biaryl coupling reactions of aryl bromides 273, 274 and 285 with 3-furaldehyde

*6.5.6.1 Model reaction: Attempted preparation of [4-tolyl]benzylmethanol (**290**)* via *a Grignard reaction*

p-Bromotoluene (200 mg, 1.17 mmol) in dry ether (0.5 mL) was added dropwise to clean, dry Mg turnings (31 mg, 1.29 mmol) and a crystal of iodine in dry ether (3 mL). This mixture was sonicated briefly to initiate the reaction. When all of the Mg turnings had been used up, benzaldehyde (137 mg, 1.29 mmol) in ether (0.5 mL) was added dropwise with cooling of the reaction flask in an ice-salt bath. After stirring for 10 min, the reaction mixture was poured into ice-water (10 mL). Acid was added to dissolve the pale yellow precipitate formed, and the aqueous solution was extracted with ether (3 x 10 mL). The combined ether layers were dried and concentrated. ^{1}H NMR spectroscopy of the crude reaction mixture showed a mixture of compounds including benzaldehyde, and no product peaks could be confidently assigned.

*6.5.6.2 Preparation of [4-tolyl]benzylmethanol (**290**) via lithiation of p-bromotoluene*

A solution of *p*-bromotoluene (200 mg, 1.17 mmol) in dry THF (5 mL) was cooled to -78°C in a Cardice cooling bath before *n*-BuLi (1.6 M in hexane, 806 μL, 1.29 mmol) was added during 5 min. After allowing the mixture to stir for 1 h, a solution of benzaldehyde (132 μL, 1.29 mmol) in dry THF (1 mL) was added dropwise. The reaction mixture was left to stir for a further 4 h as it warmed to room temperature. The reaction was quenched by the addition of 75 % EtOH (8 mL). The aqueous solution obtained after concentration of the reaction mixture was extracted with CH_2Cl_2 (3 x 15 mL). The combined organic layers were dried and concentrated to yield 250 mg of crude product (**290**) which was not purified further since ^{1}H NMR spectroscopy showed the presence of only the desired product; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.34 (3H, s), 5.79 (1H, s), 2.58 (br s), 7.16 (2H, d, J = 8 Hz), 7.27 (3H, d, J = 8 Hz), 7.37 (4H, m) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.0 (q), 76.0 (d), 126.4 (d), 126.4 (d), 127.3 (d), 127.3 (d), 128.4 (d), 128.4 (d), 129.1 (d), 129.1 (d), 137.1 (s), 141.0 (s), 144.0 (s) ppm.

6.5.6.3 Preparation of [4-tolyl](furan-3-yl)methanol (291) via *lithiation of p-bromotoluene*

The immediately preceding procedure was repeated replacing benzaldehyde with 3-furaldehyde. Quantities used were *p*-bromotoluene (400 mg, 2.34 mmol) in dry THF (6 mL), *n*-BuLi (1.6 M in hexane, 1.61 mL, 2.57 mmol), 3-furaldehyde (224 μL, 2.57 mmol). The crude product (486 mg) was applied to 10 g Si gel in 17:3 hex/EtOAc to yield crystalline **291** (350 mg, 79 %); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.19 (br d, J = 5 Hz), 2.36 (3H, s), 5.73 (1H, d, J = 5 Hz), 6.32 (1H, d, J = 1 Hz), 7.18 (2H, d, J = 8 Hz), 7.29 (2H, d, J = 8 Hz), 7.30 (1H, s), 7.37 (1H, t, J = 2 Hz) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.1 (q), 69.4 (d), 109.2 (d), 126.3 (d), 129.1 (s), 129.2 (d), 137.5 (s), 139.7 (d), 140.2 (s), 143.4 (d) ppm; HRFABMS *m/z* 190.099380 (190.099403 calcd for $C_{12}H_{14}O_2$).

6.5.6.4 Preparation of compounds 292, 293 and 294 from 273, 274 and 285 respectively via *lithiation in the presence of a Lewis base*

While the following procedure is representative, the successful coupling of **273** (80 mg, 0.38 mmol) with 3-furaldehyde to give **292** (21 mg, 24 %) was first achieved in the presence of the Lewis base DABCO (47 mg, 0.42 mmol).

A solution of **273** (300 mg, 1.41 mmol) and TMEDA (193 μL, 1.28 mmol) in dry THF (5 mL) was cooled to –78°C in a Cardice cooling bath. *n*-BuLi (1.6 M in hexane, 800 μL, 1.28 mmol) was added over several minutes and the reaction mixture was left to stir for 3.5 h. More dry ice was added to the cooling bath to maintain a bath temperature of -78°C before 3-furaldehyde (111 μL, 1.28 mmol) was added dropwise in dry THF (1 mL). The reaction mixture was again left to stir and warm up to room temperature over 4.5 h before the reaction was quenched with water and the THF was removed *in vacuo*. Brine was added and the aqueous solution extracted with CH_2Cl_2 (4 x 20 mL). The CH_2Cl_2 layers were combined, dried and concentrated to yield a crude reaction mixture (223 mg) that was chromatographed over Si gel. The crude product (135 mg), eluted in 8:2 hex/EtOAc and identified by ^{1}H NMR, was then subjected to normal phase HPLC (8:2

hex/EtOAc) to give **292** (80 mg, 27 %) as a colorless oil. Similar biaryl coupling of **274** (304 mg, 1.43 mmol) and **285** (420 mg, 1.97 mmol) with 3-furaldehyde yielded the regioisomers **293** (32 %) and **294** (15 %) respectively. The reaction was subsequently modified. Coupling of **273** and 3-furaldehyde in the presence of two equivalents of TMEDA (386 μL, 2.56 mmol) gave **292** in 49 % yield, while the exchange of *n*-BuLi for *t*-BuLi (1.7 M in pentane) in the reaction, again using two equivalents of TMEDA, gave **291** in 22 % yield.

[2-Isopropyl-5-methylphenyl](furan-3-yl)methanol (292): colorless oil; IR ν_{max} 3351, 2964, 1501, 1158, 1026, 874, 818, 600 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.13 (3H, d, *J* = 7 Hz, H_3-14), 1.22 (3H, d, *J* = 7 Hz, H_3-13), 2.08 (1H, d, *J* = 4 Hz, O<u>H</u>), 2.32 (3H, s, H_3-15), 3.19 (1H, sept, *J* = 7 Hz, H-12), 6.06 (1H, d, *J* = 4 Hz, H-7), 6.33 (1H, d, *J* = 1 Hz, H-11), 7.11 (1H, d, *J* = 8 Hz, H-6), 7.20 (1H, d, *J* = 8 Hz, H-5), 7.21 (1H, s, H-9), 7.31 (1H, s, H-2), 7.36 (1H, t, *J* = 2 Hz, H-10) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.9 (q, C-15), 23.8 (q, C-14), 24.2 (q, C-13), 27.9 (d, C-12), 65.8 (d, C-7), 109.7 (d, C-11), 125.4 (d, C-5), 126.8 (d, C-2), 128.7 (d, C-6), 129.2 (s, C-8), 135.3 (s, C-1), 139.4 (s, C-3), 139.8 (d, C-9), 142.9 (s, C-4), 143.2 (d, C-10) ppm; EIMS *m/z* (rel. int.) 230 (16), 212 (21), 197 (93), 187 (28), 183 (57), 169 (60), 162 (100), 143 (31); HRFABMS *m/z* 231.138574 (M+1, 231.138505 calcd for $C_{15}H_{19}O_2$).

[2-Isopropyl-4-methylphenyl](furan-3-yl)methanol (293): colorless crystals, mp 39-41°C; IR ν_{max} 3351, 2964, 1501, 1158, 1026, 875, 774, 600 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.13 (3H, d, *J* = 7 Hz, H_3-14), 1.22 (3H, d, *J* = 7 Hz, H_3-13), 1.97 (1H, d, *J* = 4 Hz, O<u>H</u>), 2.34 (3H, s, H_3-15), 3.21 (1H, sept, *J* = 7 Hz, H-12), 6.05 (1H, d, *J* = 4 Hz, H-7), 6.31 (1H, s, H-11), 7.02 (1H, d, *J* = 8 Hz, H-5), 7.10 (1H, s, H-2), 7.21 (1H, s, H-9), 7.36 (1H, br s, H-10), 7.36 (1H, d, *J* = 8 Hz, H-6) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.3 (q, C-15), 23.9 (q, C-14), 24.2 (q, C-13), 28.2 (d, C-12), 65.9 (d, C-7), 109.5 (d, C-11), 126.3 (d, C-2), 126.4 (d, C-6), 126.8 (d, C-5), 129.2 (s, C-8), 136.6 (s, C-4), 137.7 (s, C-1), 140.0 (d, C-9), 143.3 (d, C-10), 145.9 (s, C-4) ppm; EIMS *m/z* (rel. int.) 230 (24),

201 (29), 197 (79), 187 (47), 183 (56), 169 (59), 162 (100), 147 (39), 143 (40), 128 (32); HRFABMS *m/z* 231.138458 (M+1, 231.138505 calcd for $C_{15}H_{19}O_2$).

[5-Isopropyl-2-methylphenyl](furan-3-yl)methanol (294): white amorphous powder (64 mg, 15 %); IR ν_{max} 3351, 2960, 1501, 1459, 1158, 1025, 874, 771 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.23 (6H, d, J = 7 Hz, H_3-13, H_3-14), 2.01 (1H, d, J = 4 Hz, O<u>H</u>), 2.25 (3H, s, H_3-15), 2.89 (1H, sept, J = 7 Hz, H-12), 5.94 (1H, d, J = 4 Hz, H-7), 6.34 (1H, s, H-11), 7.07 (2H, s, H-3, H-5), 7.21 (1H, s, H-9), 7.36 (1H, s, H-10), 7.40 (1H, s, H-6) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.6 (q, C-15), 24.0 (q, C-14), 24.1 (q, C-13), 33.9 (d, C-12), 66.5 (d, C-7), 109.5 (d, C-11), 123.9 (d, C-6), 125.5 (d, C-5), 128.2 (s, C-8), 130.5 (d, C-3), 132.2 (s, C-1), 140.1 (d, C-9), 140.7 (s, C-2), 143.4 (d, C-10), 146.9 (s, C-4) ppm; EIMS *m/z* (rel. int.) 212 (42), 197 (58), 179 (18), 169 (21), 162 (100), 147 (24), 141 (24), 95 (15); HRFABMS *m/z* 231.138511 (M+1, 231.138505 calcd for $C_{15}H_{19}O_2$).

6.5.7 Dehydroxylation Reactions

6.5.7.1 Model reaction: Preparation of 4-benzyltoluene (295) by acidic reduction of ***290***

Trifluoroacetic acid (1.5 mL, solvent) was added dropwise to **290** (100 mg, 0.50 mmol) under a N_2 atmosphere. This dark orange solution became pale pink during the dropwise addition of triethylsilane (240μL, 1.50 mmol). After stirring for 10 min, the reaction mixture was poured into 1M NaOH solution (20 mL) and this aqueous solution was extracted with EtOAc (3 x 20 mL). The combined EtOAc layers were washed with brine, dried and concentrated. After crude fractionation on 10 g Si (8:2 hex/EtOAc), 45 mg of crude product was injected on normal phase HPLC (hexane) to obtain **295** as a colorless oil (16 mg, 17 %); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.31 (3H, s), 3.94 (2H, s), 7.08 (2H, d, J = 8 Hz), 7.18 (3H, m), 7.26 (4H, m) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.0 (q), 76.0 (d), 126.4 (d), 127.3 (d), 128.4 (d), 129.1 (d), 137.1 (s), 141.0 (s), 144.0 (s) ppm; HRFABMS *m/z* 182.109498 (182.109551 calcd for $C_{14}H_{14}$).

*6.5.7.2 Model reaction: Preparation of 4-[(Furan-3-yl)methyl]-toluene (**296**) by reductive dehydroxylation of **291***

Chlorotrimethylsilane (613 μL, 4.78 mmol) was added dropwise to a stirred suspension of NaI (720 mg, 4.78 mmol) in dry MeCN (2 ml) under a N_2 atmosphere. Compound **291** (150 mg) was then added dropwise in dry MeCN (5 mL) and the reaction mixture was left to stir for 10 min at ambient temperature. The reaction was quenched with 30 mL water. This aqueous phase was extracted with ether (3 x 25 mL), and the combined ether extracts were washed with a 20% $Na_2S_2O_3$ solution (2 x 30 mL) and water (30 mL) before being dried and concentrated to give **296** as a yellow oil (125 mg, 90 %); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.35 (3H, s), 3.76 (2H, s), 6.25 (1H, s), 7.13 (4H, s), 7.22 (1H, s), 7.36 (1H, t, J = 1 Hz) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.0 (q), 30.7 (t), 111.2 (d), 124.5 (s), 128.4 (d), 129.1 (d), 135.6 (s), 137.3 (s), 139.5 (d), 143.00 (d) ppm; HRFABMS *m/z* 172.088796 (172.088815 calcd for $C_{12}H_{12}O$).

*6.5.7.3 Preparation of compounds **266**, **267** and **297** by reductive dehydroxylation of compounds **292**, **293** and **294***

The following procedure is representative. Chlorotrimethylsilane (99 μL, 0.52 mmol) was added dropwise to a stirred suspension of NaI (117 mg, 0.78 mmol) in dry MeCN (2 mL) under a N_2 atmosphere. The hydroxylated compound **292** (30 mg) was then added dropwise as a 1 mL solution in dry MeCN and the reaction mixture was left to stir for 10 min at ambient temperature, before being quenched by pouring into water (10 mL). This aqueous phase was extracted with ether (3 x 25 mL), and the combined ether extracts were washed with a 20% $Na_2S_2O_3$ (3 x 25 mL) solution and water (30 mL) before being dried and concentrated. The ^{1}H NMR spectrum of the reaction mixture showed 100 % conversion to the dehydroxylated product **266**. Regioisomers **267** and **297** were similarly prepared, also in quantitative yield, from **293** and **294** respectively.

3-[(Furan-3-yl)methyl]-*p*-cymene (266): colorless oil; IR, MS and NMR data were consistent with those reported above for the isolated natural product tsitsikammafuran; HRFABMS 214.135694 (calcd for $C_{15}H_{18}O$, 214.135765).

4-[(Furan-3-yl)methyl]-*m*-cymene (267): colorless oil; IR ν_{max} 2962, 1500, 1459, 1023, 874, 771, 599 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.33 (1H, s, H-10), 7.09 (1H, s, H-2), 7.06 (1H, s, H-9), 7.03 (1H, d, J = 8 Hz, H-5), 6.93 (1H, d, J = 8 Hz, H-6), 6.21 (1H, s, H-11), 3.76 (1H, s, H-7), 3.14 (1H, sept, J = 7 Hz, H-12), 2.32 (3H, s, H_3-15), 1.18 (3H, d, J = 7 Hz, H_3-13), 1.18 (3H, d, J = 7 Hz, H_3-13) ppm; ^{13}C NMR data see Table 6.1; EIMS *m/z* (rel. int.) 215 (21), 214 (100), 185 (31), 181 (24), 171 (38), 143 (51), 133 (26), 128 (46); HREIMS *m/z* 214.135786 (calcd for $C_{15}H_{18}O$, 214.135765).

2-[(Furan-3-yl)methyl]-*p*-cymene (297): colorless oil; IR ν_{max} 2925, 1497, 1459, 1383, 1021, 872, 598 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.35 (1H, s, H-10), 7.09 (1H, s, H-9), 7.08 (1H, d, J = 8 Hz, H-5), 7.01 (1H, d, J = 8 Hz, H-6), 7.01 (1H, s, H-3), 6.23 (1H, s, H-11), 3.72 (1H, s, H-7), 2.84 (1H, sept, J = 7 Hz, H-12), 2.25 (3H, s, H_3-15), 1.21 (3H, d, J = 7 Hz, H_3-13), 1.21 (3H, d, J = 7 Hz, H_3-13) ppm; EIMS *m/z* (rel. int.) 215 (16), 214 (100), 199 (82), 171 (71), 143 (38), 131 (31), 128 (22), 81 (30); HRFABMS *m/z* 214.135702 (calcd for $C_{15}H_{18}O$, 214.135765).

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322 Crystallographic data for the structure reported here has been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge, CB21EZ, UK (Fax: +44-(0)1223-336033 or E-mail: deposit@ccdc.cam.ac.uk).

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Appendix 1

Table 1. Sample and crystal data for algoane (**121**)

Identification code	X1963A1
SB number	N/A
SB project/programme/F.S.	P00180
Chemist's labbook	N/A
X-ray labbook	RC97953-161
Crystallization labbook	N/A
Crystallization solvents	Ethyl acetate and hexane
Crystallization method	Slow evaporation
Empirical formula	$C_{17}H_{27}Br_2ClO_4$
Formula weight	490.66
Temperature	150(2) K
Wavelength	1.54178 Å
Crystal size	0.50 x 0.43 x 0.37 mm
Crystal habit	Colourless block
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 8.2536(5) Å
	b = 11.4458(7) Å
	c = 21.063(2) Å
Volume	1989.8(2) $Å^3$
Z	4
Density (calculated)	1.638 Mg/m^3
Absorption coefficient	6.554 mm^{-1}
F(000)	992

Table 2. Data collection and structure refinement for algoane (**121**)

Diffractometer	Nonius MACH3
Radiation source	Fine-focus sealed tube, CuK_α
Data collection method	ω–2θ scans
Theta range for data collection	4.20 to 69.93°.
Index ranges	$0 \leq h \leq 10, -13 \leq k \leq 1, -25 \leq l \leq 25$
Reflections collected	4650
Independent reflections	3762 [R(int) = 0.0398]
Variation in check reflections	10.51%
Absorption correction	Psi-scan
Max. and min. transmission	0.1380 and 0.0317
Structure solution technique	Direct methods
Structure solution program	SHELXTL v5.03 (Siemens, 1994)
Refinement technique	Full-matrix least-squares on F^2
Refinement program	SHELXTL v5.03 (Siemens, 1994)
Function minimized	$\Sigma w(F_o^2 - F_c^2)^2$
Data / restraints / parameters	3762 / 2 / 229
Goodness-of-fit on F^2	1.078
Δ/σ_{max}	0.000
Final R indices	
3733 data; $I>2\sigma(I)$	R1 = 0.0365, wR2 = 0.0965
all data	R1 = 0.0372, wR2 = 0.1008
Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0579P)^2 + 2.2595P]$ where $P = [MAX(F_o^2, 0) + 2F_c^2]/3$
Absolute structure parameter	0.02(2)
Largest diff. peak and hole	0.603 and -0.609 e.Å^{-3}

Refinement summary:

Ordered Non-H atoms, XYZ	Freely refining
Ordered Non-H atoms, U	Anisotropic
H atoms (on carbon), XYZ	Idealized positions riding on attached atom
H atoms (on carbon), U	Appropriate multiple of U(eq) for bonded atom
H atoms (on heteroatoms), XYZ	Refining, with O-H distance restained to 0.84(2)Å
H atoms (on heteroatoms), U	Appropriate multiple of U(eq) for bonded atom
Disordered atoms	No disorder
Disordered atoms, XYZ	
Disordered atoms, U	

Table 3. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å ^{2} x 10^3) for algoane (**121**); U(eq) is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x/a	y/b	z/c	U(eq)
Br1	7144(1)	7801(1)	2386(1)	55(1)
Br2	4119(1)	3115(1)	-1155(1)	56(1)
Cl1	3933(2)	9644(1)	2021(1)	50(1)
O1	4999(3)	7205(2)	397(1)	33(1)
O2	7401(3)	5859(3)	90(1)	38(1)
O3	2103(3)	5861(2)	1446(1)	33(1)
O4	-366(3)	6261(3)	1059(2)	43(1)
C1	2803(5)	6693(3)	1004(2)	31(1)
C2	2780(5)	7907(3)	1298(2)	35(1)
C3	3880(5)	8071(3)	1873(2)	36(1)
C4	5581(5)	7686(4)	1684(2)	37(1)
C5	5597(4)	6436(3)	1445(2)	32(1)
C6	4544(4)	6308(3)	841(2)	28(1)
C7	4752(4)	5056(3)	539(2)	29(1)
C8	6554(4)	4851(3)	312(2)	33(1)
C9	6439(5)	3973(4)	-236(2)	41(1)
C10	4655(5)	3670(3)	-302(2)	37(1)
C11	3720(4)	4761(3)	-75(2)	32(1)
C12	3771(5)	5717(4)	-586(2)	38(1)
C13	1918(5)	4445(4)	40(2)	42(1)
C14	4491(5)	4106(3)	1052(2)	36(1)
C15	3238(5)	7503(4)	2485(2)	40(1)
C16	473(4)	5761(4)	1439(2)	35(1)
C17	-110(5)	4984(4)	1950(2)	47(1)

Table 4. Selected bond lengths (Å) for algoane (**121**)

Br1-C4	1.967(4)	Br2-C10	1.957(4)
Cl1-C3	1.827(4)	O1-C6	1.439(4)
O2-C8	1.428(5)	O3-C16	1.350(5)
O3-C1	1.451(4)	O4-C16	1.203(5)
C1-C2	1.521(5)	C1-C6	1.542(5)
C2-C3	1.525(5)	C3-C4	1.524(5)
C3-C15	1.537(5)	C4-C5	1.517(5)
C5-C6	1.547(5)	C6-C7	1.578(5)
C7-C14	1.548(5)	C7-C8	1.580(5)
C7-C11	1.584(5)	C8-C9	1.534(6)
C9-C10	1.519(6)	C10-C11	1.543(5)
C11-C12	1.535(6)	C11-C13	1.549(5)
C16-C17	1.477(6)		

Table 5. Selected bond angles (°) for algoane (**121**)

C16-O3-C1	116.4(3)	O3-C1-C2	109.5(3)
O3-C1-C6	109.0(3)	C2-C1-C6	111.3(3)
C1-C2-C3	115.3(3)	C4-C3-C2	107.8(3)
C4-C3-C15	114.5(3)	C2-C3-C15	114.1(3)
C4-C3-Cl1	107.9(3)	C2-C3-Cl1	105.7(3)
C15-C3-Cl1	106.3(3)	C5-C4-C3	111.6(3)
C5-C4-Br1	107.8(3)	C3-C4-Br1	112.9(3)
C4-C5-C6	110.9(3)	O1-C6-C1	100.6(3)
O1-C6-C5	108.7(3)	C1-C6-C5	108.3(3)
O1-C6-C7	111.0(3)	C1-C6-C7	116.8(3)
C5-C6-C7	110.9(3)	C14-C7-C6	110.0(3)
C14-C7-C8	103.8(3)	C6-C7-C8	111.0(3)
C14-C7-C11	110.2(3)	C6-C7-C11	117.7(3)
C8-C7-C11	103.2(3)	O2-C8-C9	108.2(3)
O2-C8-C7	116.1(3)	C9-C8-C7	105.5(3)
C10-C9-C8	106.1(3)	C9-C10-C11	105.8(3)
C9-C10-Br2	112.1(3)	C11-C10-Br2	115.7(3)
C12-C11-C10	110.2(3)	C12-C11-C13	107.6(3)
C10-C11-C13	109.8(3)	C12-C11-C7	113.9(3)
C10-C11-C7	99.0(3)	C13-C11-C7	116.0(3)
O4-C16-O3	122.7(4)	O4-C16-C17	125.7(4)
O3-C16-C17	111.6(4)		

Table 6. Selected torsion angles (°) for algoane (**121**)

C16-O3-C1-C2	84.6(4)	C16-O3-C1-C6	-153.4(3)
O3-C1-C2-C3	67.0(4)	C6-C1-C2-C3	-53.7(4)
C1-C2-C3-C4	53.4(4)	C1-C2-C3-C15	-74.9(4)
C1-C2-C3-Cl1	168.6(3)	C2-C3-C4-C5	-56.7(4)
C15-C3-C4-C5	71.4(4)	Cl1-C3-C4-C5	-170.5(3)
C2-C3-C4-Br1	-178.3(2)	C15-C3-C4-Br1	-50.2(4)
Cl1-C3-C4-Br1	67.9(3)	C3-C4-C5-C6	62.1(4)
Br1-C4-C5-C6	-173.4(2)	O3-C1-C6-O1	178.6(3)
C2-C1-C6-O1	-60.5(3)	O3-C1-C6-C5	-67.5(3)
C2-C1-C6-C5	53.4(4)	O3-C1-C6-C7	58.5(4)
C2-C1-C6-C7	179.4(3)	C4-C5-C6-O1	49.9(4)
C4-C5-C6-C1	-58.5(4)	C4-C5-C6-C7	172.2(3)
O1-C6-C7-C14	172.0(3)	C1-C6-C7-C14	-73.5(4)
C5-C6-C7-C14	51.1(4)	O1-C6-C7-C8	57.8(4)
C1-C6-C7-C8	172.2(3)	C5-C6-C7-C8	-63.1(4)
O1-C6-C7-C11	-60.7(4)	C1-C6-C7-C11	53.7(4)
C5-C6-C7-C11	178.4(3)	C14-C7-C8-O2	-150.7(3)
C6-C7-C8-O2	-32.6(4)	C11-C7-C8-O2	94.4(4)
C14-C7-C8-C9	89.5(4)	C6-C7-C8-C9	-152.4(3)
C11-C7-C8-C9	-25.4(4)	O2-C8-C9-C10	-126.4(3)
C7-C8-C9-C10	-1.5(4)	C8-C9-C10-C11	29.1(5)
C8-C9-C10-Br2	156.1(3)	C9-C10-C11-C12	75.9(4)
Br2-C10-C11-C12	-48.8(4)	C9-C10-C11-C13	-165.7(4)
Br2-C10-C11-C13	69.5(4)	C9-C10-C11-C7	-43.8(4)
Br2-C10-C11-C7	-168.5(2)	C14-C7-C11-C12	174.3(3)
C6-C7-C11-C12	47.2(4)	C8-C7-C11-C12	-75.4(4)
C14-C7-C11-C10	-68.7(4)	C6-C7-C11-C10	164.2(3)
C8-C7-C11-C10	41.5(3)	C14-C7-C11-C13	48.6(4)
C6-C7-C11-C13	-78.5(4)	C8-C7-C11-C13	158.9(3)
C1-O3-C16-O4	5.5(5)	C1-O3-C16-C17	-174.2(3)

Table 7. Anisotropic displacement parameters ($Å^2 \times 10^3$) for algoane (**121**)
The anisotropic displacement factor exponent takes the form: $-2\pi^2 [h^2a^{*2} U_{11} + ... + 2 h k a^* b^* U_{12}]$

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
Br1	41(1)	74(1)	49(1)	-24(1)	-11(1)	-6(1)
Br2	64(1)	57(1)	46(1)	-21(1)	-7(1)	2(1)
Cl1	63(1)	39(1)	49(1)	-12(1)	4(1)	-2(1)
O1	35(1)	32(1)	33(1)	4(1)	3(1)	-3(1)
O2	30(1)	46(2)	38(1)	-2(1)	0(1)	-4(1)
O3	23(1)	42(1)	33(1)	5(1)	1(1)	-1(1)
O4	26(1)	57(2)	47(2)	2(1)	-5(1)	2(1)
C1	26(2)	36(2)	29(2)	2(1)	0(1)	0(1)
C2	33(2)	37(2)	34(2)	-4(2)	2(2)	4(2)
C3	40(2)	32(2)	36(2)	-4(2)	6(2)	0(2)
C4	31(2)	45(2)	34(2)	-7(2)	0(2)	-7(2)
C5	26(2)	42(2)	30(2)	-2(2)	-1(1)	-1(1)
C6	25(2)	33(2)	25(2)	3(1)	2(1)	-1(1)
C7	25(2)	32(2)	29(2)	3(1)	1(1)	0(1)
C8	26(2)	40(2)	33(2)	-1(2)	0(2)	1(1)
C9	34(2)	39(2)	50(2)	-12(2)	2(2)	5(2)
C10	39(2)	36(2)	36(2)	-7(2)	-4(2)	-2(2)
C11	29(2)	35(2)	33(2)	-5(2)	-4(1)	0(1)
C12	41(2)	41(2)	31(2)	-1(2)	-7(2)	3(2)
C13	30(2)	47(2)	49(2)	-12(2)	-3(2)	-7(2)
C14	41(2)	36(2)	31(2)	6(2)	1(2)	3(2)
C15	39(2)	49(2)	30(2)	-4(2)	2(2)	-1(2)
C16	25(2)	41(2)	39(2)	-3(2)	2(2)	-3(2)
C17	32(2)	50(2)	59(3)	8(2)	6(2)	-4(2)

Table 8. Hydrogen atom coordinates (x 10^4) and isotropic displacement parameters ($Å^2$ x 10^3) for algoane (**121**)

	x/a	y/b	z/c	U
H1	5723(49)	6892(42)	198(23)	50
H2	8132(54)	6042(51)	359(22)	57
H1A	2142(5)	6701(3)	607(2)	37
H2A	1654(5)	8090(3)	1426(2)	42
H2B	3098(5)	8480(3)	969(2)	42
H4A	5961(5)	8205(4)	1332(2)	44
H5A	5179(4)	5911(3)	1781(2)	39
H5B	6725(4)	6201(3)	1348(2)	39
H8A	7184(4)	4491(3)	667(2)	40
H9A	6853(5)	4323(4)	-634(2)	49
H9B	7080(5)	3263(4)	-140(2)	49
H10A	4415(5)	3022(3)	3(2)	44
H12A	3172(35)	5454(13)	-961(6)	57
H12B	4899(6)	5875(21)	-702(12)	57
H12C	3273(38)	6432(10)	-420(6)	57
H13A	1444(13)	4142(30)	-353(5)	63
H13B	1326(10)	5144(8)	175(17)	63
H13C	1846(6)	3848(23)	373(12)	63
H14A	3342(8)	4077(19)	1168(11)	54
H14B	5140(30)	4294(15)	1427(6)	54
H14C	4825(37)	3345(6)	883(6)	54
H15A	3267(38)	6651(4)	2441(6)	59
H15B	2120(15)	7758(23)	2559(9)	59
H15C	3918(25)	7739(24)	2844(3)	59
H17A	-1294(6)	5030(25)	1976(12)	70
H17B	364(36)	5227(21)	2355(4)	70
H17C	213(40)	4178(6)	1857(9)	70