1992

Chemical and biochemical studies on metabolites from Australian marine sponges

Doreen K. Bali
University of Wollongong

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CHEMICAL AND BIOCHEMICAL STUDIES ON METABOLITES FROM AUSTRALIAN MARINE SPONGES

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

of

MASTER OF SCIENCE (HONOURS)

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UNIVERSITY OF WOLLONGONG

by

DOREEN K. L. BALI, BScGCEd, MSc

DEPARTMENT OF CHEMISTRY 1992
To my son Alvin
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Abstract

A large number of bioactive sponge metabolites are known. The purpose of this project was to separate novel compounds from local sponges and determine whether they were products of the sponge or symbionts.

Seven novel bromoterpenes were isolated from Cacospongia sp. These metabolites were all related to the known Cacospongia compounds tribromo- and tetrabromocacoxanthene, and the cymopols isolated from a green alga Cymopolia barbata. Their structures were determined using various spectroscopic methods including 1D and 2D n.m.r. techniques. The major bromoterpenes tribromo- and tetrabromocacoxanthene were found to be biologically inactive.

Verongid sponges are well known for producing biologically active halogenated phenols, hence the metabolite content of a local verongid sponge Psammaplysilla species was studied. The bulk of this Psammaplysilla sp. consisted of the known brominated compounds aeroplysinin-1, aerothionin and homoaerothionin. The separation of the C 26 alkylated sterols was also undertaken, and it was found that aplysterol made up to sixty percent of the sterol fraction of this sponge.

The two dendroceratid sponges Aplysilla var pallida and Aplysilla violacea contained the known compounds aplysulphurin, membranolide, tetrahydrosulphurin 1, and aplyviolene and
aplyviolacene. A third compound isolated from *A. violacea* was most likely cheloviolene B but this could not be confirmed as the sample decomposed during an attempt to acetylate it.

Cell separation studies on *Cacospongia* sp. were done to determine if the bromoterpenes were of sponge or symbiont origin. *Psammaplysilla* sp. was used as a known system and showed that the brominated metabolites were concentrated in the more denser ficoll fractions, possibly spherulous cells as previously obtained from cell separation on *Aplysina fistularis*. However the cell separation on *Cacospongia* sp. was unsuccessful. This was probably due to the use of proteinase for dissociation of the sponge, resulting in cell lysis, as well as a poor fixative used for electron microscopic sampling. Thus the origin of the bromoterpenes could not be determined.
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LIST OF ABBREVIATIONS

ED  Minimum effective dose
IC  Minimum inhibitory concentration
VLFA Very long chain fatty acid
$^1$H n.m.r. Proton nuclear magnetic resonance
$^{13}$C n.m.r. Carbon nuclear magnetic resonance
h.r.e.i.m.s. High resolution electron impact mass spectrometry
u.v. Ultraviolet
i.r. Infrared
h.p.l.c. High performance liquid chromatography
N.O.E Nuclear Overhauser Effect
HMOC Proton detected Heteronuclear Multiple Quantum Coherence
HMBC Proton detected long-range Heteronuclear Multiple Bond Correlation
PDC Pyridinium dichromate
DEPT Distortionless Enhancement by Polarisation Transfer
GCMS Gas Chromatography Mass Spectrometry
ECL Equivalent chain length
M. wt Molecular weight
CDC13 Deuterated chloroform
CHCl3 Chloroform
CH2Cl2 Dichloromethane
EtOAc Ethyl acetate
MeOH Methanol
Na2SO4 Sodium sulphate
ATP Adenosine triphosphate
c.i.m.s. Chemical ionization mass spectrometry
H$_2$SO$_4$  Sulphuric acid

CMF-ASW  Calcium-magnesium free artificial sea water

EM  Electron microscopy

[α]D  Specific Optical rotation

ε  Extinction coefficient

ECL  Equivalent Chain Length

FAME  Fatty Acid Methyl Ester
CHAPTER 1

GENERAL INTRODUCTION
1.1 Intriguing Aspects of Marine Chemistry

Like terrestrial plants marine organisms are also known to produce a wide variety of metabolites. However, there still exists some differences between marine and terrestrial metabolism. Firstly halogens and isocyanide functions are more common in products of marine metabolism and rarely found as terrestrial metabolites. Secondly the marine environment provides a fairly competitive habitat for many organisms. The pH of the marine environment is normally between 8.2 - 8.5, maintained by the buffering action of sodium carbonate and bicarbonate. Seawater contains up to 4% salt and has an osmotic pressure between 15 - 25 atm. Hence marine organisms need specialized cell structures and membrane components to cope with such an environment. Further there are complex food chains operating where almost every organism is in danger of being a prey to another organism. This has led most organisms to evolve powerful biological and chemical defence mechanisms. For example a few have developed shells to protect them from the hostile environment, while some others produce toxic chemicals to make them unpalatable. The nudibranchs have often attracted the attention of biochemists and biologists as they lack the protective shells of other gastropods, but are infrequently preyed upon. It has now been documented that many nudibranchs employ secondary metabolites acquired from sponges in their diet as defensive allomones to thwart predation.

Thirdly, more caution is required when deciding the origin of products of marine metabolism. Often marine metabolites are
products from the association of two or more organisms. That is a natural product may be formed by metabolic elaboration of a precursor provided by the symbiont or obtained from dietary sources, or it maybe completely produced by the symbiont. In the last few years several macrolides and polyethers have been isolated from marine invertebrates. These unique metabolites in all cases were present as minor constituents. Their low concentration together with their structural characteristics has often led one to believe that such metabolites are the products of micro-organisms living in or on the invertebrates. The metabolite tetrodotoxin is known to be produced by symbiotic bacteria from the puffer fish and okadaic acid found in the extracts of sponge Haliclona okadai is produced by a dinoflagellate. The diketopiperazines found in extracts of Tedania ignis have been also isolated from a bacteria (Micrococcus sp.), associated with T. ignis in a laboratory media culture. Moreover, the origin of a potent antineoplastic agent tedanolide and some aromatic metabolites obtained from the sponge Tedania ignis is questionable. The fact that all were present in minute amounts and were structurally similar to known microbial metabolites, indicated that these were either produced by the microbes associated with T.ignis or from host degradation of microbial compounds, or maybe obtained by the filter feeding sponge from its diet.
1.2 Biologically-active Sponge Metabolites

The sponges are very primitive multicellular organisms. The phylum Porifera consists of more than 5000 described species that can be subdivided into 4 large classes. The class Demospongiae with a mineral skeleton or a skeleton made up of strong collagen fibres or both are the most common, most widespread and most diverse class of sponges. A few genera have neither mineral nor fibrous skeleton. Table 1.1 shows the classification of sponges as outlined by Wells and Bergquist, this classification has been used for the work throughout this thesis. Marine sponges have continually produced interesting and biologically active marine natural products. Although some of these metabolites may be produced through association with symbionts, a large number of true sponge metabolites are known. The increasing awareness of chemical defence mechanisms of sponges that are fragile and unprotected have encouraged biologists and chemists to isolate biologically active sponge metabolites. Cyclotheonamides isolated from *Theonella* sp. proved to be good leads in the search of serine protease inhibitors, which are important in development of drugs for treatment of diseases related to ageing.

Deoxytedanolide (1) isolated from the sponge *Mycale adhaerens* was found to be a good antitumour agent with T/C 190% at 0.125 mg/kg for P-388 (T/C is the ratio of tumour weights of treated and control animals, T/C values < 50 are insignificant).
A large number of biologically active marine alkaloids are known. Ptilomycalin, an antiviral and antifungal alkaloid, was obtained from a red sea sponge *Hemimycale* sp. A derivative of this alkaloid (di(trifluoroacetyl)ptilomycalin trifluoroacetate) possesses remarkable affinity for fatty acids.\(^{12}\)

Discorhabdin C (2), a dibromotyrosine derivative presumably derived from tyrosine and tryptophan, contains a new skeleton with a spiro 2,6-dibromocyclohexadienone ring. This compound was isolated from a sponge of the genus *Latrunculia* and is a highly cytotoxic agent (ED\(_{50}\) in P-388 is 0.03 ug/ml).\(^{13}\)

While, duryne, a metabolite related to the polyacetylene class of marine natural products, was obtained from the sponge *Cribrochalina dura*.\(^{14}\) It was found to be a potent cytotoxic agent with IC\(_{50}\) of 0.07 ug/ml against P-388 murine leukemia and
minimum inhibitory concentrations of 0.1 μg/ml against colon (HCT-8), lung (A-549) and mammary (MCF-7) human tumour cell lines.

Long chain fatty acids (VLFA's) (C\textsubscript{24} - C\textsubscript{34}) are increasingly being isolated from sponges. Some very long chain fatty acids especially ω6 and ω3 are of importance, since these are reported to accumulate in the brains of patients with Zellweger Syndrome, a severe neurodegenerative disorder due to deficiency in the peroxisomal β-oxidation pathway. Omega-3-fatty acids in recent years has been shown to lower blood fats, making hormones like prostaglandins and decreasing the tendency towards blood clotting.\textsuperscript{15,16}

1.3 Biosynthetic Studies on Sponges

Secondary metabolites as opposed to primary metabolites (needed for the survival and well being of the organism) have been suggested to be of no apparent utility to the organism.\textsuperscript{17} Studies on biosynthetic pathways is providing insights into the role of the secondary metabolites in the organism. It is fairly difficult otherwise to describe the role of secondary metabolites, as each metabolite may play a different role in a different organism. The ability to synthesize an array of secondary metabolites which may repel or attract other organisms has evolved as one facet of the organism's strategy for survival.\textsuperscript{18} In conducting biosynthetic studies on marine organisms, a number of factors have to be taken into consideration:\textsuperscript{19}
i) The small amounts of compounds produced in complex mixtures by marine organisms makes it difficult to isolate pure compounds in reasonable amounts for biosynthetic studies.
ii) The metabolite content of an organism may depend on time of year (seasonal variation especially in algae) or stage of development or amount of nutrients available, thus this may cause difficulty where long term biosynthetic studies are needed.

iii) Also the slow rate of synthesis in many marine organisms requires long term biosynthetic studies to get labelled material, which has to be observed against a high level of unlabelled background.

iv) Organisms used for biosynthetic work are often removed from their natural environment, and thus production of metabolite under stressed conditions may not be the same.

A large number of novel metabolites have been isolated from sponges and this indicates the operation of complex biosynthetic pathways. The major difficulty involved in study of biosynthetic pathways of sponges is the maintenance of sponges under stress free aquarium conditions for long periods. The preferred method of introducing precursors to the filter feeding sponges is the use of liposomes. Precursors are encapsulated in a membrane of cholesterol and phosphatidylcholine to resemble bacterial particle. The study of biosynthetic pathways in sponges has helped in determining in a few cases whether the metabolites are of bacterial or sponge origin. Sponge metabolites such as aerothionin and homoaerothronin are known to be located in specialized cells and thus are more likely to be products of sponge metabolism. It is hoped the isolation, identification and culturing of symbiont types will provide detailed study of translocation of nutrients between sponge and symbionts, and thus lead to the identification of the biological role and location of secondary metabolites. Cell
fractionation studies using differential gradients have now made the isolation of individual cell types easier and provided more success with study of biosynthetic pathways in sponges.

1.4 Future Outlook

There has been a phenomenal expansion in the research efforts in the field of marine invertebrates during the last decade. The motivation was derived from the fact that many novel compounds expressed biological activity and were thus of interest to the pharmaceutical industry at a time when new discoveries stemming from terrestrial sources were diminishing. The recognition of the useful pharmacological activity of marine metabolites is of great interest to organic chemists as these compounds are normally produced in minute amounts. Extraction of compounds in reasonable quantity can prove to be very difficult and costly, thereby synthesizing similar or related compounds having similar pharmacological activities can be very useful. Moreover the study of biosynthetic pathways will be of interest as many compounds produced by marine organisms are structurally and stereochemically complex, hence such compounds may need to be produced from genetic manipulation of fermentable microorganisms to enable bulk drug production.19

Finally, the hope of locating the potential antiviral, anticancer and anti-aids drug from the marine source still exists and this has led to the testing of most marine metabolites for anticancer, antiviral or anti-AIDS activity.
### Table 1.1 Classification of Demospongiae as described by Bergquist and Wells

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CHAPTER 2

THE CHEMISTRY OF METABOLITES
FROM A SPONGE OF THE GENUS
CACOSPONGIA
2.1 Introduction

Terpenes are the most abundant, nonsteroidal secondary metabolites isolated from sponges. The terpenes are classified depending on the number of isoprene (C5) units in their structure:

- Monoterpenes (C10)
- Sesquiterpenes (C15)
- Diterpenes (C20)
- Sesterterpenes (C25)
- Triterpenes (C30)
- Carotenoids (C40)
- Rubber (C5)n

The monoterpenes, sesquiterpenes, diterpenes and sesterterpenes contain the isoprene unit linked in a head to tail fashion. The triterpenoids and carotenoids are made up of two C15 and C20 units respectively linked in the middle head to head.

The earliest terpenes isolated were plant essential oils particularly oil of turpentine, known to the ancient Egyptians. Astringent and toxic properties of sesquiterpenoids and diterpenoid bitter principles figure in many folk medicines. The name terpene derived from turpentine, appears to have originated in the writings of Kekule in 1866. As extractives of terrestrial plants, the terpenoids were prized for their pleasant aroma.

With the development of sophisticated modern spectroscopic techniques terpenes have been isolated from other more varied
natural sources, including micro-organisms, insects, marine plants and animals. There is a fairly wide distribution of terpenes in marine organisms such as algae, coelenterates, echinoderms, mollusks and sponges.22

2.1.1 Terpenes from Algae

The terpenes isolated from algae are mostly associated with the red and the brown algae.23,24 Both non-halogenated and halogenated terpenes have been isolated and it is beyond the scope of this work to discuss all of them. Only halogenated terpenes isolated from algae will be briefly discussed, since the chemistry reported in this chapter describes halogenated terpenes.

Many algal monoterpenes appear to be active in pharmaceutical and agrochemical testings25,26 and have considerable activity in the natural environment of the plants.27 These range from ichthyotoxicity, fish antifeedant, antimicrobial and antifungal activities. Cyclic monoterpenoids of the red alga ochtodes crockeri were found to function as herbivore feeding deterrents, compounds related to (3) and (4) were reported.
Sesquiterpenes of the α and β chamigrene type have been isolated from red algae\textsuperscript{27,28} and some marine mollusks of the genus \textit{Aplysia}.\textsuperscript{1,23} Compounds such as nidificene (5) and the epoxide (6) are typical examples of these.

\begin{center}
\begin{tabular}{cc}
\includegraphics[width=0.4\textwidth]{image1} & \includegraphics[width=0.4\textwidth]{image2} \\
(5) & (6)
\end{tabular}
\end{center}

It had been presumed that halogenated metabolites in algae were only associated with the red and brown algae, until recently, when Hogberg \textit{et al} isolated a group of prenylated bromo-hydroquinones from a tropical green alga \textit{Cymopolia barbata}.\textsuperscript{29} Ether extracts of this algae was shown to possess antibiotic and antifungal properties. Compounds typical of cymopol (7) and cyclocymopol (8) were isolated from the extract.

\begin{center}
\begin{tabular}{cc}
\includegraphics[width=0.4\textwidth]{image3} & \includegraphics[width=0.4\textwidth]{image4} \\
(7) & (8)
\end{tabular}
\end{center}

The cymopols are likely to be products of mixed biogenetic origin, arising from bromination and geranylation of a shikimate derived quinol moiety. Some related meroterpenoids, panicein C (9), isolated
from a red sponge *Halichondria panicea* and zonarol (10), from brown algae *Dictyopteris zonarioides* may have similar biosynthetic pathways.30,31

In the formation of cymopols, bromination and cyclization proceeds through an enzyme catalysed reaction. Attack by the bromine cation on the double bond remote from the aromatic ring would give bromonium ion (11), which on cyclization would give the stable cymopol skeleton (12), with the bulky groups in the equatorial position (Scheme 2.1).

Both (7) and (8) have been synthesized using a biomimetic approach. Cymopol (7) was synthesized by alkylation of the bromohydroquinone with geraniol using boron trifluoride-ether as
catalyst\textsuperscript{29} The cyclocymopol (8) has been synthesized by Tanaka et al\textsuperscript{32} (Scheme 2.2).

Later, two new cymopols, cymobarbatol (14) and 4-isocymobarbatol (15) were isolated from the same alga\textsuperscript{33} The complete structural elucidation of these compounds was carried out using 2D n.m.r. techniques and x-ray crystallography, to confirm the cis ring fusion (of AB) in (14). The absolute configuration of (14) and (15) was also determined from the x-ray data.
Both compounds were found to be non-toxic over a broad concentration range to Salmonella typhimurium strains T-98 and T-100 and also strongly inhibited mutagenecity of 2-aminoanthracene and ethylmethanesulfonate towards the T-98 and T-100 strains respectively.

2.1.2 Terpenes from Sponges

Numerous terpene compounds have been isolated from dictyoceratid, dendroceratid and a few other sponges, and all cannot be discussed in this work. Terpenes isolated from the genus Cacospongia are reviewed, since this sponge forms the subject of this chapter, while one or two examples of each structural type from other dictyoceratid sponges are given.

The first monoterpenoid to be isolated from a sponge was adriadysiolide (16), and was isolated from a North Adriatic species of Dysidea. The structure proposed for adriadysiolide (16) was confirmed by a stereorational total synthesis. The novel skeleton of (16) was suggested to be derived biogenetically from 4-oxogeranic acid via C8-C4 cyclization followed by lactonization.
Dictyoceratid sponges are rich sources of both linear and cyclic-furanoterpenes, furanosesquiterpenes and sesquiterpenes. Linear furanoterpenes containing 21 carbon atoms mainly occur in the genera *Spongia*. Most of them terminate at both ends with a furan ring. Nitenin (17) and dihydronitenin were the first two such compounds isolated from *Spongia nitens*.35

\[
\text{(17)}
\]

The C 21 furanoterpenes are interesting from a biosynthetic point of view since the formation of these compounds either by degradation of C 25 linear sesterterpenes or biosynthesis by addition of a C 1 unit to a diterpenoid precursor, still remains to be explored.36 The first hypothesis, that these are degradation products is preferred on the grounds that C 25 sesterterpenes have been isolated from same sponges in several cases.

Sponges of the genus *Dysidea* are known to contain sulphur containing furanosesquiterpenes.37,38 A Palauan sp. of *Dysidea* was found to contain 15-acetylthioxyfurodysin lactone (18).39 The structure of (18) was determined by interpretation of its spectral data and confirmed by a synthesis that employed photo-oxidation of 15-acetylthioxyfurodysin, which co-occurs with (18) in the sponge. The first reported isolation of an unacetylated thiol was the
isolation of sesquiterpene thiofurodysinin (19) from Australian Dysidea avara.\textsuperscript{38}

![Chemical structure of sesquiterpene thiofurodysinin (19)](image)

Drimane sesquiterpenoids arenarol (20) and arenarone (21), with \textit{cis}-decalin rather than \textit{trans} stereochemistry have been obtained from Dysidea arenaria.\textsuperscript{40} Both (20) and (21) are cytotoxic with ED\textsubscript{50}'s against P-388 lymphocytic leukemia 17.5 and 1.7 μg/ml respectively ("Active" materials display an ED\textsubscript{50} < 20 μg/ml).

![Chemical structure of arenarol (20) and arenarone (21)](image)

A halogenated β-chamigrene-type sesquiterpene rogiolol acetate (22) was isolated from Spongia zimocca.\textsuperscript{41} This sponge grows in an area colonised by red alga of the genus Laurencia. Characterization of both Laurencia and the sponge metabolites showed that the 9-bromochamigrene metabolites found in the sponge were common to Laurencia, whereas rogiolol acetate (22) was only found in the
sponge, perhaps obtained from modification of a dietary sesquiterpene of the β-chamigrene type.

Two novel diterpenes rotalin A (23) and rotalin B (24) were obtained from a Mediterranean sponge *Mycale rotalis* (order Poecilosclerida). Their structures were determined from their spectral data, 2D n.m.r. experiments and a series of chemical reactions.

Diterpenes with the "spongian" skeleton have been isolated from various sponges of the genus *Spongia* and related species. These will be discussed in the section on Dendroceratid sponges (chapter 4).

Dictyoceratid sponges are also rich in the C 25 terpenoids formed by the head to tail addition of 5 isoprene units (sesterterpenes). The C 25 sesterterpenes isolated from these sponges fall into two main groups: (i) The linear series of sesterterpenes terminated by a
furan ring at one end and (ii) Tetra or pentacyclic sesterterpenes found mostly in the Spongidae family and some taxonomically related genera of the Thorectidae.

The linear C 25 sesterterpenes typified by variabilin (25), isolated from *Ircinia variabilis*, *Faciospongia fovea*, and more recently from a *Sacotragus* sp., are found in a number of dictyoceratid sponges. The stereochemistry of the 20,21 bond in variabilin was determined by examination of the 20-0-methyl derivative of variabilin and isolation of variabilin isomer with 20E configuration from a sponge of the genus *Sarcotragus*. The complete stereochemistry of variabilin (25) was established to be 7E, 12E, 20Z.

![Variabilin](image)

Cyclic sesterterpenes have been isolated from *Cacospongia, Hyrtios, Carteriospongia, Lendenfeldia, Hyatella* and some *Spongia* species. *Spongia officinalis* collected in the Tyrrhenian sea contained deoxoscalarin (26). Deoxoscalarin (26) and 12-epideoxoscalarin (27) were also found in *Spongia nitens* and recently 16-deacetyl-12-episcalarafuranacetate (28), deoxo-scalarin acetate (29) and (-)-12-epideoxoscalarin have been isolated from a North Adriatic *Spongia officinalis*. 24-Acetoxy-12-desacetyl-12-epideoxoscalarin (30) was obtained from *Hyatella intestinalis*, together with
heteronemin (31), which was initially isolated from *Hyrtios erecta*\textsuperscript{51,52}

\begin{align*}
(26) & \quad R_1 = \alpha \text{OAc}, \ R_2 = H, \ R_3 = CH_3 \\
(27) & \quad R_1 = \beta \text{OAc}, \ R_2 = H, \ R_3 = CH_3 \\
(29) & \quad R_1 = \alpha \text{OAc}, \ R_2 = Ac, \ R_3 = CH_3 \\
(30) & \quad R_1 = \beta \text{OH}, \ R_2 = H, \ R_3 = CH_2 \text{OAc}
\end{align*}

Sponges of the genus *Carteriospongia* and *Phyllospongia* have gained interest recently because these are unique in producing scalaranes with additional methyl groups at C 24 (24-methyl scalaranes)\textsuperscript{53} and C 19 (or 20).\textsuperscript{54,55} The first bisalkylated scalarane was assigned as having the 4\(\beta\)-ethyl substituent\textsuperscript{55} i.e. methylated at C 19. The origin of these extra methyl groups is yet to be determined.

25-Norscalaranes are rare and until recently hyrtial isolated from *Hyrtios erecta*\textsuperscript{52} and norscalaran-24-one from *Carteriospongia foliascens*\textsuperscript{56} were the only reported examples. Two novel bisalkylated norscalaranes isolated from *Carteriospongia foliascens* (32) and (33) are new additions to this group.\textsuperscript{54}
2.1.2.1 Terpenes from *Cacospongia* species

Scalarin (34) was the first sesterterpene compound isolated and was obtained from *Cacospongia scalaris*. The structure of scalarin was determined by $^1$H n.m.r. and i.r. data and a series of chemical reactions. Support for the lactol ring was obtained from the n.m.r spectrum of (34), taken using pyridine-d$_5$/NaOD, which showed evidence for ring opening to an aldehyde. Conversion of (34) to a known compound (38) (Scheme 2.3), indicated the stereochemistry of the AB ring junction was *trans*. Base catalysed deuterium exchange of ketolactone (36) was used to decide the position of the acetoxy group as C 12, since deuterium atoms were incorporated on C 11 and C 17. The acetoxy group was established to be axial from $^1$H n.m.r. coupling data.
Scheme 2.3 Chemical correlation of scalarin

12-Episcalarin (39) was later isolated from *Spongia nitens*, showing the close taxonomic relationship between the two genera. Related sesterterpenes scalaradial (40), episcalaradial (41), a cytotoxic metabolite desacetyl scalaradial (42) and heteronemin (31) have all been isolated from *Cacospongia mollior* and *Cacospongia scalaris*.58,59
Three molliorins obtained from *Cacospongia mollior* are probably products of reactions of the scalaradials with primary amines. The structure of molliorin-a (43), -b (44) and -c (45) were deduced from spectral properties.\textsuperscript{60,61,62}

Moreover, furoscalarol (46) with an alternate cyclization in D ring was also isolated from *Cacospongia mollior*.\textsuperscript{62,63} It was suggested at the time of isolation that furoscalarol might arise through a scalarin-type cyclization with a different closure of ring D, or from a scalarane nucleus by a methyl migration. Recent isolation of 25-norscalaranes from *Carteriospongia foliascens* has led to the suggestion by Tucker and Quinn,\textsuperscript{54} that (46) may be of the class 24-methyl-25 norscalarane. Thus (46) could represent the first 25-norscalarane isolated from a sponge.
Another sesterterpene cacosphionolide (47) with a new carbon skeleton was isolated from *Cacospongia mollior* of Northern Adriatic. The structure of cacosphionolide (47), a potent antitumour agent, was determined by 2D n.m.r. techniques and by conversion of (47) into its diastereoisomeric acetates.

Very few C 21 furans have been isolated from sponges of the genus *Cacospongia*, cacosphionone A (48), cacosphionenone A (49) and B (50) are metabolites of *Cacospongia scalaris*.

So far the terpenes isolated from sponges of the genus *Cacospongia* all are non-halogenated. Halogenated terpenes have been restricted to the algae. However, in 1988, our group working at Wollongong
University, isolated two novel meroterpenoids tribromocacoxanthene (51) and tetrabromocacoxanthene (52) from a *Cacospongia* sp. From the same sponge the known non-halogenated terpene metabolite 12-epideoxoscalarin (27) was also obtained. The structure of (51) was determined by $^1$H, $^{13}$C n.m.r. and single crystal x-ray diffraction analysis. The stereochemistry of the AB ring junction in (51) was established to be *trans*. The structure of (52) was decided from its $^1$H, $^{13}$C n.m.r., mass spectroscopic data and by comparison with (51).

The traces of red algae found on the sponge was analysed, but no brominated compounds were found, suggesting the brominated metabolites were of sponge origin and represent the first examples of brominated terpenes to be isolated from marine sponges. Subsequently Pietra *et al* reported rogiolol acetate (22) from *Spongia zimocca*.

![Chemical structures](image-url)

(51) $X = H$

(52) $X = Br$
2.1.3 Fatty acids and Sterols from Demospongiae

2.1.3.1 Fatty Acids

Sponges of the class Demospongiae have high levels of C\textsubscript{24}-C\textsubscript{34} fatty acids. The accumulation of C\textsubscript{24}-C\textsubscript{34} acyl chains in the membrane may reduce its flexibility and make reactions with the membrane more difficult than with their C\textsubscript{14}-C\textsubscript{22} counterparts. Some members of the demospongiae, e.g. *Jaspis Stellifera* contain a number of these novel very long chain fatty acids (VLFA's). These are known to be biosynthesized from the short-chain (C 15 and C 16) fatty acids. The short chain precursors are obtained from bacteria then homologated and desaturated by the sponge.

A 19,22,25,28,31-triacontapentaenoic acid (53) was among the VLFA's reported from *Petrosia pellasarca*. These VLFA's were associated with the phosphatidyl-ethanolamine which indicates their participation in the membrane lipid bilayers. The extra bulk of their longer chain probably makes the bilayer thicker.

Brominated fatty acids though uncommon in marine organisms, have been found in sponges of a few genera, especially *Xestospongia* and *Petrosia*. The first dibrominated straight-chain...
C16 acetylenic acid \((7E,13E,15Z)-14,16\text{-dibromo-3,13,15\text{-hexadecatrien-5-ynoic acid}}\) (54) was identified from the sponge *Xestospongia muta*.\(^{73}\) Later, other brominated acids were isolated from some *Xestospongia* sp.\(^{74,75}\)

\[
\text{Br-CH=CH-CBr=CH-(CH}_2\text{)}_4\text{-CH=CH-C=-(CH}_2\text{)}_3\text{-COOH} \quad (54)
\]

6-Bromo-25-methylhexacosa-5,9-dienoic acid (55) and its 24-methyl homologue (56) were obtained from a *Petrosia* sp.,\(^{76}\) while two new brominated fatty acids, \((5E,9Z)-6\text{-bromo-5,9-tetracosadienoic acid}\) and \((5E,9Z)-6\text{-bromo-5,9-pentacosadienoic acid}\) have been isolated from a tropical marine sponge *Amphimedon terpenensis*.\(^{77}\)

\[
\begin{align*}
\text{HO}_2\text{C}(\text{CH}_2)_2 & \text{ Br} & \text{ (CH}_2\text{)}_2 & \text{ (CH}_2\text{)}_{14} \\
\text{HO}_2\text{C}(\text{CH}_2)_2 & \text{ Br} & \text{ (CH}_2\text{)}_2 & \text{ (CH}_2\text{)}_{13}
\end{align*}
\]

The bromo function of the 6-Br-\(\Delta^{5,9}-26:2\) acid in a *Hymeniacidon* sponge has been shown to be introduced in the final step of biosynthesis\(^{78}\) by use of [1-\(^{14}\)C] sodium salt of \(\Delta^{5,9}-26:2\) acid. It was earlier showed that the \(\Delta^{5,9}-26:2\) acid was biosynthesized by chain elongation of precursors with seven or more two-carbon units, followed by desaturation starting at either the \(\Delta^5\) or \(\Delta^9\) positions.\(^{79}\) Therefore, bromination takes place after chain desaturation in the formation of these novel brominated fatty acids.
2.1.3.2 Sterols

The sterol content of sponges varies in that some sponges contain complex mixtures of over seventy sterols while others have one or two major sterols making up 50-100% of the sterol fraction. \( \Delta^5 \) sterols are the most widely distributed and sponges are capable of converting \( \Delta^5 \) sterols to \( \Delta^5,7 \) sterols.\(^{80}\) It has been suggested that sponges biosynthesize their sterols either from acetate and mevalonate, or obtain them from either symbiotic relationships or dietary sources, with or without modification.\(^{19}\) By use of lipid precursors such as squalene it was shown that many sponges are capable of \textit{de novo} sterol biosynthesis though to different extents.\(^{81}\)

A significant number of sponges possess novel sterols. The unusually alkylated sterols in \textit{Aplysina (=Verongia) fistularis} were found as components of spherulous cell membrane.\(^{82}\) Cholesterol may function as a membrane stabilizer by maintaining the integrity of cell membranes.\(^{68}\) In some sponges cholesterol is completely replaced by unconventional sterols. Strongysterol (57) is the sole sterol of \textit{Strongylophora purissima},\(^{83}\) hence the functional role of cholesterol may be fulfilled by the unconventional sterols together with the VLFA's, present in the cell membrane of certain sponges.

![Sterol Structure](image)
Polyhydroxylated sterols have been isolated from some *Ircinia*, *Dysidea*, *Hippospongia* and *Spongia* sp.\textsuperscript{84,85,86} \(9,11\)-Secosterol (58) was established to be ichthyotoxic and antibacterial by Faulkner and Capon.\textsuperscript{87} In four dictyoceratid sponges containing polyhydroxylated sterols, \(\Delta^5,7\) sterols predominate in their monohydroxy sterol fractions. There is speculation that \(3\beta,6\alpha\)-dihydroxy-\(\Delta^7\)-sterols and \(3\beta,5\alpha,6\beta\)-trihydroxy-\(\Delta^7\)-sterols are formed from the \(\Delta^5,7\)-3\(\beta\)-hydroxy sterols.\textsuperscript{85}

![Chemical structures](58) ![Chemical structures](59)

Certain sponges such as *Amphimedon* sp. contain only the conventional \(\Delta^5,7\) sterols and these are most likely obtained through dietary sources.\textsuperscript{88}

It has been indicated that the unusual terpene diisocyanoadociane (59) may substitute for sterols in the *Amphimedon* sp.\textsuperscript{89} The structure of diisocyanoadociane (59) mimics the rigid tetracyclic ring of sterols and possesses suitable polar substituents. Sterol formation is known to be a biosynthetic alternative to terpene biosynthesis.\textsuperscript{89} It appears certain sponges have not evolved the
ability to synthesize sterols *de novo* and thus retain the terpene pathway.

The isolation of the novel brominated terpenes (51) and (52) from the *Cacospongia* sp. led us to further investigate the crude extract of this sponge, to see if there were any other brominated metabolites. Thus the work described in this chapter continues our chemical investigation of *Cacospongia* sp.

Seven minor brominated meroterpenoids related to (51) and (52) were isolated and their structure determination is described below. Other brominated metabolites are also currently being isolated and analysed and it is interesting to note that this *Cacospongia* sp. is a rich source of brominated terpenes. The brominated metabolites appear to constitute the bulk (60%) of the crude extract.

Brominated and very long chain fatty acids are being discovered in ever increasing number of sponges. Given the ability of *Cacospongia* sp. to make brominated compounds, it was of interest to see if this sponge had any novel brominated fatty acid or VLFA's and this was done by analysis of the fatty acid components using G.C.M.S. The rich terpene chemistry of this sponge was in contrast with its low sterol content. Thus an analysis of the sterols was undertaken by h.p.l.c. and n.m.r. to see if there were any unconventional sterols present. Finally the biological function of the brominated meroterpenoids was investigated and this will be discussed in chapter 5.
2.2 Results and Discussion

Tribromocacoxanthene (51) and tetrabromocacoxanthene (52) were isolated by our group as major components of a sponge of the genus *Cacospongia*. Structure elucidation of seven minor metabolites from the same sponge is reported in this section. These are related to compounds (51) and (52) and hence where possible chemical correlations have been made through comparison with these.

An analysis of the fatty acids and sterols of *Cacospongia* sp. crude extract is also discussed in this section.

2.2.1 Minor Metabolites of *Cacospongia* species

Seven minor metabolites (60-66) were isolated by extraction of wet sponge with ethanol (95%), partitioned into ethyl acetate and water, and separated by silica flash column chromatography followed by preparative high performance liquid chromatography. Two different structural types were obtained: (i) hydroxy derivatives of tribromocacoxanthene (60)-(63) and (ii) Spiro-fused metabolites possessing a 3'-oxygenated substituent (64)-(66).
2.2.1.1 Hydroxy Derivatives of Tribromocacoxanthene (60)-(63)

2.2.1.1.1 9R-Hydroxytribromocacoxanthene (60)

Compound (60) systematically named \((2S,4aS,9R,9aS)2,7\text{-dibromo-}4a\text{-bromomethyl-}1,1\text{-dimethyl-}2,3,4,4a,9,9a\text{-hexahydro-}1H\text{xanthen-9-ol}\) was obtained by h.p.l.c. using 4% ethyl acetate in hexane in a yield of 0.007%. The \(^1\text{H}\) n.m.r. spectrum of the u.v. active (60) showed two methyl singlets at \(\delta1.42\) and 1.12, six protons in the region \(\delta1.5-2.5\), 4 deshielded protons geminal to either oxygen or bromine and 3 aromatic protons (Figure 2.1). The cluster of peaks in the mass spectrum at 480-486 suggested the presence of 3 bromines. The molecular formula was established as \(\text{C}_{16}\text{H}_{19}\text{Br}_3\text{O}_2\) from high resolution electron impact mass spectrometry (h.r.e.i.m.s.), therefore the compound was tricyclic. The infrared spectrum showed the presence of a hydroxyl group \((\nu_{\max}3583\text{ cm}^{-1}\) sharp) and this was confirmed by the disappearance of \(\delta1.49\) signal in the \(^1\text{H}\) n.m.r. spectrum after shaking with \(\text{D}_2\text{O}\) shake. The ether bands in the i.r. spectrum were seen at 1147 and 1005 cm\(^{-1}\). \(^{13}\text{C}\) n.m.r. data (table 2.1) showed six aromatic signals as in tribromocacoxanthene (51) and ten other carbon signals. The ultra-violet spectrum gave the aromatic ring chromophore absorption of 228 and 285 nm.
The structure of (60) was determined from $^1H-^1H$ decoupling experiments and Nuclear Overhauser Enhancement (N.O.E.) studies. In the $^1H$ n.m.r. two mutually coupled signals at $\delta 2.70$ and 2.81 which were present in (51), were replaced in (60) by a deshielded proton signal at $\delta 4.81$ coupled to protons at $\delta 1.49$ (d, J 9.8 Hz, exchanges with $D_2O$) and $\delta 2.16$ (d, J 10.0 Hz) (Figure 2.1). This coupling data suggested that the hydroxyl group was present on C 9, in the equatorial position, as H 9 and H 9a show a diaxial coupling of 10.0 Hz. The proximity of the hydroxyl group caused a downfield shift of H 8 signal by 0.3 ppm compared to (51). The $^{13}C$ n.m.r. signal for C 9 was shifted downfield to 66.1 ppm together with that of C 4a, C 8a, C 9a and C 12 relative to their chemical shift values in (51). Assignment of the bromo-cyclohexane ring signals was carried out as follows: Irradiation of the double doublet at $\delta 4.04$ assigned to H 2 (methine signal) affected the proton signals at $\delta 2.00$ (J 12.7 Hz) and $\delta 2.30$ (J 4.0 Hz). The signal at $\delta 2.00$ (H 3ax) was coupled to proton signals at $\delta 2.30$ (H 3eq, J 14.3 Hz), $\delta 1.61$ (H 4ax, J 13.7 Hz) as well as to $\delta 2.49$ (H 4eq, J 3.4 Hz). The $\delta 2.00$ signal was assigned to H 3ax position consistent with diaxial coupling of 12.7 Hz to H 2 ($\delta 4.04$) and the $\delta 2.30$ signal to H 3eq respectively. Irradiation of $\delta 2.49$ (H 4eq) signal caused the quartet of doublets at $\delta 1.61$ (H 4ax) to change to a quartet (J 13.7 Hz, typical of a geminal coupling),
Figure 2.1 400 MHz $^1$H n.m.r. spectra of (60). Solutions in CDCl$_3$; $\delta$ values in ppm, referenced at $\delta$ 7.25 for CHCl$_3$. 
while the doublet of quartets at $\delta 2.00$ changed to a quartet ($J$ 3.4 Hz, coupling constant which is eliminated by double irradiation) and the doublet of quartets at $\delta 2.30$ changed to a doublet of triplets ($J$ 3.8 Hz). The $\delta 2.00$ and $\delta 2.30$ signals appeared to be the axial-equatorial and equatorial-equatorial coupled protons vicinal to the $\delta 2.49$ signal. The doublet at $\delta 3.66$ ($J$ 11.7 Hz) was coupled to a double doublet at $\delta 3.48$. Both of these are the CH$_2$ protons on C 13, and the coupling observed ($J$ 11.7 Hz) was consistent for a germinal coupling, where the CH$_2$ is attached to an electronegative group. A W-coupling of 2.5 Hz was observed between H 4ax at $\delta 1.61$ and the $\delta 3.48$ signal (assigned for CH$_2$Br), indicating a restricted rotation of the bromo-methyl group. The W-coupling of (60) is shown in Figure 2.2 (bold line).

![Figure 2.2 W-coupling in (60)](image)

In the mass spectrum of (60) the ions at m/z 201 and 121 could be attributed to the fragmentation (Scheme 2.4).

![Scheme 2.4 Fragmentation of ions m/z 201 and 121 in h.r.e.i.m.s.](image)
Table 2.1. $^1$H and $^{13}$C n.m.r. data for compound (60).
Solutions in CDCl$_3$; $\delta$ values based on $\delta$ 7.26 ($^1$H) and 77.0 ($^{13}$C) for
CHCl$_3$. In $^1$H data, coupling constants are in parentheses.

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta$ $^{13}$C</th>
<th>$\delta$ $^1$H</th>
<th>Responses in HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.9</td>
<td>-</td>
<td>11,12, H 9a, H 2</td>
</tr>
<tr>
<td>2</td>
<td>65.2</td>
<td>4.04, dd (12.7, 4.0)</td>
<td>Me 11&amp;12</td>
</tr>
<tr>
<td>3</td>
<td>31.1</td>
<td>2.00, dddd (14.3, 13.7, 12.7, 3.4)</td>
<td>Me 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$eq$ 2.30, dddd (14.3, 4.0, 3.8, 3.8)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>36.2</td>
<td>1.61, dddd (13.7, 13.7, 3.8, 2.5)</td>
<td>H 13 ($\delta$ 3.48)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$eq$ 2.49, ddd (13.7, 3.8, 3.4)</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>78.3</td>
<td>-</td>
<td>H 9a</td>
</tr>
<tr>
<td>5</td>
<td>119.6</td>
<td>6.74, d (8.6)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>130.6</td>
<td>7.31, dd (8.6, 2.2)</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>113.9</td>
<td>-</td>
<td>H 5</td>
</tr>
<tr>
<td>8</td>
<td>132.5</td>
<td>7.59, d (2.2)</td>
<td>H 6</td>
</tr>
<tr>
<td>8a</td>
<td>128.1</td>
<td>-</td>
<td>H 5</td>
</tr>
<tr>
<td>9</td>
<td>66.1</td>
<td>$ax$ 4.81, t (10.0, 9.8)</td>
<td>H 9a</td>
</tr>
<tr>
<td>9a</td>
<td>55.9</td>
<td>2.16, d (10.0)</td>
<td>Me 11&amp;12</td>
</tr>
<tr>
<td>10a</td>
<td>149.6</td>
<td>-</td>
<td>H 6 &amp; H 8</td>
</tr>
<tr>
<td>11</td>
<td>18.8</td>
<td>1.12, s (CH$_3$)</td>
<td>Me 12 &amp; H 9a</td>
</tr>
<tr>
<td>12</td>
<td>31.1</td>
<td>1.42, s (CH$_3$)</td>
<td>Me 11 &amp; H 2</td>
</tr>
<tr>
<td>13</td>
<td>32.7</td>
<td>3.66, d (11.7)</td>
<td>H 9a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.48, dd (11.7, 2.5)</td>
<td></td>
</tr>
<tr>
<td>OH</td>
<td>1.49</td>
<td>d (9.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OH</td>
<td></td>
</tr>
</tbody>
</table>
The relative stereochemistry of (60) was established by $^1$H n.O.e. experiments. The nuclear Overhauser effect (n.O.e) is the change in intensity observed when a nucleus is irradiated with a particular frequency ($\omega_2$), and this causes an increase or decrease in the intensity of another nucleus close in space recorded with observing frequency ($\omega_1$). The ($\omega_1$) effect is proportional to $r^{-6}$, where $r$ is the distance between the nuclei, implying that the effect gets reduced rapidly with increasing $r$, and is not normally observed for $r > 0.3$ nm. Since the n.O.e. effect is occurring through-space, it does not depend on whether the two nuclei are spin-spin coupled or not. Since small n.O.e.s are not easily detected from just the spectrum of the irradiated sample, a difference method is employed. Nuclear Overhauser effect difference spectroscopy is obtained by accumulating a number of scans with the system perturbed, with an equal number obtained without the perturbation, by shifting the irradiating frequency away from the resonance. Subtraction of the unperturbed from the perturbed free induction decay (FID), followed by Fourier Transformation (on Jeol) generates a spectrum showing only the difference between the two. The difference spectra retains the increase in signal-to-noise produced by n.O.e. while at the same time giving a fully coupled spectrum, with cancellation of unwanted peaks.

The n.O.e.s obtained in our experiments were not quantified because of small sample size and poor signal to noise ratio. Irradiation of H 9 at $\delta$ 4.81 gave n.O.e. enhancement of H 8 at $\delta$ 7.59, w-coupled H 13 at $\delta$ 3.48 and H3C 11 at $\delta$ 1.12 (Fig. 2.3), while irradiation of H 2 ($\delta$ 4.04) resulted in n.O.e. enhancement of H 9a at $\delta$ 2.16, H 3eq at $\delta$ 2.30 and H3C 12 at $\delta$ 1.42 (Fig. 2.4). The above
n.O.e. data allowed assignment of the $^1$H n.m.r. values for $\text{H}_3\text{C} 11$ and $\text{H}_3\text{C} 12$ which otherwise were ambiguous. Irradiation of the $\text{H} 9a$ signal at $\delta 2.16$ gave n.O.e.'s onto $\text{H} 2$ ($\delta 4.04$) and $\text{H} 4ax$ at $\delta 1.61$.

![Figure 2.3 n.O.e. on irradiation of H 9](image1)

![Figure 2.4 n.O.e. on irradiation of H 2](image2)

The assignment of the aromatic $^{13}\text{C}$ n.m.r signals was clarified firstly by synthesis of model compound (68) and secondly by theoretical calculations.

$o$-Cresol was converted into 2-methyl-4-bromoanisole (68) by bromination using bromine in carbon tetrachloride, followed by methylation using dimethyl sulphate.$^{92}$ (Scheme 2.5).

![Scheme 2.5 Synthesis of 2-methyl-4-bromo-anisole from $o$-cresol](image3)
The $^{13}$C n.m.r. values of (68) (table 2.2) and (51) were compared and used as an initial guide to assign the $^{13}$C n.m.r values of (60). For compound (68) the carbon assignments have been clarified from literature values, assigned from experimental and calculated values on evidence of steric effects.\textsuperscript{93}

![Chemical structures of compounds (68) and (51)](#)

**Table 2.2. $^1$H and $^{13}$C n.m.r. data for compound (68).**

Solutions in CDCl$_3$ (90 MHz).

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Position</th>
<th>$\delta^{13}$C</th>
<th>$\delta^{1}$H</th>
</tr>
</thead>
<tbody>
<tr>
<td>(68)</td>
<td>1</td>
<td>156.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>129.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>133.2</td>
<td>7.28, s</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>112.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>129.3</td>
<td>7.30, d (9.2)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>111.5</td>
<td>6.71, d (9.2)</td>
</tr>
<tr>
<td>OMe</td>
<td>55.4</td>
<td>3.83, s</td>
<td></td>
</tr>
<tr>
<td>Me</td>
<td>15.9</td>
<td>2.22, s</td>
<td></td>
</tr>
</tbody>
</table>

In compound (60), C 5 was assigned the carbon value 119.6 ppm and 113.9 ppm value was assigned to C 7. These were consistent
with the signal intensities in $^{13}$C n.m.r. (C 7 non-protonated carbon therefore signal is weaker). The signals at 132.5 and 130.6, for C 6 and C 8 could not be assigned further at this stage.

The above assignments for (60) were compared to the theoretical values calculated using the equation provided by Wehrli and Wirthlin\textsuperscript{94} for substituted benzenes.

$$\delta c(k) = 128.5 + Ai(R) \quad \text{(Equation 2.1)}$$

where $\delta c(k)$ is the chemical shift of a carbon k and Ai(R) represents the chemical shift increment for a substituent R in the "i" th position (Cl, ortho, meta or para). The results are tabulated in table 2.3.

**Table 2.3** Comparison of measured $^{13}$C n.m.r data of (60) with that calculated using equation (2.1). Solutions in CDCl\textsubscript{3} (400 MHz).

<table>
<thead>
<tr>
<th>Carbon No.</th>
<th>Theoretical Values</th>
<th>Measured Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>115.8</td>
<td>119.6</td>
</tr>
<tr>
<td>6</td>
<td>130.0</td>
<td>130.6\textsuperscript{A}</td>
</tr>
<tr>
<td>7</td>
<td>115.3</td>
<td>113.9</td>
</tr>
<tr>
<td>8</td>
<td>133.7</td>
<td>132.5\textsuperscript{A}</td>
</tr>
<tr>
<td>8a</td>
<td>125.1</td>
<td>128.1</td>
</tr>
<tr>
<td>10a</td>
<td>159.1</td>
<td>149.6</td>
</tr>
</tbody>
</table>

\textsuperscript{A} Assignment may be interchanged.
From the data for compound (68) (Table 2.2), and theoretical calculations (Table 2.3), C 6 and C 8 have approximate carbon values of 130 and 133 ppm respectively. However compound (51) has C 6 at 132.5 and C 8 at 130.6 ppm, and these assignments were confirmed from 2D n.m.r. experiments.

Therefore the assignments for C 6 and C 8 for (60) were subsequently confirmed by HMQC and HMBC data. HMQC is the inverse detected direct Heteronuclear Multiple Quantum Coherence experiment used to correlate protons to their respective carbon signals. Thus all the protonated carbon signals can be assigned from the HMQC data. In the HMQC spectrum, the δ 7.31 double doublet assigned for H 6 was coupled to the 132.5 ppm carbon signal and the δ 7.59 doublet to the carbon signal at 130.6 ppm.

The HMBC is used to detect long range Heteronuclear Multiple Bond Correlations. Information about non-protonated (quaternary) carbons can be obtained. The HMBC result for compound (60) is summarized in table 2.1 and this further confirms the proton-carbon assignments for (60). The absolute stereochemistry of (60) was assumed to be same as that of (51) and (52), established by x-ray crystallography.

2.2.1.1.2 9S-Hydroxytribromocacoxanthene (61)

Compound (61) was eluted from the preparative h.p.l.c. column using 2% ethyl acetate in hexane in a yield of 0.002%. The 1H n.m.r. data resembled that of (60), except the triplet in (60) at δ 4.81 (J 10.0, 9.8 Hz) for C 9 was replaced by a triplet at δ 5.02 (J 3.7, 2.2
Hz), so epimeric at C 9. This change at C 9 caused the H 8 proton signal to move upfield by 0.2 ppm, while the w-coupled H 13 and the H3C 11 signals moved downfield by 0.85 and 0.3 ppm respectively. Again the presence of the hydroxyl group was seen by the disappearance of the δ 1.81 signal in the 1H n.m.r. on D2O shake. In the mass spectrum m/z 465 indicated M+ - H2O peak. The hydroxy signal (δ 1.81) in the 1H n.m.r. was coupled to δ 5.02 (J 2.2 Hz) proton. Thus δ 5.02 proton and the hydroxy signal (δ 1.81) were assigned to the same carbon (C 9). The δ 5.02 signal was further coupled to the proton at δ 2.08 (J 3.7 Hz), assigned to H 9a. Due to the small coupling between H 9 and H 9a protons, they were expected to be arranged in an equatorial-axial fashion rather than the diaxial arrangement as in (60), thus placing the OH substituent in the axial position on C 9. The position of the hydroxyl group explains the deshielding of the H3C 11 and H 13 (due to the proximity of the OH substituent). The mass spectra data did not give a molecular ion. 9α-Hydroxytribromocacoxanthene (61) was found to be unstable and decomposing, thus the 13C n.m.r. and the mass spectral data could not be obtained for pure (61). Due to the cis orientation of the OH and the CH2Br substituents, further reaction was taking place. In fact, (61) was found to be decomposing to (62) by a SN2 displacement reaction.
2.2.1.1.3 **Dibromocyclocacoxanthene (62)**

2,7-Dibromo-1,1-dimethyl-1,2,3,4,9,9a-hexahydro-9,4a-epoxymethano-4aH-xanthene (62), though isolated from natural sources by h.p.l.c. (2% ethylacetate in hexane) in a yield of 0.001%, could be an artefact, considering the ease of conversion of (61) into (62). The $^1$H n.m.r. spectrum of (62) contained four deshielded protons between $\delta$ 3.9-4.8, three aromatic signals at $\delta$ 6.63, 7.15 and 7.25, two methyl singlets at $\delta$ 1.14 and 1.17 and five other high field protons between $\delta$ 1.1-2.5 (Table 2.4). The aromatic proton arrangement appeared similar to (60) and (61), and the $\delta$ 4.03 signal from decoupling experiment was also consistent for C 2, as in (51) or (60) and (61). The $\delta$ 4.16 and $\delta$ 3.93 doublets were the geminally coupled CH$_2$ protons (C 13) and the $\delta$ 4.73 signal was for a proton on an oxygenated carbon as in (60) and (61). The absence of a hydroxyl group was confirmed by the absence of the OH stretch in the i.r. spectrum, and further no signal being affected by D$_2$O shake in the $^1$H n.m.r. From the $^{13}$C n.m.r. data it was found that the C 9 signal at $\delta$ 66.1 ppm in (51) and the C 13 signal at 32.7 ppm were replaced by the 77.7 ppm and 76.2 ppm signals in (62). The h.r.e.i.m.s. data gave the molecular formular C$_{16}$H$_{18}$Br$_2$O$_2$, i.e. the absence of HBr compared to (58) or (61). Therefore (62) was considered to be a $S_N2$ cyclization product of (61), consistent with the absence of the hydroxyl signal and the presence of deshielded $^{13}$C n.m.r. signals at 85.3, 77.7 and 76.2 ppm. The W-coupling present in (60) and (61) was also missing in (62) ($S_N2$ inversion of the C13 centre). Displacement of bromine by oxygen has resulted in
deshielding of H 4ax (trans) signal and shielding of H 4eq (cis) proton relative to (61).\textsuperscript{96} The $^{13}$C n.m.r. data showed downfield shift for C 4a (85.3 ppm), while C 4 (γ to substituent) shifted upfield to 31.0 ppm. relative to (60).

The HMQC experiment was used to confirm the C-H correlations, that is the protonated carbon signals were assigned, while the HMBC (proton-detected long range heteronuclear chemical shift correlation) experiment gave information about the quaternary carbons (Appendix 1). In the HMQC experiment each proton signal was correlated to its respective carbon signal. The methyl signal at 17.5 ppm was correlated to the proton signal at δ 1.17, (H$_3$C 11) clarifying the carbon data ambiguity for the methyl signals. The 30.6 ppm signal in the $^{13}$C spectrum was associated with the H 3ax and the H 3eq protons at δ 2.03 and 2.25, while the 31.0 ppm signal was correlated to H 4ax and H 4eq signals at δ 2.14 and 2.09 respectively. The 53.9 ppm carbon signal corresponded to δ 2.19 signal in the proton spectra assigned to H 9a. The HMQC correlation in this instance was useful in distinguishing between the otherwise overlapping H 3, H 4 and H 9a signals in the proton spectra of (62). Further the carbon signal at 76.2 ppm was correlated to the -CH$_2$-O-protons at δ 3.93 and 4.16, while the 77.7 ppm signal corresponded to δ 4.73 proton for H 9, clearly assigning the $^{13}$C n.m.r. data for both these carbons. Moreover it was difficult to distinguish between C 6 and C 8 signals in the $^{13}$C n.m.r. as both these carbons had very similar chemical environment. Using the HMQC data, it was clear that the H 6 proton at δ 7.25 was coupled to the signal at 132.6 ppm in the carbon spectrum, and the H 8 proton at δ 7.15 was coupled to 129.2 ppm signal. From the HMBC experiment quaternary
assignments could be verified. The correlations obtained from the HMBC experiment performed are listed in Table 2.4. In particular the C 4a signal at 85.3 ppm was long-range correlated to both H 3 signals, one of the methylene protons at δ 4.16 and the H 9 signal (Fig. 2.5), while C 1 was correlated to the proton signals of both methyls, H 3eq, H 2 and H 9a (Fig. 2.6). The HMQC and the HMBC experiments together confirmed the arrangement of the carbon skeletal moiety, consistent with the proposed structure.

Figure 2.5 and Figure 2.6 HMBC correlations for C 4a and C 1 in (62)
Table 2.4. $^1$H and $^{13}$C n.m.r. data, and long range correlation responses (HMBC) for (62). Solutions in CDCl$_3$ (400 MHz).

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta^{13}$C</th>
<th>$\delta^{1}$H</th>
<th>Responses in HMBC (H)</th>
</tr>
</thead>
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<td>_</td>
</tr>
<tr>
<td></td>
<td></td>
<td>eq 2.25, dddd (13.4, 6.6, 3.4, 3.0)</td>
<td>_</td>
</tr>
<tr>
<td>4</td>
<td>31.0</td>
<td>ax 2.14, ddd (13.7, 10.7, 4.6)</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td></td>
<td>eq 2.09, ddd (13.7, 4.9, 3.0)</td>
<td>_</td>
</tr>
<tr>
<td>4a</td>
<td>85.3</td>
<td>_</td>
<td>3(\beta), 4(\alpha/\beta), 9, 13 (4.16)</td>
</tr>
<tr>
<td>5</td>
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<td>6.63, d (8.6)</td>
<td>_</td>
</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>112.1</td>
<td>_</td>
<td>5, 8</td>
</tr>
<tr>
<td>8</td>
<td>129.2</td>
<td>7.15, d (2.3)</td>
<td>6, 9</td>
</tr>
<tr>
<td>8a</td>
<td>129.1</td>
<td>_</td>
<td>5, 9a</td>
</tr>
<tr>
<td>9</td>
<td>77.7</td>
<td>eq 4.73, s</td>
<td>8, 13 (3.93)</td>
</tr>
<tr>
<td>9a</td>
<td>53.9</td>
<td>2.19, s</td>
<td>4(\alpha/\beta), 9, 11, 12</td>
</tr>
<tr>
<td>10a</td>
<td>152.2</td>
<td>_</td>
<td>5, 6, 8, 9</td>
</tr>
<tr>
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<td>29.7</td>
<td>1.17, s (CH$_3$)</td>
<td>2(\beta), 9(a), 12</td>
</tr>
<tr>
<td>12</td>
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<td>3(\beta), 9(a), 11</td>
</tr>
<tr>
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<td>76.2</td>
<td>4.16, d (10.7)</td>
<td>4(\alpha/\beta), 9, 9a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.93, d (10.7)</td>
<td></td>
</tr>
</tbody>
</table>
The relationship between (60)-(62) was confirmed by chemical comparison. Compound (60) was oxidized to the ketone (69) by pyridinium dichromate in dichloromethane.

The i.r. spectrum of (69) showed an absorption band for a carbonyl at 1700 cm$^{-1}$, and the u.v. spectrum showed an additional absorption at 329 n.m. for the conjugated carbonyl chromophore. In the $^1$H n.m.r. spectra, the H 4ax and H 4eq protons, $\gamma$ to the carbonyl shifted downfield by 0.3 and 0.1 ppm respectively. The H 8 proton was shifted downfield to $\delta$ 7.91 from $\delta$ 7.59 in (60), and the H 9a proton was deshielded to $\delta$ 3.20 (s). Further the H 11 signal shifted downfield by 0.4 ppm to $\delta$ 1.54, due to the anisotropy of the carbonyl group.

Reduction of (69) with sodium borohydride in methanol gave a mixture of two compounds, which were separated by h.p.l.c. using 5% ethyl acetate in hexane. The major product was (62), by comparison of the $^1$H n.m.r. and optical rotation data. The minor product appeared to be different from compound (60), in that there appeared to be additional aromatic signals in the n.m.r. as if a mixture of compounds, as well as additional signals at $\delta$ 4.52 (1H, dd), a double doublet at $\delta$ 3.48, and triplets at $\delta$ 2.81 and $\delta$ 4.81. The CH$_2$Br signals at $\delta$ 3.48 and $\delta$ 3.66 shifted to $\delta$ 3.15 and $\delta$ 3.25 in the
1H n.m.r spectra. The minor compound could not be further identified owing to the small sample size. The formation of (62) as the major product instead of (60) or (61) can be explained as follows. The bottom face of the ketone (69) is sterically hindered, hydride attack occurs from the top face, giving (61) as a major product, which would then cyclize to (62), due to the spatial arrangement of the OH and the CH₂Br groups. Moreover, (61) was also oxidized using PDC to give the same ketone (69) confirming that (60) and (61) are stereoisomers.

2.2.1.1.4. 3R-Hydroxytribromocacoxanthene (63)

3β-Hydroxybromocacoxanthene (63) was eluted from the h.p.l.c. column in a 0.003% yield using 1.5% ethyl acetate in hexane. Thin layer chromatography showed a single u.v. active spot. The 1H n.m.r. data (table 2.5) showed 3 aromatic protons in the region δ 6.6-7.3, six high field protons (δ 1.5-3.1), four deshielded protons next to either bromine or oxygen (δ 3.5-4.0) and two methyl singlets at δ 1.04 and 1.22. The i.r. spectrum showed absorption for a hydroxyl group (v_max 3566 cm⁻¹). The molecular formula was established by h.r.e.i.m.s. as C₁₆H₁₉O₂Br₃. A cluster of peaks in the mass spectrum in the region 480-486 confirmed the presence of three bromines. The 13C n.m.r. spectrum contained 16 carbon signals as expected (Table 2.5). The structure of (63) was established by 1H-1H decouplings and proton-detected long range heteronuclear correlation (HMBC) and HMQC experiments.
Irradiation of the proton signal at $\delta$ 2.69 caused the aromatic signal at $\delta$ 7.20 to sharpen and the double doublet at $\delta$ 2.81 to change to a doublet ($J$ 16.9 Hz). The large coupling suggested that $\delta$ 2.69 and $\delta$ 2.81 protons are on the same carbon, as 16.9 Hz is consistent for a geminal coupling in absence of electronegative substituents. The sharpening of the $\delta$ 7.20 signal further indicated that both these protons are next to the benzene ring, that is on C 9. Moreover, the proton signal at $\delta$ 2.17 (dd, $J$ 13.3, 5.6 Hz) was also affected ($J$ 13.3 Hz); this signal was assigned to H 9a. The H 9a proton at $\delta$ 2.17 and H 9 proton at $\delta$ 2.69 are in a diaxial arrangement ($J$ 13.3 Hz).

Irradiation of the proton signal at $\delta$ 3.81 sharpened the broad singlet at $\delta$ 2.56 (OH, exchanges with D$_2$O) and affected the doublet at $\delta$ 3.93 (J 10.4 Hz) as well as $\delta$ 1.60 (J 11.4 Hz) and $\delta$ 3.07 (J 4.3 Hz) signals. These data suggested fragment (70), that is the $\delta$ 3.81 signal was assigned to the H 3ax position, showing a diaxial coupling of 10.4 Hz to H 2 ($\delta$ 3.93) and 11.4 Hz to H 4ax ($\delta$ 1.60), and the hydroxyl group was placed in the H 3eq position.
In the HMQC spectra δ 3.81 and δ 2.56 signals for H 3 were correlated to the 67.8 ppm carbon signal for C 3, while the δ 3.93 proton signal corresponded to the signal at 75.5 ppm for C 2, deshielded compared to (60) due to the oxygen on C 3. Thus the 74.9 ppm carbon signal could be assigned to C 4a. Other proton-carbon correlations were also confirmed by the HMQC data. The methyl signals at δ 1.22 and 1.04 were each correlated to their respective carbon signals at 29.9 and 19.1 ppm. The proton signals at δ 1.60 and 3.07 were both correlated to C 4 signal, H 9a signal at δ 2.15 was correlated to 47.7 ppm carbon signal and the methylene protons were associated with 33.3 ppm carbon signal. The aromatic carbon signals for C 6 and C 8 was also clarified from the HMQC data, the H 6 proton at δ 7.22 was correlated to the signal at 132.0 ppm in the carbon spectra, while the H 8 signal at δ 7.20 was coupled to 131.1 ppm signal. From the HMBC data similar correlations to (62) were seen for the quaternary C 1 (Table 2.5), while the correlations for C 4a are shown in Figure 2.7.

![Figure 2.7 HMBC correlations for C 4a in (63)](image-url)
Table 2.5. $^1$H and $^{13}$C n.m.r. data and long range correlations (HMBC) for (63). Solutions in CDCl$_3$ (400 MHz).

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta^{13}$C</th>
<th>$\delta^{1}$H</th>
<th>Responses in HMBC (H)</th>
</tr>
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<td>2$\beta$, 9$\alpha$, 11, 12</td>
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<td>2</td>
<td>75.5</td>
<td>3.93, d (10.4)</td>
<td>4$\alpha$/$\beta$, 9$\alpha$, 11, 12</td>
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<td>67.8</td>
<td>2.56, bs (OH)</td>
<td>2$\beta$, 4$\alpha$/$\beta$</td>
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<td>1.60, ddd (13.4, 11.4, 1.8)</td>
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<td>74.9</td>
<td></td>
<td>3$\alpha$, 9$\beta$, 13</td>
</tr>
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<td>5</td>
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<td>6.77, d (8.7)</td>
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<td>132.0</td>
<td>7.22, dd (8.7, 2.3)</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
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<td></td>
<td>5, 6, 8</td>
</tr>
<tr>
<td>8</td>
<td>131.1</td>
<td>7.20, d (2.3)</td>
<td>6, 9$\beta$</td>
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<tr>
<td>8a</td>
<td>122.9</td>
<td></td>
<td>5, 9$\alpha$/$\beta$</td>
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<td>24.0</td>
<td>2.69, dd (16.9, 13.3)</td>
<td>8, 9$\alpha$</td>
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<td>9a</td>
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<td>2.17, dd (13.3, 5.6)</td>
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<td>150.4</td>
<td></td>
<td>5, 6, 8, 9$\beta$</td>
</tr>
<tr>
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<td>29.9</td>
<td>1.22, s (CH$_3$)</td>
<td>2$\beta$, 9$\alpha$, 12</td>
</tr>
<tr>
<td>12</td>
<td>19.1</td>
<td>1.04, s (CH$_3$)</td>
<td>2$\beta$, 9$\alpha$, 11</td>
</tr>
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<td>13</td>
<td>33.3</td>
<td>3.50, dd (2H, 1.8)</td>
<td>4$\alpha$/$\beta$, 9$\alpha$</td>
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</tbody>
</table>
Compound (63) represents an unusual oxidation pattern, i.e. oxidation at C 9 as in (60)-(62) is expected as it is benzylic, but oxidation at an un-activated carbon, as at C 3 (in 63) is unexpected and may occur prior to ring closure. The biosynthetic pathway of compounds (60-62) appears to be similar to that of tribromocacoxanthene (51) and tetrabromocacoxanthene (52). That is alkylation of a polyketide derived bromophenol (71) generates (72), *trans* addition across double bond of which gives the tricyclic system (73). Oxidation takes place either prior (in 63) to or after the cyclization (60-62) (Scheme 2.6).

![Scheme 2.6 Proposed biosynthetic pathway for formation of compounds (60)-(63)]
2.2.1.2 **Spiro-fused Metabolites (64)-(66)**

Compounds (64)-(66) appeared to be closely related from their $^1$H n.m.r. spectra. All of these were obtained in fairly low yields and the similarities of their spectra certainly helped in determination of their structure (Appendix 2).

The molecular formular obtained by h.r.e.i.m.s for (64) and (65) were $C_{16}H_{19}Br_3O_2$ and $C_{16}H_{18}Br_4O_2$ respectively. From the $^1$H n.m.r., the only difference between (64) and (65) appeared in the aromatic region, which was in agreement with an extra bromine in the mass spectra of (65), thus detailed structural analysis was only carried out on (65).

2.2.1.2.1 **Tetrabromospiro-fused (65)**

The $^1$H n.m.r. spectra of (65) showed 2 aromatic signals indicating 2 aromatic bromine substituents. Thus the benzene ring is present as in compounds (60-63) but with an additional Br substituent. An isolated AB system at $\delta$ 3.57 and $\delta$ 3.60 integrated for two protons, and this was assigned for (H 3)$_2$. Two other deshielded proton signals at $\delta$ 4.07 and $\delta$ 4.71 were expected to be next to either bromine or oxygen (Table 2.6). From the i.r. spectrum the hydroxyl group was identified ($v_{max}$ 3567 cm$^{-1}$) and the signal for this was at $\delta$ 3.20 in the $^1$H n.m.r. (exchanges with D$_2$O). In the $^1$H n.m.r there were three methyl signals with one being deshielded at $\delta$ 1.69, while the CH$_2$Br signal was missing (C 13). The $\delta$ 1.69 signal was
either on an unsaturated carbon or next to an electronegative group.

Decoupling experiments established that one of the non-aromatic bromines was in a similar environment as in (60)-(63), with the H 5' ax (δ 4.71 dd, $^{13}$C 55.8 ppm). The deshielding of H 5' compared to H 2 in (58)-(61) is probably due to the 1,3-diaxial shielding effect of the 3' OH and the 1' ether group. The remaining bromine was on an unprotonated carbon as there were no other deshielded proton signals except for the one at δ 4.07, assigned for the H 3'eq position, next to the hydroxyl group. The three low field signals in the $^{13}$C n.m.r. at 45.4, 66.2 and 99.2 ppm analogous with 45.4, 66.8 and 96.3 ppm signals in (66) were shown by the DEPT to belong to quaternary carbons. The signal at 45.4 ppm was assigned to the gem-dimethyl containing carbon (C 6'), while the other two signals at 66.2 and 99.2 ppm were that for the bromine and the ether bearing carbons. While the 99.2 ppm carbon signal was consistent with the bridged carbon signal of (63), the 66.2 ppm signal was assigned to the quaternary carbon bearing the bromine and the methyl group. To satisfy the molecular formula, the remaining rings were the aromatic system and the 5-membered tetrahydrofuran ring, instead of the six membered ring.
An alternative structure (74) was eliminated because chemical shift values for C 9a and C 4a did not fit this structure. As C 4a in (74) would have a value of approximately 75 ppm, the same as cymopols (14) and (15), whereas in our compound the value is much higher (99.2 for C 1').
Table 2.6. $^1$H (400 MHz) and $^{13}$C n.m.r. data for compound (65). Solutions in CDCl₃.

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<thead>
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<th>Position</th>
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</tr>
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</tr>
<tr>
<td>2'</td>
<td>66.2</td>
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</tr>
<tr>
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<tr>
<td>4'</td>
<td>37.9$^A$</td>
<td>$ax$ 3.00, ddd (14.1, 13.6, 2.9) $eq$ 2.52, ddd (14.1, 3.8, 3.5)</td>
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<tr>
<td>5'</td>
<td>55.8</td>
<td>$ax$ 4.71, dd (13.6, 3.8)</td>
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<td>6'</td>
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<tr>
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<td>3.60 (Ha) 3.57 (Hb)</td>
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<td>4</td>
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<td>OH</td>
<td>3.20</td>
<td>d (11.2)</td>
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</table>

$^A$-$^D$ Assignments may be interchanged.
The correct relative stereochemistry of (65) was established by $^1$H n.O.e experiments. Four structures (65), (75)-(77) were compatible from the spectral data obtained so far. That is either the ether oxygen is $\beta$ to the cyclohexane ring as in (65) and (75), or alternatively the ether oxygen is $\alpha$ (76)-(77).

Irradiation of the H$_3$C 10 signal resulted in n.O.e. enhancement of H 3b ($\delta$ 3.57), H 3'eq at $\delta$ 4.07, while no n.O.e. was observed onto H 4'ax at $\delta$ 3.00 or H$_3$C 8 at $\delta$ 1.46, which would have been expected for structures (75) or (77). Therefore structures (65) or (76) with H$_3$C 10 in the equatorial position were the preferred structures. Irradiation of (H 3)$_2$ signal gave n.O.e. onto all three methyl signals, again excluding structure (76), as only two out of three methyls in (76) would have experienced the n.O.e.

When the signal H 5' at $\delta$ 4.71 was irradiated n.O.e. enhancements were observed onto the methyl signal at $\delta$ 1.07 assigned to H$_3$C 9, the hydroxy signal at $\delta$ 3.20 and the proton at $\delta$ 2.52 for H 4'eq. No n.O.e. was observed onto (H 3)$_2$ as would be expected for structure (76) and (77). Irradiation of the H$_3$C 9 at $\delta$ 1.07 gave n.O.e.s onto $\delta$
1.46 singlet assigned to H$_3$C 8, H 5' at $\delta$ 4.71, $\delta$ 3.60 signal for H 3a and the methyl signal at $\delta$ 1.69 assigned to H$_3$C 10. This suggested that H$_3$C 10 was in equatorial position as in (65) and (76) and not axial as in (75) or (77), while n.O.e. effects observed onto one of the (H 3)$_2$ signals suggests the more likely structure to be (65) and not (76).

Finally, irradiation of the H$_3$C 8 at $\delta$ 1.46 gave n.O.e. enhancement onto H 3a ($\delta$ 3.60), H$_3$C 9 and H 4'ax but not onto H$_3$C 10, confirming structure (65). The n.O.e. data also allowed assignment of H 3a and H 3b protons. From the above n.O.e. data the H 3a proton is $\beta$ while H 3b is in the $\alpha$ position.

Considering the relative stereochemistry (64)-(66) the biogenetic origin of these metabolites appears to be through the double bond isomerisation of the appropriately substituted cymopol derivative (Scheme 2.7). The oxygen at Cl' and bromine at C2' are predicted to be $trans$ on biogenetic grounds. It appears from (65) that double bond isomerisation of appropriately substituted cymopol derivative (78) produces (79). Bromination takes place on less hindered face to give a $trans$ diaxial double bond addition (away from the bulky methyl groups), followed by attack of the aryl oxygen produces the spiro-fused cyclized product (80). Hydroxylation may occur by insertion of an oxygen radical into the C-H bond or by epoxidation of a double bond followed by ring opening.
Scheme 2.7 Proposed biosynthetic pathway for the formation of spiro-fused compounds

2.2.1.2.2 Tribromospiro-fused (66)

Compound (66) was shown by h.r.e.i.m.s. to be the acetate derivative of (64) (C$_{18}$H$_{21}$Br$_3$O$_3$), and this was confirmed by the carbonyl absorption in the i.r spectrum ($v_{\text{max}}$ 1741 cm$^{-1}$, C=O), and the $^1$H n.m.r. data ($\delta$ 2.12, 3H, s, OAc). The $^{13}$C n.m.r. (Table 2.7) showed the signal for the carbonyl carbon at 169.7 ppm and the methyl at 21.4 ppm. In the $^1$H n.m.r. the anisotropy of the carbonyl group deshielded the signal for H 3' to $\delta$ 5.41, while the H 4'eq signal shifted upfield to $\delta$ 2.32 by 0.2 ppm compared to (66). The relative stereochemistry of (66) was shown to be as for (65) by n.O.e. experiments. Irradiation of (H 3)$_2$ gave n.O.e.'s onto the three methyl signals at $\delta$ 1.05, 1.45 and 1.52, consistent with (66). An inverse (relay) n.O.e was also detected onto H 4'ax at $\delta$ 2.97.8. Irradiation of H 4'ax resulted in n.O.e. enhancements of H 3'eq ($\delta$
5.41), H 4'eq (δ 2.32) and H₃C 8 (δ 1.45), while irradiation of H 5' resulted in n.O.e's onto H₃C 9 (δ 1.05), H 4'eq but not (H 3)₂, consistent with relative stereochemistry for (66). Acetylation of (64) using acetic anhydride in pyridine converted it to a compound with spectroscopic properties and optical rotation identical to those of (66) in a yield of 92%, further confirming the inter-relationship between these three compounds.
Table 2.7. $^1$H and $^{13}$C n.m.r. data for (66). Solutions in CDCl$_3$ (400 MHz). In $^{13}$C data, multiplicities (by DEPT) are in parenthesis.

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta^{13}$C</th>
<th>$\delta^1$H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'</td>
<td>96.3 (s)</td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>66.8 (s)</td>
<td></td>
</tr>
<tr>
<td>3'</td>
<td>77.2 (d)</td>
<td>eq 5.41, t (3.1, 2.9)</td>
</tr>
<tr>
<td>4'</td>
<td>37.7 (t)$^A$</td>
<td>ax 2.97, ddd (14.8, 13.4, 2.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>eq 2.32, ddd (14.8, 3.5, 3.1)</td>
</tr>
<tr>
<td>5'</td>
<td>56.4 (d)</td>
<td>ax 4.73, dd (13.4, 3.5)</td>
</tr>
<tr>
<td>6'</td>
<td>45.4 (s)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>34.8 (t)$^A$</td>
<td>3.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.47</td>
</tr>
<tr>
<td>3a</td>
<td>128.7 (s)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>131.3 (d)$^B$</td>
<td>7.24, d (2.0)</td>
</tr>
<tr>
<td>5</td>
<td>110.5 (s)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>127.2 (s)$^B$</td>
<td>7.20, dd (8.4, 2.0)</td>
</tr>
<tr>
<td>7</td>
<td>110.1 (d)</td>
<td>6.58, d (8.4)</td>
</tr>
<tr>
<td>7a</td>
<td>158.7 (s)</td>
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</tr>
<tr>
<td>8</td>
<td>24.7 (q)$^C$</td>
<td>1.45, s (CH$_3$)</td>
</tr>
<tr>
<td>9</td>
<td>21.0 (q)$^C$</td>
<td>1.05, s (CH$_3$)</td>
</tr>
<tr>
<td>10</td>
<td>25.4 (q)$^C$</td>
<td>1.52, s (CH$_3$)</td>
</tr>
<tr>
<td>OCOCCH$_3$</td>
<td>169.7 (s)</td>
<td></td>
</tr>
<tr>
<td>OCOCCH$_3$</td>
<td>21.4 (q)$^C$</td>
<td>2.12, s</td>
</tr>
</tbody>
</table>

$^A-C$ Assignments may be interchanged.
2.2.2. G.C.M.S. analysis of Fatty acids and Sterols

Since the *Cacospongia* sp. was found to be a rich source of brominated terpenes. It was of interest to find out whether this sponge might contain brominated fatty acids, as brominated fatty acids have been obtained from a few other sponges.\(^{73-79}\)

The fatty acids components in an extract of *Cacospongia* sp. was analysed by G.C.M.S at University of California, Mass Spectrometry Facility, Department of Pharmaceutical Chemistry, San Francisco, California. The *Cacospongia* crude extract was methylated with 1.4N hydrochloric acid in dry methanol. A portion of the product was cleaned up by florisil chromatography to remove pigments, followed by a short "pipette" column using anasil in hexane-ether. The G.C.M.S results are summarised in Table 2.8.

**Table 2.8. Fatty acid components of *Cacospongia* sp Crude extract. Molecular wt. in g/mole.**

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>ECL</th>
<th>M wt.</th>
<th>Fatty Acid</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.00</td>
<td>477</td>
<td>14:0</td>
<td>7.2</td>
</tr>
<tr>
<td>2</td>
<td>14.62</td>
<td>527</td>
<td>15:0 iso</td>
<td>17.7</td>
</tr>
<tr>
<td>3</td>
<td>15.45</td>
<td>602</td>
<td>16:1 cis 9</td>
<td>10.4</td>
</tr>
<tr>
<td>4</td>
<td>16.00</td>
<td>634</td>
<td>16:0</td>
<td>36.8</td>
</tr>
<tr>
<td>5</td>
<td>18.00</td>
<td>782</td>
<td>18:0</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Note only fatty acid components > 5% are reported.
A purified fatty acid fraction was also analysed for its fatty acid components. The crude extract was cleaned by column chromatography using silica followed by h.p.l.c. (μ-porasil), using 3% ethyl acetate in hexane to provide a cleaner FAME sample. The sample was analysed as for the crude extract. (Table 2.9).

Table 2.9 Fatty acid components in a partly purified sample of Cacospongia sp.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>ECL</th>
<th>M Wt.</th>
<th>Fatty Acid</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.00</td>
<td>476</td>
<td>14:0</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>14.62</td>
<td>527</td>
<td>15:0 iso</td>
<td>14.3</td>
</tr>
<tr>
<td>3</td>
<td>14.71</td>
<td>547</td>
<td>15:0 ANTEISO</td>
<td>12.8</td>
</tr>
<tr>
<td>4</td>
<td>15.63</td>
<td>605</td>
<td>16:0 iso</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>16.00</td>
<td>633</td>
<td>16:0</td>
<td>21.1</td>
</tr>
<tr>
<td>6</td>
<td>16.42</td>
<td>666</td>
<td>17:1 iso F</td>
<td>8.5</td>
</tr>
<tr>
<td>7</td>
<td>16.63</td>
<td>680</td>
<td>17:0 iso</td>
<td>2.8</td>
</tr>
<tr>
<td>8</td>
<td>16.72</td>
<td>686</td>
<td>17:0 ANTEISO</td>
<td>4.8</td>
</tr>
<tr>
<td>9</td>
<td>17.82</td>
<td>761</td>
<td>18:1 cis11/t,9/t,6</td>
<td>10.6</td>
</tr>
<tr>
<td>10</td>
<td>18.00</td>
<td>780</td>
<td>18:0</td>
<td>4.1</td>
</tr>
<tr>
<td>11</td>
<td>18.73</td>
<td>817</td>
<td>19:0 ANTEISO</td>
<td>2.5</td>
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<tr>
<td>12</td>
<td>18.90</td>
<td>837</td>
<td>19:0 cyclo C11-12</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Only fatty acid components > 1% are reported
In both extracts according to tables 2.8 and 2.9 there were only standard fatty acids such as 13-methyltetradecanoic (iso C 15:0) and hexadecanoic (C 16:0 palmitic) acids which are known as the short chain fatty acids. *Cacospongia* sp. does not appear to have any C_{24-34} fatty acids (VLFA's). The VLFA's are suggested to be derived from short chain fatty acids,\(^7\) however the *Cacospongia* sp. does not appear to need the VLFA's.

Also the low sterol content of this sponge led to the analysis of sterols to see if there were any unconventional sterols which may play a role in the cell membrane structure. A sterol extract from the sponge was passed through reverse phase HPLC using methanol. Individual sterols were collected and analysed by \(^1\)H n.m.r.

\[\text{Δ-22 Dehydrocholesterol} \quad (10.7\%)\]

\[\text{24-Methylene cholesterol} \quad (7.1\%)\]

\[\text{Crinosterol/Brassicasterol} \quad (7.1\%)\]
All the sterols obtained were conventional. The high level of cholesterol in this sponge was also notable.
2.2.3 Summary and Conclusion

The seven minor metabolites of *Cacospongia* sp were the oxygenated (either at C 9 or C 3 or C 3') derivatives of tribromo- and tetrabromocacoxanthene. Dibromocyclocacoxanthene (62) may be an artefact formed from (61), but since it was also isolated from the sponge, it is possibly produced by the sponge as well. Other brominated terpenes have been isolated by our group, (M. J. Garson and K. Field, unpublished work) and some of the structures are (81)-(84).

![Chemical structures](image)

These bromoterpenes have the same basic structure as the reported compounds (60)-(66). Compound (81) is the bromo derivative of (63), and (82) is the bromo derivative of (60), while (84) is the epimeric form of (65). Compound (83) is unique in that it has a *cis* ring junction similar to cymobarbatol (14) isolated from the tropical green alga. This structural similarity might further suggest the possibility of a dietary or a symbiont source of the *Cacospongia* compounds.
The bromoterpenes could be synthesized by a procedure similar to that used for the synthesis of cyclocymopol (8).\textsuperscript{32} In fact initial attempts by Tanaka \textit{et al.} during synthesis of (8), resulted in a tricyclic compound (85), which most likely mimics the biosynthetic route for the bromoterpenes of \textit{Cacospongia} sp. Therefore using appropriately substituted hydroquinone derivative, tribromo- and tetrabromocacoxanthene and their derivatives may be prepared.
2.3 Experimental

2.3.1 Instruments used

Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. High resolution electron impact mass spectra were recorded on a V.G. micromass 7070F or an AEI-902 mass spectrometer at 70 eV.

$^1$H and $^{13}$C n.m.r. spectra were recorded for solutions in deuterated chloroform with internal chloroform (δ 77.0 for $^{13}$C and δ 7.27 for $^1$H) as a standard on Jeol GX400 or Jeol FX90Q or Bruker AMX500 Fourier transform N.M.R. spectrometers.

Infrared spectra were recorded on BioRad FTS 20/80 or Perkin Elmer 783 infrared spectrometers, for solutions in chloroform.

Ultraviolet spectra were obtained for solutions in dichloromethane or methanol on a Shimadzu UV-265 spectrometer.

Optical rotations were measured on a Perkin Elmer PE141 polarimeter in chloroform solution, unless otherwise stated.

Silica gel (Merck Art 7734) was used for flash chromatography under a pressure of nitrogen gas.

Thin layer chromatography was carried out by using aluminium or plastic-backed silica gel (F$_{254}$) plates. The plates were visualized
either under u.v. light or by spraying with a solution of vanillin (10 mg) in concentrated sulphuric acid (50 ml). High Performance liquid chromatography used a Waters Associates u-porasil semipreparative column (5 mm by 30 cm) and a Waters R403 Refractive Index detector.

2.3.2 Method

2.3.2.1 Sponge Collection and Extraction

*Cacospongia* sp. (80.5 g wet weight, 16 g dry weight) was collected in November, 1987 from the shady walls of a rocky tide pool at North Wollongong. A voucher specimen (No. Z5031) identified by Professor P. R. Bergquist, was lodged at the Australian Museum, Sydney. The sponge was cut into small pieces and extracted with 95% ethanol (3x250 ml). The combined ethanol extracts were filtered and concentrated under vacuum to give an aqueous solution, which was partitioned between ethyl acetate (3x50 ml) and water (50ml). The combined ethyl acetate extracts were dried over anhydrous sodium sulphate and evaporated to give a brown oil (453 mg, 2.8%).

2.3.2.2 Chromatography of the Crude Extract

The crude extract (453 mg) was dissolved in a minimum volume of dichloromethane and applied to the top of a column packed with silica (15 cm by 2 cm), topped with sand (0.5 cm). The column was eluted with dichloromethane (150 ml) changing slowly to ethyl acetate and finally methanol. Fractions (15-20 ml) were collected,
checked by t.l.c. (3:1 dichloromethane/ethyl acetate) and combined as appropriate to give seven major fractions.

2.3.2.3 Separations of Bromo Metabolites

From the $^1$H n.m.r. only the first four fractions appeared to contain the bromo compounds of interest (1.6%), the other fractions were rich in scalaranes, fatty acids and sterols.

Fractions (1-4) were combined and further purified by a second flash chromatography using a silica column (15 by 1 cm), eluting with hexane/dichloromethane (6:1) changing to (3:1), (1:1) and dichloromethane. Each fraction (20 ml) was examined by t.l.c. using hexane/dichloromethane (1:1) and similar fractions combined together. Fractions left over from a previous collection (September, 1987) of Cacospongia sp. (dry weight 158 g) were combined with appropriate fractions and rechromatographed to give the bromo compounds (60)-(66).

**Compound (60)**

$(2S,4aS,9R,9aS)\text{-}2,7$-dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthen-9-ol (60) (12.0 mg, 0.007%), was obtained from the flash column with dichloromethane and purified by h.p.l.c (4% ethyl acetate in hexane). Recrystallization from dichloromethane/hexane (2:1) gave shiny white needles, m.p. 187-189°, $[\alpha]_D^{11.8\circ}$ (c, 0.321 in CH$_3$COCH$_3$) (Found: M$^+$, 479.8936. C$_{16}$H$_{19}$Br$_2$O$_2$ requires M$^+$, 479.8935). $\lambda_{max}$ (CH$_3$OH) 228
(2980), 285 nm (580). $v_{\text{max}}$ 3583 (OH), 2957, 2929, 2859, 1580, 1475, 1256, 1147, 1005, 823 cm$^{-1}$.

Mass Spectrum: m/z 486/484/482/480 (16, 57, 58, 16%, M), 373 (60), 371 (100), 305 (16), 303 (18), 203 (24), 201 (60), 199 (37), 187 (14), 185 (10), 121 (20), 91 (35), 81 (31), 69 (54%). $^1$H n.m.r. (CDCl$_3$) δ 1.12 (3H, s, CH$_3$), 1.42 (3H, s, CH$_3$), 1.49 (1H, d, J 9.8 Hz, OH), 1.61 (1H, dddd, J 13.7, 13.7, 3.8, 2.5 Hz), 2.00 (1H, dddd, J 14.3, 13.7, 12.7, 3.4 Hz), 2.16 (1H, d, J 10.0 Hz), 2.30 (1H, dddd, J 14.3, 4.0, 3.8, 3.8 Hz), 2.49 (1H, ddd, J 13.7, 3.8, 3.4 Hz), 3.48 (1H dd, J 11.7, 2.5 Hz), 3.66 (1H, d, J 11.7 Hz), 4.04 (1H, dd, J 12.7, 4.0 Hz), 4.81 (1H, dd, J 10.0, 9.8 Hz), 6.74 (1H, d, J 8.6 Hz), 7.31 (1H, dd, J 8.6, 2.2 Hz), 7.59 (1H, d, J 2.2 Hz). $^{13}$C n.m.r. 18.8 (C 11), 31.1 (C 12), 31.1 (C 3), 32.7 (C 13), 36.2 (C 4), 39.9 (C 1), 55.9 (C 9a), 65.2 (C 2), 66.1 (C 9), 78.3 (C 4a), 113.9 (C 7), 119.6 (C 5), 128.1 (C 8a), 130.6 (C 6), 132.5 (C 8), 149.6 (C 10a) ppm.

Elution of the flash column with hexane/dichloromethane (3:1/1:1) gave compounds (61)-(66), these were further purified by h.p.l.c. using ethyl acetate (1-2%) in hexane.

**Compound (61)**

(2S,4aS,9S,9aS)-2,7-Dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthen-9-ol (61), obtained as a clear oil in a yield of 3.2 mg (0.002%), $[\alpha]_D$ - 0.830 (c 0.120). $\lambda_{\text{max}}$ (CH$_2$Cl$_2$) 232 (ε 5531), 288 nm (1179). $v_{\text{max}}$ 2960, 2858, 1580, 1480, 1243, 1011, 823 cm$^{-1}$, the OH signal was obscured by machine noise. $^1$H n.m.r. δ 1.32 (3H, s, CH$_3$), 1.38 (3H, s, CH$_3$), 1.61 (1H, dddd, J 13.9, 12.4, 3.9, 2.3 Hz), 1.81 (1H, d, J 2.2 Hz, OH), 2.08 (1H, d, J 3.7 Hz),
2.16 (1H, dddd, J 14.7, 12.4, 12.4, 3.7 Hz), 2.21 (1H, dddd, J 14.7, 3.9, 3.9, 3.7 Hz), 2.68 (1H, ddd, J 13.9, 3.9, 3.7 Hz), 3.82 (1H, d, J 12.2 Hz), 4.02 (1H, dd, J 12.4, 3.9 Hz), 4.33 (1H, dd, J 12.2, 2.3 Hz), 5.02 (1H, dd, J 3.7, 2.2 Hz), 6.79 (1H, d, J 9.5 Hz), 7.32 (1H, d, J 9.5 Hz), 7.33 (1H, s).

Compound (62)

2,7-Dibromo-1,1-dimethyl-1,2,3,4,9,9a-hexahydro-9,4a-epoxymethano-4aH-xanthene (62), clear oil (2.6 mg, 0.001%), [α]D -45.1° (c, 0.324) (Found: M+, 399.9664. C16H₁₈₇Br₂O₂ requires M+, 399.9674). λmax (CH₂Cl₂) 238 (ε 8243), 287 (2008), 295 nm (1803). νmax 2930, 2856, 1605, 1475, 1373, 1268, 1237, 1180, 1145, 1045, 810 cm⁻¹. Mass Spectrum: m/z 404/402/400 (4, 7, 4%, M), 373 (4), 371 (7), 369 (3), 323 (35), 321 (32), 253 (6), 251 (6), 201 (15), 199 (16), 121 (10), 107 (15), 81 (14), 79 (9), 69 (100), 55 (19), 43 (14%). ¹H n.m.r. δ 1.14 (3H, s, CH₃), 1.17 (3H, s, CH₃), 2.03 (1H, dddd, J 13.4, 12.2, 10.7, 4.9 Hz), 2.09 (1H, ddd, J 13.7, 4.9, 3.0 Hz), 2.14 (1H, ddd, J 13.7, 10.7, 4.6 Hz), 2.19 (1H, s), 2.25 (1H, dddd, J 13.4, 4.6, 3.4, 3.0 Hz), 3.93 (1H, d, J 10.7 Hz), 4.03 (1H, dd, J 12.2, 3.4, Hz), 4.16 (1H, d, J 10.7 Hz), 4.73 (1H, s), 6.63 (1H, d, J 8.6 Hz), 7.15 (1H, d, J 2.3 Hz), 7.25 (1H, dd, J 8.6, 2.3 Hz). ¹³C n.m.r. 17.5 (C 12), 29.7 (C 11), 30.6 (C 3), 31.0 (C 4), 37.6 (C 1), 53.9 (C 9a), 63.3 (C 2), 76.2 (C 13), 77.7 (C 9), 112.1 (C 7), 117.7 (C 5), 129.1 (C 8a), 129.2 (C 8), 132.6 (C 6), 152.2 (C 10a) ppm.
**Compound (63)**

2,7-Dibromo-4a-bromomethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthen-3-ol (63) (5.2 mg, 0.003%), (Found: M⁺, 479.8938. C₁₆H₁₉⁷⁹Br₃O₂ requires M⁺, 479.8935). λₑₓₘₐₓ (CH₂Cl₂) 234 (ε 5432), 288 nm (1966). νₑₓₘₐₓ 3566 (OH), 2993, 2942, 2850, 1480, 1295, 1224, 1147, 1004, 810 cm⁻¹. Mass spectrum: m/z 486/484/482/480 (3, 8, 8, 3%, M), 385 (2), 371 (2), 303 (8), 225 (8), 209 (5), 185 (27), 137 (21), 119 (21), 107 (19), 91 (20), 81 (17), 79 (17), 69 (28), 55 (74), 43 (99), 41 (100%). ¹H n.m.r. δ 1.04 (3H, s, CH₃), 1.22 (3H, s, CH₃), 1.60 (1H, ddd, J 13.4, 11.4, 1.8 Hz), 2.17 (1H, dd, J 13.3, 5.6 Hz), 2.56 (1H, bs, OH), 2.69 (1H, dd, J 16.9, 13.3 Hz), 2.81 (1H, dd, J 16.9, 5.6 Hz), 3.07 (1H, dd, J 13.4, 4.3 Hz), 3.50 (2H, d, J 1.8 Hz), 3.81 1H, ddd, J 11.4, 10.4, 4.3 Hz), 3.93 (1H, d, J 10.4 Hz), 6.77 (1H, d, J 8.7 Hz), 7.20 (1H, d, J 2.3 Hz), 7.22 (1H, dd, J 8.7, 2.3 Hz). ¹³C n.m.r. 19.1 (C 12), 24.0 (C 9), 29.9 (C 11), 33.3 (C 13), 38.9 (C 1), 41.8 (C 4), 47.7 (C 9a), 67.8 (C 3), 74.9 (C 4a), 75.5 (C 2), 113.4 (C 7), 119.9 (C 5), 122.9 (C 8a), 131.1 (C 8), 132.0 (C 6), 150.4 (C 10a) ppm.

**Compound (64)**

2',5,5'-Tribromo-2',6',6'-trimethylspiro[benzofuran-2(3H),1'-cyclohexan]-3'-ol (64) (2.1 mg, 0.001%), obtained as a clear oil, [α]ₒ - 32.4° (c, 0.153) (Found: M⁺, 479.8936. C₁₆H₁₉⁷⁹Br₃O₂ requires M⁺, 479.8935). λₑₓₘₐₓ (CH₂Cl₂) 237 (ε 7490), 289 (2906), 296 nm (2686). νₑₓₘₐₓ 3562 (OH), 2928, 2856, 1590, 1477, 1252, 1175, 1004, 810 cm⁻¹. Mass spectrum: m/z 586/584/582/580 (4, 7, 8, 3%, M), 323
(18), 321 (18), 239 (13), 225 (10), 223 (10), 187 (12), 185 (10), 115 (11), 99 (28), 81 (83), 79 (17), 69 (100), 55 (23%). \(^1\)H n.m.r. \(\delta\) 1.06 (3H, s, CH\(_3\)), 1.45 (3H, s, CH\(_3\)), 1.70 (3H, s, CH\(_3\)), 2.49 (1H, ddd, J 14.0, 3.8, 3.5 Hz), 2.97 (1H, ddd, J 14.0, 13.6, 2.9 Hz), 3.11 (1H, d, J 11.0 Hz, OH), 3.47 (1H), 3.51 (1H), 4.06 (1H, ddd, J 11.0, 3.5, 2.9 Hz), 4.69 (1H, dd, J 13.6, 3.8 Hz), 6.61 (1H, d, J 8.4 Hz), 7.22 (1H, dd, J 8.4, 2.0 Hz), 7.28 (1H, d, J 2.0 Hz).

*Compound (65)*

\(2',5,5',7\)-Tetrabromo-\(2',6',6'\)-trimethylspiro[benzofuran-2(3\(H\)),1'-cyclohexan]-3'-ol (65), obtained as a white solid (6.25 mg, 0.004%), \([\alpha]_D \pm 33.7^\circ\) (c, 0.601) (Found: M\(^+\), 559.8003. \(\text{C}_{16}\text{H}_{18}\text{Br}_7\text{O}_2\) requires M\(^+\), 559.8020). \(\lambda_{\text{max}}\) (CH\(_2\)Cl\(_2\)) 230 (e 15534), 291 (4167), 299 nm (4167). \(\nu_{\text{max}}\) 3567 (OH), 2928, 2856, 1581, 1457, 1263, 1166, 1004, 952 cm\(^{-1}\). Mass spectrum: m/z 566/564/562/560/558 (<1, 4, 6, 4, <1%, M), 484 (3), 482 (3), 480 (2), 403 (4), 401 (6), 400 (3), 385 (7), 383 (8), 305 (4), 303 (6), 267 (4), 265 (7), 263 (4), 189 (9), 187 (15), 185 (7), 137 (12), 99 (29), 81 (72), 79 (7), 69 (100), 55 (22%). \(^1\)H n.m.r. \(\delta\) 1.07 (3H, s, CH\(_3\)), 1.46 (3H, s, CH\(_3\)), 1.69 (3H, s, CH\(_3\)), 2.52 (1H, ddd, J 14.1, 3.8, 3.5 Hz), 3.00 (1H, ddd, J 14.1, 13.6, 2.9 Hz), 3.20 (1H, d, J 11.2 Hz, OH), 3.57 (1H), 3.60 (1H), 4.07 (1H, ddd, J 11.2, 3.5, 2.9 Hz), 4.71 (1H, dd, J 13.6, 3.8 Hz), 7.22 (1H, d, J 4.3 Hz), 7.44 (1H, d, J 4.3 Hz). \(^1^3\)C n.m.r. 22.4, 24.6, 26.3 (C 8, C 9, C 10), 37.3, 37.9 (C 3, C 4'), 45.4 (C 6'), 55.8 (C 5'), 66.2 (C 2'), 99.2 (C 1'), 102.6, 113.9 (C 5, C 7), 126.5, 133.6 (C 4, C 6), 129.0 (C 3a), 154.2 (C 7a) ppm.
Compound (66)

2',5,5'-Tribromo-2',6',6'-trimethylspiro[benzofuran-2(3H),1'-cyclohexan]-3'-yl acetate (66), clear oil (5.8 mg, 0.003%), [α]D -21.6° (c, 0.473) (Found: M+, 523.9012. C18H2179Br281BrO3 requires M+, 523.9020). λmax (CH2Cl2) 235 (ε 8058), 291 (2880), 298 nm (2623). νmax 2929, 2857, 1741 (C=O), 1477, 1373, 1237, 1180, 1025, 810 cm⁻¹. Mass spectrum: m/z 528/526/524/522 (1, 2, 2, 1%, M), 445 (<1), 365 (1), 363 (1), 239 (10), 237 (11), 81 (6), 69 (100%). ¹H n.m.r. δ 1.05 (3H, s, CH3), 1.45 (3H, s, CH3), 1.52 (3H, s, CH3), 2.12 (3H, s, CH3), 2.32 (1H, ddd, J 14.8, 3.5, 3.1 Hz), 2.97 (1H, ddd, J 14.8, 13.4, 2.9 Hz), 3.47 (1H), 3.52 (1H), 4.73 (1H, dd, J 13.4, 3.5 Hz), 5.41 (1H, t, J 3.1, 2.9 Hz), 6.58 (1H, d, J 8.4 Hz), 7.20 (1H, dd, J 8.4, 2.0 Hz), 7.24 (1H, d, J 2.0 Hz). ¹³C n.m.r. (DEPT) 21.0 (q), 21.4 (q), 24.7 (q), 25.4 (q) (C 8, C 9, C 10, OAc), 34.8 (t), 37.7 (t), (C 3, C 4'), 45.4 (s) (C 6'), 56.4 (d) (C 5'), 66.2 (s) (C 2'), 77.2 (d) (C 3'), 96.3 (s) (C 1'), 110.1 (d) (C 7), 110.5 (s) (C 5), 127.2 (d), 131.3 (d) (C 4, C 6), 128.7 (s) (C 3a), 158.7 (s) (C 7a), 169.7 (s) (OAc) ppm.

2.3.2.4 Preparation of Model Compounds (67)-(68)

Preparation of 4-Bromo-o-cresol (67)

Bromine (1 ml) 0.02 mole was dissolved in carbon tetrachloride (10 ml) and added dropwise for four hours to a solution of o-cresol (2.16 g, 0.02 mole) in carbon tetrachloride (20 ml), while stirring at 0°. The solution was further stirred at room temperature overnight. Water (100 ml) was added to the reaction mixture and extracted with ether (3x30 ml). The combined ether layer was dried over
anhydrous sodium sulphate and evaporated in vacuo to give 4-bromo-o-cresol (67), 3.25 g, 85%, m.p. 61-63° (Lit. 64°). $v_{\text{max}}$ 3500-3300 (br, OH), 1600, 1490, 810, 670 cm$^{-1}$. $^1$H n.m.r. (CDCl$_3$) $\delta$ 6.61 (1H, bs, OH), 6.77 (1H, d, J 9.2 Hz), 7.30 (1H, d, J 9.2 Hz), 7.35 (1H, s).

Preparation of 2-Methyl-4-bromoanisole (68)

4-Bromo-o-cresol (67) (0.186 g, 0.001 mole) was dissolved in toluene (3 ml) and dimethyl sulphate (0.25 ml, 0.0025 mole) was added. Potassium carbonate (0.50 g) was added to the flask. The mixture was refluxed gently and the reaction followed by t.l.c. After six hours the reaction appeared to be complete, the heating was stopped and the reaction mixture allowed to cool to room temperature. The solution was partitioned between toluene (5 ml) and water (5 ml), the toluene layer was washed with sodium hydroxide (10%) (5 ml), water (5 ml), dried over anhydrous sodium sulphate and evaporated to give crude 2-methyl-4-bromoanisole (68). The product was purified by flash chromatography using a column (5 cm by 30 cm) packed with silica and topped with sand (0.5 cm). Elution with hexane gave (68) as a white solid, recrystallized from hexane as white shiny crystals (0.043 g, 22%) m.p. 65-67° (Lit. 67-68°). $^1$H n.m.r. (CDCl$_3$) $\delta$ 2.22 (3H, s, CH$_3$), 3.83 (3H, s, OCH$_3$), 6.71 (1H, d, J 9.2 Hz), 7.28 (1H, s), 7.30 (1H, d, J 9.2 Hz). $^{13}$C n.m.r. $\delta$ 15.9 (CH$_3$), 55.4 (OCH$_3$), 111.5 (C 6), 112.4 (C 4), 129.0 (C 2), 129.3 (C 5), 133.2 (C 3), 156.9 (C 1).
Oxidation of (60)

Pyridinium dichromate \(((\text{C}_5\text{H}_5\text{NH}^+)\text{Cr}_2\text{O}_7^{2-})\) (6.0 mg, 6.25 x 10^{-6} mole) was added to a small round-bottomed flask containing (60) (4.0 mg) in dichloromethane (2 ml). The reaction mixture was stirred for 5 hours at room temperature and the reaction followed by t.l.c. It was then diluted with ether (3 ml), filtered and evaporated to dryness. The pyridinium dichromate residue was removed by filtration of the crude product through a small silica column using hexane/dichloromethane (1:1). Evaporation of the eluate gave 2,7-dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthen-9-one (69) (4.0 mg, 99.5%), \([\alpha]_D -11.8^\circ\) (c, 0.103). \(\lambda_{\text{max}}\) (CH$_2$Cl$_2$) 229 (\(\Sigma\) 5843), 292 (643), 329 nm (614). \(v_{\text{max}}\) 2958, 2928, 2855, 2361, 2337, 1700 (C=O), 1458, 1263, 670 cm$^{-1}$. Mass spectrum: m/z 484/482/480/478 (all <5%, M), 389 (30), 387 (65), 385 (38), 319 (10), 277 (8), 201 (100), 199 (95), 145 (18), 97 (60), 85 (14), 83 (80), 81 (42), 79 (29), 63 (85), 56 (45%). \(^1\)H n.m.r. \(\delta\) (CDCl$_3$) 1.24 (3H, S, CH$_3$), 1.54 (3H, s, CH$_3$), 1.87 (1H, ddd, J 13.7, 13.4, 3.8 Hz), 2.02 (1H, dddd, J 14.3, 13.7, 12.7, 3.4 Hz), 2.29 (1H, dddd, J 14.4, 3.8, 3.2 Hz), 2.60 (1H, ddd, J 13.4, 3.4, 3.2 Hz), 3.20 (1H, s), 3.69 (2H, s), 3.92 (1H, dd, J 12.7, 3.8 Hz), 6.87 (1H, d, J 8.7 Hz), 7.57 (1H, dd, J 8.7, 2.4 Hz), 7.91 (1H, d, J 2.4 Hz).
Reduction of (69)

Compound (69) (3.96 mg) was dissolved in chloroform (4 drops) and diluted with methanol to 1 ml. The solution was cooled in ice (0-5°) and sodium borohydride (NaBH₄) was added in excess (10 mg) over an hour. The reaction mixture was stirred under nitrogen for a further 5 hours at 0° and the reaction followed by t.l.c. (dichloromethane). The solvent was evaporated under vacuum and the residue was extracted with dichloromethane (3x3 ml), dried over anhydrous sodium sulphate and evaporated. The oily product was purified by h.p.l.c. using ethyl acetate (2%) in hexane to give (62) (1.4 mg, 35.0%) [α]D - 41.6° (c, 0.172). A second product isolated in 26.5% yield was not identified.

Acetylation of (64)

To a small round bottom flask containing (64) (0.8 mg) was added acetic anhydride (0.5 ml) and pyridine, and the reaction mixture was stirred at room temperature overnight. It was then poured into water and extracted with dichloromethane (3x2 ml). The combined dichloromethane extract was dried (Na₂SO₄) and evaporated under vacuum to give (66) (0.8 mg, 89.8%) [α]D - 23.6° (c, 0.062).
CHAPTER 3

THE CHEMISTRY OF METABOLITES
FROM *PSAMMAPLYSILLA* SPECIES
3.1 Introduction

3.1.1 Brominated Metabolites from Verongid sponges

Marine sponges of the order Verongida are unique in their ability to give a vast array of brominated or chlorinated metabolites derived from dibromo- or dichloro-tyrosine. These are seen as the biogenetic precursors of 3,5-dichloroverongiaquinol (86),\textsuperscript{101} some macrocyclic bastadins (87) and (88),\textsuperscript{102} 5-bromocarvenicolin (89),\textsuperscript{103} and a number of other halogenated metabolites. The parent amino acids such as dibromotyrosine and lysine have been found in sponge proteins.\textsuperscript{104}

![Chemical structures](image)

There is some experimental evidence to support the conversion of phenylalanine and tyrosine via their dibromo analogues into dienone (90) and the rearranged dibromohomogentisamide (91) (Scheme 3.1).\textsuperscript{20}
Biosynthetic studies was conducted using liposome encapsulated labelled precursors, introduced into the sponge in a fresh running sea water bath. "Enzymes, presumably bromoperoxidases, present in the sponge convert tyrosine into dibromotyrosine by utilizing bromine (as Br\textsuperscript{-}) from the sea water." Both phenylalanine and tyrosine were shown to be biosynthetic precursors of (90). The presence of comparable radioactivity in both (90) and (91) indicated that (91) was formed from (90) by skeletal
rearrangement, similar to that in mammalian biosynthesis of homogentisic acid.

The most spectacular feature of verongid sponges is that they undergo a rapid change in colour from yellow to dark purple when they are exposed to air. This colour change is due to the oxidation of the yellow pigment uranidine to a blue quinone which then polymerizes to produce a black precipitate.

![Chemical structure of uranidine and quinone](image)

The order Verongida includes the genera *Aplysina, Verongula, Pseudoceratina, Psammaplysilla* and *Ianthella*. The majority of the dibromotyrosine derived natural products from verongid sponges have been reported to possess significant antimicrobial activity, and this may be attributable to the presence of phenolic groups.

Arene oxides have been proposed to be possible precursors of some of the dibromotyrosine derived metabolites found in verongid sponges. Anderson and Faulkner suggested that compounds such as (92) and (93) could be derived from an unstable metabolite related to α-oximino acid (94).
Other more complex molecules such as the cavernicolins and the bastadins are suggested to have originated from phenol oxidative couplings of their monomeric amino acids, which involves a free-radical process that is catalysed by enzymes. Isolation of racemic and nearly racemic cavernicolins suggests ortho-para orientation appears to be favoured in the oxidative coupling and a racemic or nearly racemic spirolactone (95) may be the intermediate, derived from a halotyrosine precursor. Hydrolysis of the spirolactone (95), followed by conjugate attack of amino acid N-atom and decarboxylative oxidation may convert (95) to the cavernicolins, for example (89).
3.1.2 Bromo-metabolites of *Psammaplysilla* species

Brominated compounds isolated from sponges of the genus *Psammaplysilla* will be discussed in some detail as the compounds isolated from *Psammaplysilla* sp. discussed in this section are all brominated, with the exception of sterols.

Several dibromotyrosine derivatives have been reported from the genus *Psammaplysilla*. (+)-Aeroplysinin-1 (96), epimeric aeroplysinin-2 (97) and an aromatic amide (98) were reported from *Psammaplysilla purpurea* collected from Enewetak.\(^{106}\) Both aeroplysinin-1 and aeroplysinin-2 have been previously obtained from a number of verongid sponges\(^1\) and (98) was initially formed by treatment of (96) with base, followed by acid.\(^{109}\)

![Chemical structures](image)

*Psammaplysilla purpurea* from the Gulf of Eilat was reported to contain two antibiotic psammaplysins A (99) and B (100).\(^{110}\) Psammaplysin A and B were reisolated by Roll et al\(^{111}\) from a Palauan *Psammaplysilla purpurea* and their structures were determined by extensive \(^{13}\)C-\(^{13}\)C connectivity and single-crystal x-ray diffraction studies. The psammaplysins were found to contain a spiro[4,6]-dioxaundecane and not a spiro[4,5]-oxazadecane
skeleton. The psammaplysins A and B are expected to be derived from dibromotyrosine via benzene oxide-oxepin intermediates.

A *Psammaplysilla* sp. collected from Tonga contained cytotoxic compounds 3-bromo-4-hydroxyphenylacetonitrile (101), a compound previously isolated from *V. aurea*, and a dimeric disulphide psammaplin A (102). Psammaplin A showed *in vitro* activity (IC$_{50}$ = 0.3 µg/ml) against P-388 cells and its structure was determined by combination of 1D and 2D n.m.r. techniques. Psammaplin A was also isolated from the marine sponge *Thorectopsamma xana* collected in Guam.

Purealin (103) was reported from the Okinawan *Psammaplysilla purea*. It moderates the reactions of enzyme ATPase. The
structure of (103) was determined by $^1$H-$^1$H homonuclear and $^1$H-$^{13}$C heteronuclear n.m.r. chemical shift correlations and CD spectra. Three related ATPase inhibitors, lipopurealins A, B and C were obtained as minor metabolites of the same sponge.$^{115}$

![Diagram of (103)](image)

Recently, two new brominated tyrosine derivatives 14-debromoaraplysillin (104) and 14-debromoprearaplysillin (105)$^{116}$ have been obtained from *Psammaplysilla purpurea*, together with the known compound araplysillin (106).$^{117}$

![Diagram of (104) and (106)](image)

![Diagram of (105)](image)
14-Debromoaraplysillín (104) and araplysillín (106) are presumed to be biosynthesized from 14-debromoprearaplysillín (105) (Scheme 3.2).

Scheme 3.2 Proposed biosynthetic pathway from the prearaplysillins to the araplysillins

3.1.3 Sterols from Verongid Sponges

Initial studies conducted by De Rosa et al on several species of sponges (twenty five from seven different orders) from the same habitat as Verongia aerophoba, which contains aplysterol (107) and 24 (28) dehydroaplysterol (108),\textsuperscript{118} showed that only the verongid sponges contained the unconventional sterols (107) and (108).

Later, isolation of 24 (28) dehydroaplysterol (108) from the Australian sponge Jaspis stellifera\textsuperscript{119} and the occurrence of 25 (26) dehydroaplysterol (109) with verongulasterol (110) in Verongia cauliformis,\textsuperscript{120} and further isolation of 24 (28) dehydroaplysterol (108), verongulasterol (110) and mutasterol (111) from Xestospongia muta,\textsuperscript{121} indicated a more wide distribution of sterols with aplystane skeleton than once believed. Stelliferasterol
(112)\textsuperscript{122}, isostelliferasterol (113),\textsuperscript{123} verongulasterol (110)\textsuperscript{120} and strongysterol (114)\textsuperscript{122} all are characterised by an extra carbon atom attached to C 26, while 25-methylxestosterol (115) has extra methyl groups at C 24, C 25, C 26 and C 27.\textsuperscript{124} These unusual sterols in several cases have been found to be the major component of the sterol mixture of sponges. Aplysterol has been found to be 50-70% of sterol fraction in some verongid sponges. Sutinasterol (116) produced from four biomethylations was found to be the major sterol (94% of sterol mixture) of \textit{Xestospongia} sp. from the Caribbean. Another sterol (117) found in trace amounts in \textit{Xestospongia} sp. is a product of five biomethylations, representing the largest free sterol isolated.\textsuperscript{125} Strongysterol was the sole sterol of the sponge \textit{Strongylophora purissima}.\textsuperscript{122} The presence of unconventional sterols in such high concentrations possibly indicates that sterols have a functional role in stabilization of the sponge cell membrane. Sponges possessing unconventional sterols are known to have a softer (less dense) consistency.\textsuperscript{121}
Psammaplysilla sp. are known to contain both simple and some very complex bromotyrosine compounds. Our work on an Australian Psammaplysilla sp. involved isolation of brominated metabolites to see if there were any novel brominated compounds in this verongid sponge. Analysis of the sterol fraction was carried out to separate the unusually alkylated sterols expected to be present in all verongid sponges and to note the percentage of these sterols in this sponge. A large number of unusually alkylated sterols
are known and it would be interesting to note which of these are present in this *Psammaplysilla* sp.
3.2 Results and Discussion

3.2.1 Brominated Metabolites

3.2.1.1 Isolation of Aeroplvisinin-1 (96)

A dark grey Psammaplysilla sp. (mustard interior) (wet weight 465 g, dry weight 93.6 g) was collected at North Wollongong, N.S.W., in October 1988 and extracted with ethanol. The ethyl acetate soluble portion was evaporated to give the crude extract (2.01 g, 2.15%). The crude extract was purified by flash chromatography using hexane/dichloromethane (1:1), changing to ethyl acetate and finally methanol. Elution of the flash column with dichloromethane/ethyl acetate (5:1) gave a major u.v. active brown spot with Rf 0.4 on t.l.c. (dichloromethane/ethyl acetate (3:1), in a yield of 0.52%. Recrystallization from chloroform gave aeroplysinin-1 (96), melting point 118-119° (Lit. 120°). The 1H n.m.r. spectrum showed the expected signal for an OCH$_3$ group at $\delta$ 3.70, a methylene group at $\delta$ 2.89 (2H, s) and the C=CH system at $\delta$ 6.41. The $\delta$ 6.41 signal showed a long range coupling (J 1.1 Hz) to $\delta$ 4.25 signal assigned to CHOH. The $\delta$ 4.25 proton signal was further coupled to the $\delta$ 5.26 signal for the hydroxyl proton ($\delta$, J 7.7 Hz, OH, exchanges with D$_2$O), indicating that they were on the same carbon. The $^{13}$C n.m.r. spectrum contained 9 carbon signals and the chemical ionization mass spectra showed a cluster of peaks in the region 337-342 (M+, M+H$^+$) for the molecular ion. Aeroplysinin-1 is known to exist in nature in both enantiomeric forms. From the optical rotation ([$\alpha$]$_D$ +194 (c, 0.219)) and melting point (118-119°), our sample was confirmed to
be the (+)-isomer, the (-)-isomer is known to have a lower melting point (112-116°).\textsuperscript{127}

Acetylation of (96) using acetic anhydride in pyridine\textsuperscript{128} gave aeroplysinin-1 diacetate (118), recrystallized from hexane/ chloroform (2:1), m.p. 110-112° (Lit. 114°). The infrared spectrum contained the carbonyl signal ($\nu_{\text{max}}$ 1755 cm$^{-1}$). The $^1$H n.m.r. spectrum of (118) showed two additional methyl singlets at $\delta$ 2.22 and 2.08 and a downfield shift (1.96 ppm) for the CHOAc signal to $\delta$ 6.21. The $^{13}$C n.m.r. contained 13 carbon signals with two at 169.1 and 169.4 ppm for the carbonyl containing carbons in agreement with structure (118).

Acid treatment of (+)-aeroplysinin-1 has been known to generate three rearranged dienones.\textsuperscript{129} Therefore acid treatment of (96) was undertaken to confirm that (96) is indeed (+)-aeroplysinin-1. Treatment of (96) with trifluoroacetic acid\textsuperscript{129} gave a mixture of four products, which were separated by h.p.l.c. (35% ethyl acetate in hexane). The least polar compound (119) was eluted in a yield of 4.25%. The $^1$H n.m.r. spectrum showed an aromatic proton singlet at $\delta$ 7.52 integrating for one H, a one proton singlet at $\delta$ 5.85 (assigned for an OH or NH, exchangeable with D$_2$O), a three proton singlet at $\delta$ 3.88 (OCH$_3$) and a two proton singlet at $\delta$ 3.70 (CH$_2$). By comparison
with literature data\textsuperscript{109} compound (119) was assigned to be the known compound 3,5-dibromo-2-hydroxy-4-methoxyphenyl-acetonitrile.\textsuperscript{109} The sample (119) decomposed before an optical rotation was obtained, therefore it was not determined whether it was the (+) or the (-) enantiomer.

The other three compounds were the expected dibromonitriles (120) and (121) in a mixture of 3:1 and the dienone (122) which was obtained in a yield of 50\% of the total mixture. The signal for OCH\textsubscript{3} was absent in the $^1$H n.m.r. of (120), compared to (96) and (119). The signal at $\delta$ 7.30 (1H, d, J 2.1 Hz) was assigned to the olefinic proton coupled to $\delta$ 4.43 proton. The $\delta$ 4.43 signal was also coupled to the hydroxyl signal at $\delta$ 5.96 (d, J 5.6 Hz, OH) indicating a CHO\textsubscript{2}H group. By comparison with literature data\textsuperscript{129} the structure for (120) was assigned.

Similarly, compound (121) showed the expected ABq signal, integrating for two protons (CH\textsubscript{2}) at $\delta$ 3.15, the olefinic proton at $\delta$ 7.52 (s) and the two hydroxyl protons at $\delta$ 5.78 and 6.04 in the $^1$H n.m.r. The doublet at $\delta$ 5.05 (H 5') was coupled to the $\delta$ 4.28 signal (d, J 11.1 Hz) assigned to H 6', consistent for a diaxial coupling in (121). Finally, the $^1$H n.m.r. spectrum of the dienone (122) contained two olefinic proton signals at $\delta$ 7.62 (2H, s), a single hydroxyl proton at $\delta$ 6.12 (bs, exchanges with D\textsubscript{2}O) and a CH\textsubscript{2} group at $\delta$ 3.11 consistent with structure (122).

Treatment of aeroplysinin-1 with trifluoroacetic acid previously had resulted in either dibromoketone (123)\textsuperscript{127} or compounds (120)-(122)\textsuperscript{129} only, and not (119). Aromatic (119) was isolated initially on base treatment of aeroplysinin-1.\textsuperscript{109} Therefore, it is surprising
that acid treatment of aeroplysinin-1 should also produce (119), thus it is more likely that (119) in this case is formed by acid catalysed dehydration of aeroplysinin-1 (Scheme 3.3).

![Scheme 3.3 Formation of compounds (119)-(122) on acid treatment of aeroplysinin-1](image)

3.2.1.2 Isolation of Aerothionin and Homoaerothionin (124)-(125)

Two more polar compounds aerothionin (124) and homoaerothionin (125) were obtained from the flash column on a *Psammaphlysilla* sp. extract using ethyl acetate. Aerothionin (124) crystallized out of ethyl acetate in a yield of 0.19%, while homoaerothionin (125) was further purified by h.p.l.c. using 70% ethyl acetate in hexane in a yield of 0.15%. The infrared spectra of both compounds were very similar, showing the presence of OH ($\nu_{\text{max}}$ 3335 cm$^{-1}$), NH ($\nu_{\text{max}}$ 3165 cm$^{-1}$), carbonyl ($\nu_{\text{max}}$ 1725 cm$^{-1}$) and olefinic stretch ($\nu_{\text{max}}$ ...
Their u.v. spectra confirmed the presence of a homocyclic conjugated diene conjugation ($\lambda_{\text{max}}$ 283, $\varepsilon$ 11633). The $^1$H n.m.r. spectrum of homoaerothionin (125) had a broad triplet at $\delta$ 7.63 (2H, NHCH$_2$) and the olefinic signal at $\delta$ 6.52 (2H, s, C=CH). Irradiation experiments confirmed that the two proton signal at $\delta$ 5.48 (bs) was coupled to the $\delta$ 4.16 proton (2H, bs, CHOH). Therefore the hydroxyl proton was assigned the $\delta$ 5.48 signal which was exchangeable with D$_2$O. The signal at $\delta$ 3.82 showed an AB coupling of 18.3 Hz to the $\delta$ 3.17 signal, each assigned for a CH$_2$ group. A six proton singlet at $\delta$ 3.72 was expected to be two methoxy signals. When the multiplet at $\delta$ 3.31 (4H, m, NHCH$_2$) was irradiated, it caused the broad triplet at $\delta$ 7.63 (NH) to collapse to a singlet and also affected the multiplet at $\delta$ 1.60 (6H, CH$_2$-CH$_2$-CH$_2$). The $^{13}$C spectrum had 13 carbon signals, twelve due to 2 carbons each because of the symmetry of the molecule. Acetylation of (125) using acetic anhydride in pyridine gave the diacetate (127), recrystallized from ethanol (m.p. 160-162°, Lit. 166-167°). The i.r. spectrum showed the ester stretch ($v_{\text{max}}$ 1755 cm$^{-1}$, OAc). The $^1$H n.m.r. spectrum contained two additional methyls at $\delta$ 2.15 (6H, s), while the $^{13}$C n.m.r. spectrum had four additional signals, two each at 169.7 ppm and 20.4 ppm. In the $^1$H n.m.r. the methine proton ($\delta$ 4.16) in (125) shifted downfield to $\delta$ 5.82 in (127).

Aerothionin (124) (m.p. 130-134°, Lit. 134-137°) had very similar spectroscopic data to (125), except in the $^1$H n.m.r. spectrum the signal at $\delta$ 1.62 integrated for four protons instead of six and the $^{13}$C n.m.r. showed only 24 carbon signals. The diacetate (126) was prepared in the same way as (127) (m.p. 201-204°, Lit. 206-208°) and showed the expected additional ester stretch in the i.r.
spectrum ($v_{\text{max}}$ 1755 cm$^{-1}$), while the $^1$H and $^{13}$C n.m.r. data were as expected for aerothionin diacetate.

Recently dihydroxy and monohydroxy derivatives of aerothionin were isolated from two Caribbean sponges *Verongula rigida* and *Pseudoceratina durissima*. The structure of both compounds has been determined by interpretation of spectral data and comparison with aerothionin. The stereochemistry of the 11-hydroxy group in hydroxyaerothionin and 11,12-dihydroxy groups in dihydroxyaerothionin has not yet been determined. Interestingly, these compounds were found to co-occur with the known metabolites aerothionin, homoaerothionin and aerophobin-1 in the sponges. Hydroxyaerothionin was found to have similar biological activity to that of aerothionin and homoaerothionin. Therefore hydroxyaerothionin and possibly dihydroxyaerothionin are most likely to play a similar role in the sponge as aerothionin and homoaerothionin.
3.2.2 Sterols

A nonpolar fraction was eluted from the flash column on silica, using dichloromethane. The spot on t.l.c. went dark purple on spraying (H$_2$SO$_4$/vanillin), indicating the presence of sterols. Purification by reverse phase h.p.l.c. using methanol gave a number of minor peaks and a major peak. The ratio of the total minor peaks to that of the major peak was 2:3. The major peak was collected and the sterol characterized by comparison of its spectroscopic data with literature values as aplysterol (107). The molecular ion in the electron impact mass spectrum was at M$^+$ 414 in agreement with the molecular formula C$_{29}$H$_{50}$O. In the $^1$H n.m.r. a single proton multiplet at δ 5.35 was assigned to the olefinic proton and a one proton multiplet at δ 3.51 for the CHO proton. There were seven protons in the region δ 1.5-2.5 and twenty three protons in the region δ 0.8-1.5. The two singlet methyls were at 0.67 (18-H$_3$) and 1.00 (19-H$_3$), and three methyl doublets and one methyl triplet between δ 0.7-0.9.

In the mass spectra of (107), the peak at m/e 273 was due to loss of the C 10 side chain and the peak at m/e 255 was due to loss of C 10 side chain + H$_2$O. Also there were peaks at m/e 231 (loss of C 10 side chain + C$_3$H$_6$) and m/e 213 (loss of C 10 side chain + H$_2$O + C$_3$H$_6$) (Fig. 3.1). Acetylation of aplysterol gave aplysteryl acetate (128), confirmed by the presence of M-OAc (m/z 397) in the mass spectrum and the additional downfield methyl at δ 1.98 in the $^1$H n.m.r. spectrums as well as deshielding of the methine signal to δ 4.56 in (128).
Aplysterol (107) and 24 (28) dehydroaplysterol (108) differ in that (108) has an additional double bond compared to (107). Both (107) and (108) are known to occur together in a number of verongid sponges, therefore (108) may have been present as one of the minor constituents of this *Psammoplysilla* sp. However this was not investigated due to lack of time.

![Chemical structures of Aplysterol and Dehydroaplysterol](image)

Figure 3.1 Fragmentation pattern for ions m/z 273, 255, 231, 213 and 161 in e.i.m.s. of aplysterol

It has been suggested that the unconventional sterols found in sponges have a role analogous to that of cholesterol in higher animals, since cholesterol is often replaced by unconventional sterols in sponges. Also as aplysterol, dehydroaplysterol, verongulasterol *etc.* have been shown to be membrane constituents in *Aplysina fistularis*, it is reasonable to speculate that the
aplysterol in *Psammaplysilla* sp. may also serve the same functional role.
3.3 Summary and Conclusion

The order Verongida is unique in that it produces bromotyrosine derived compounds responsible for the marked antibiotic activity of these sponges. Aeroplysinin-1 showed antibacterial activity against *Staphylococcus albus, Bacillus cereus,* and *B. subtilis.*

Aeroplysinin-1 has been isolated from a number of verongid sponges in three different forms, the dextrorotatory, the levorotary and the racemic forms. The dextrorotatory enantiomer was obtained from *Verongia aerophoba, Aplysina (Verongia) fistularis, A. laevis,* *Psammaplysilla purpurea* and *Aplysina cavernicola.* The levorotatory enantiomer was obtained from the sponges *Pseudoceratina crassa, Verongula gigantea* and *Ianthella ardis,* while racemic aeroplysinin-1 was found in some Caribbean collections of *Verongula gigantea* and *Pseudoceratina crassa.* It is unusual to obtain a natural product in both the enantiomeric and the racemic forms. Racemic aeroplysinin-1 has been synthesized by Andersen and Faulkner.

We have isolated (+)-aeroplysinin-1 from a *Psammaplysilla* sp. based on the optical rotation ([α]D + 194) and melting point (118-119°). Aeroplysinin-1 was the major constituent (0.52%) of our sponge.

Two bromotyrosine metabolites, aerothionin (124) and homoaerothionin (125), were also obtained in 0.19% and 0.15% yield respectively. These have been previously isolated from a number of verongid sponges and in one case synthesized. Only aeroplysinin-1, aerothionin and homoaerothionin were isolated from the *Psammaplysilla* sp. because these were the major constituent of this sponge, other
compounds present in smaller amounts could not be analysed due to lack of time.

Aplysterol was isolated from the *Psammaplysilla* sp. under investigation. Aplysterol, 24 (28) dehydroaplysterol, 25 (26) dehydroaplysterol and verongulasterol are some of the unconventional sterols that make up the cellular membrane of the verongid sponges. The structural modification exhibited by these novel sponge sterols could suggest a structural adjustment for a "better fit" with other membrane components such as the phospholipids.
3.4 Experimental

3.4.1 Instruments used

Experimental details are as for *Cacospongia* sp. except:
1) Electron Impact and Chemical Ionization mass spectra were recorded on a Vacuum Generator V.G.-12-12 mass spectrometer.
2) Infrared spectra were recorded on a Perkin Elmer 783 Infrared spectrometer for solutions in chloroform or as nujol mulls.
3) Reverse phase High Performance Liquid Chromatography was performed using a waters C 18 reverse phase column and waters R403 refractive index detector.

3.4.2 Method

3.4.2.1 Sponge Collection and Extraction

Dark grey sponge (mustard inside) (wet weight 465 g, dry weight 93.6 g) was collected at North Wollongong and extracted with ethanol (95%) (3x250 ml). The ethanol extract was filtered and evaporated to an aqueous solution, then partitioned between ethyl acetate (3x100 ml) and water (100 ml). The combined ethyl acetate extract was dried over anhydrous sodium sulphate, filtered and reduced under vacuum to give a brown oil (2.01 g, 2.15%).
3.4.2.2 Column Chromatography of Crude Extract

A column (15 by 2 cm) was packed with silica and sand (0.5 cm) applied to the top. The column was flushed under nitrogen with hexane/dichloromethane (1:1) and the crude extract dissolved in a minimum volume of hexane/dichloromethane (1:1) was carefully introduced to the top of the sand with a pasteur pipette. The column was eluted with hexane/dichloromethane (1:1) (50 ml), changing to dichloromethane (200 ml), dichloromethane/ethyl acetate (3:1) (300 ml), ethyl acetate and finally methanol. Fractions (20 ml) were collected, checked by t.l.c. using dichloromethane/ethyl acetate (3:1) and grouped into six major fractions. From the $^1$H n.m.r. the first fraction was mainly fats (0.040 g, 0.43%). Fraction two contained some fats and sterols (0.040 g, 0.43%), while fractions (3)-(5) (1.23 g, 1.31%) contained mostly the bromo compounds.

3.4.2.3 Isolation of Bromo Compounds

Fraction 3 (0.59 g), on evaporation in vacuo, gave shiny white crystals, which were separated from the mother liquor and recrystallized from chloroform (0.36 g, 0.38%). Purification of the liquid portion by h.p.l.c. using ethyl acetate (50%) in hexane gave a further portion of crystals, recrystallized from chloroform (0.13 g, 0.14%) as aeroplysinin-1 (96), m.p. 118-119°, $[\alpha]_D + 194^\circ$ (c, 0.219, in CH$_3$COCH$_3$). $\lambda_{\text{max}}$ (CH$_3$OH) 203 (ε 4272), 233 (3240), 282 nm (5893). $v_{\text{max}}$ (nujol) 3340 (OH), 2250, 1625, 1575, 1300, 1195, 1095, 645 cm$^{-1}$. Mass spectrum: m/z 341/339/337 (M$^+$, M$^+$H$^+$), 323/321/319 (M-OH)$^+$, 297/295/293 (M-OH$^-$-H CN)$^+$, 247/245/243/241 (M-OH$^-$-Br$^-$+H)$^+$ (base peak). $^1$H n.m.r. (90 MHz)
(acetone-d₆) δ 2.89 (2H, s, CH₂), 3.70 (3H, s, OCH₃), 4.25 (1H, dd, J 7.7, 1.2 Hz), 5.26 (1H, s, OH), 5.26 (1H, d, J 7.7 Hz, OH), 6.41 (1H, d, J 1.1 Hz). ¹³C n.m.r. (acetone-d₆) 26.7, 60.0, 74.5, 78.5, 113.7, 117.9, 121.0, 133.6, 148.5 ppm.

Acetylation of (96) (0.10 g) using acetic anhydride in pyridine gave the diacetate (118) (0.093 g, 77.5%) as shiny white crystals, m.p. 110-112°, λₘₐₓ (CH₂Cl₂) 235 (ε 3424), 290 nm (5214). vₘₐₓ (nujol) 2235, 1755, 1735, 1635, 1585, 1300, 1220, 1195, 1005 cm⁻¹. Mass spectrum: m/z 425/423/421 (M⁺), 365/363/361 (M⁺-CH₃COOH), 343/341/339 (M⁺-Br), 323/321/319 (M⁺-CH₃COOH-CH₂CN), 308/306/304 (M⁺-2CH₃COOH), 241, 239 (100%). ¹H n.m.r. (CDCl₃) (400 MHz) δ 2.08 (3H, s, CH₃), 2.22 (3H, s, CH₃), 3.06 (2H, q, J 16.9 Hz, CH₂), 3.76 (3H, s, OCH₃), 6.21 (1H, s), 6.54 (1H, s). ¹³C n.m.r. (CDCl₃) 20.5, 21.2, 22.3, 60.2, 72.9, 79.6, 108.5, 114.7, 120.1, 129.2, 149.3, 169.1, 169.4 ppm.

On evaporation of fraction 5 (0.64 g), some shiny crystals appeared at the bottom of the flask. These were removed by filtration and recrystallised from ethyl acetate to give aerothionin (124) (0.18 g, 0.19%) m.p. 130-134° (Lit 134-137°).¹⁰⁷ λₘₐₓ (CH₃OH) 205 (ε 15752), 232 (17692), 283 nm (11633). vₘₐₓ (nujol) 3335, 3165, 1725, 1670, 1635, 1600, 1575, 1550, 1265, 1220, 1045, 915, 740 cm⁻¹. ¹H n.m.r. (acetone-d₆) δ 1.62 (4H, m), 3.16 (2H, d, J 18.3 Hz), 3.34 (4H, m), 3.72 (6H, s, OCH₃), 3.83 (2H, d, J 18.3 Hz), 4.16 (2H, d, J 8.1 Hz), 5.42 (2H, d, J 8.1 Hz), 6.52 (2H, s), 7.63 (2H, bt). ¹³C n.m.r. (acetone-d₆) 27.4, 39.4, 40.2, 60.2, 75.1, 91.6, 113.8, 122.0, 132.4, 148.8, 155.3, 159.9 ppm.

The diacetate (126) was prepared by adding acetic anhydride (5 ml) and pyridine (5 ml) to (124) (0.01g) in a round bottomed flask.
and stirring overnight at room temperature. Aerothionin diacetate was extracted with ethyl acetate and recrystallized from hot ethyl acetate (0.10 g, 90.7%) m.p. 201-204° (Lit 206-208°). $\lambda_{\text{max}}$ (CH$_2$Cl$_2$) 231 (ε 13472), 285 nm (7337). $\nu_{\text{max}}$ (CHCl$_3$) 3150, 2830, 1755, 1670, 1600, 1580, 1370, 985, 670 cm$^{-1}$. $^1$H n.m.r. (CDCl$_3$) δ 1.63 (4H, m), 2.15 (6H, s, CH$_3$), 3.10 (2H, d, J 18.5 Hz), 3.38 (4H, m), 3.43 (2H, d, J 18.5 Hz), 3.77 (6H, s, OCH$_3$), 5.82 (2H, s), 6.32 (2H, s), 6.67 (2H, bt).

The mother liquor of fraction 5 was purified by h.p.l.c. using 70% ethyl acetate in hexane to give homoаerothionin (125) as a pale yellow oil (0.14 g, 0.15%). $^1$H n.m.r. (acetone-d$_6$) δ 1.60 (6H, m), 3.17 (2H, d, J 18.3 Hz), 3.31 (4H, m), 3.72 (6H, s, OCH$_3$), 3.82 (2H, d, J 18.3 Hz), 4.16 (2H, bs), 5.48 (2H, bd, OH, exchanges with D$_2$O), 6.52 (2H, s), 7.63 (2H, bt). $^{13}$C n.m.r. (acetone-d$_6$) 24.8, 27.5, 39.6, 40.2, 60.2, 75.1, 91.6, 113.8, 122.0, 132.4, 145.1, 155.5, 160.0 ppm.

The diacetate (127) was prepared and recrystallized from ethanol (0.092 g, 83.6%) m.p. 160-162° (Lit. 166-167°). $\lambda_{\text{max}}$ (CH$_2$Cl$_2$) 243 (ε 9080), 261 nm (8335). $\nu_{\text{max}}$ (CHCl$_3$) 3020, 2860, 1755, 1700, 1670, 1600, 1370, 670 cm$^{-1}$. $^1$H n.m.r. (CDCl$_3$) δ 1.62 (6H, m), 2.15 (6H, s, CH$_3$), 3.09 (2H, d, J 18.5 Hz), 3.35 (4H, m), 3.42 (2H, d, J 18.5 Hz), 3.78 (6H, s, OCH$_3$), 5.82 (2H, s), 6.31 (2H, s), 6.65 (2H, bt). $^{13}$C n.m.r. (CDCl$_3$) 20.4, 23.1, 28.0, 39.9, 40.7, 60.0, 73.3, 90.0, 107.9, 122.0, 130.6, 149.9, 154.2, 158.9, 169.7 ppm. Three other compounds were isolated in a yield of 0.10 g, 0.09%, but were not identified.
3.4.2.4 Treatment of (96) with Trifluoroacetic acid

Aeroplysinin-1 (20 mg) was placed in a round bottom flask (10 ml) and trifluoroacetic acid (1 ml) was added to the flask. The reaction mixture was stirred at room temperature for 30 minutes, evaporated under vacuum. T.l.c. in dichloromethane/ethyl acetate showed a mixture of 4 compounds, which were separated by h.p.l.c. using ethyl acetate (35%) in hexane. The first compound eluted after 4.5 minutes (chart speed 30 cm/h, flow rate 4 ml/min.) was 3,5-dibromo-2-hydroxy-4-methoxyphenyl-acetonitrile (119) as a white solid (0.85 mg, 4.25%). ¹H n.m.r. (CDCl₃) δ 3.70 (2H, s, CH₂), 3.88 (3H, s, OCH₃), 5.85 (1H, s, OH), 7.52 (1H, s, CH=O).

Dienone (122) was eluted after 6.1 min as a clear oil (7.3 mg, 36.5%). ¹H n.m.r. (acetone-d₆) δ 3.20 (2H, s, CH₂), 6.12 (1H, bs, OH), 7.62 (2H, s, CH=O).

The dibromonitriles (120) and (121) were eluted after 7.6 and 9.5 minutes respectively. (1'R, 5'S, 6'S)-2-(3',5'-dibromo-1',6'-dihydroxy-4'-oxycyclohex-2'-enyl) acetonitrile (120) (4.8 mg, 24.0%) as a white solid. ¹H n.m.r. (acetone-d₆) δ 3.11 (2H, s, CH₂), 4.43 (1H, ddd, J 5.6, 2.1, 2.1 Hz), 5.72 (1H, d, J 2.1 Hz), 5.94 (1H, s, OH), 5.96 (1H, d, J 5.6 Hz), 7.30 (1H, d, J 2.1 Hz).

(1'R, 5'R, 6'S) epimer (121) (1.4 mg, 7.0%). ¹H n.m.r. (acetone-d₆) δ 3.15 (ABq, J 16.9 Hz, CH₂), 4.28 (1H, ddd, J 11.1, 5.8, 5.3 Hz), 5.05 (1H, d, J 11.1 Hz), 5.78 (1H, bs, OH), 6.04 (1H, bs, CHOH), 7.25 (1H, s).
3.4.2.5 Isolation of aplysterol

Fraction 2, eluted from the flash column using dichloromethane/ethyl acetate (5:1) appeared to contain mostly sterols from the $^1$H n.m.r. and t.l.c. The crude fraction was cleaned by a further flash chromatography using a column (5 cm by 1 cm) packed with silica and eluted with dichloromethane, dichloromethane/ethyl acetate (7:1), (5:1), (3:1) and (1:1), changing to ethyl acetate and methanol. Fractions (15 ml) were collected and combined after t.l.c. into 4 major fractions. From the t.l.c. (dichloromethane/ethyl acetate (3:1)) and $^1$H n.m.r., sterols were concentrated in the second fraction (0.11 g, 0.12%). Purification by reverse phase h.p.l.c. using methanol gave a major peak and 3-4 minor peaks (very close together). The minor peaks were combined together and not analysed further. The major compound was separated and identified to be aplysterol (107) (32.6 mg, 0.034%). $^1$H n.m.r. (CDCl$_3$) δ 0.67 (3H, s, CH$_3$), 0.79 (3H, t, CH$_3$), 0.84 (3H, d, CH$_3$), 0.91 (3H, d, CH$_3$), 1.00 (3H, s, CH$_3$), 0.70-2.50 (30H, m), 3.51 (1H, m), 5.35 (1H, bd). Mass spectrum: m/z 414 (M$^+$, 10%), 398 (12), 396 (11), 329 (3), 303 (20), 273 (18), 255 (22), 231 (20), 213 (25), 161 (33), 145 (30), 121 (42), 107 (74), 95 (80), 81 (100%).

Acetylation of (107) using acetic anhydride and pyridine resulted in the acetate (128). $^1$H n.m.r. (CDCl$_3$) δ 1.96 (3H, s, OAc), 4.53 (1H, m, CHOAc). Mass spectrum: m/z 394 (M$^+$-OAc, 20%), 393 (18), 379 (8), 310 (5), 295 (12), 255 (25), 213 (20), 147 (70), 135 (30), 133(35), 121 (42), 107 (53), 95 (64), 81 (100%).
CHAPTER 4

ISOLATION AND STRUCTURE ELUCIDATION OF COMPOUNDS FROM TWO DENDROCERATID SPONGES
The order Dendroceratida, which contains fewer species than the Dictyoceratida, includes the genus *Aplysilla, Darwinella, Dendrilla, Chelonaplysilla and Pleraplysilla*. These are exemplified by sponges with dendritic skeleton and large sac shaped choanocyte chambers, or by some forms lacking a fibrous skeleton completely. The genus *Aplysilla* contains a small number of species including *Aplysilla rosea, Aplysilla sulphurea* and *Aplysilla violacea*, which vary in colour from red-pink, tango, violet to cream-white. The terpenoid compounds are more commonly found in the sponges of the order Dictyoceratida where they appear to be the biosynthetic alternatives to sterols. The *Dysideidae* family of the dictyoceratida is known to contain sesquiterpenes typical of the genus *Pleraplysilla* (Dendroceratid) while the *Spongiidae* family has been reported to contain furanoterpenes related to furospongin (129) and diterpenes, typical of a number of aplysillid sponges.

4.1 Introduction

4.1.1 Diterpenes from Dictyoceratid sponges

*Isoagatholactone* (130), a diterpene lactone was isolated from the sponge *Spongia officinalis*. Its structure was determined by chemical correlation with grindelic acid (131). *Isoagatholactone* (130) has also been synthesised by Wakano *et al* from (+)-labda-8(20),13-dien-15-oic acid.
A number of spongian diterpenes have been isolated from *Hyatella intestinalis*\(^{143}\) (order dictyoceratida). Spongia-13(16),14-diene (132) has also been synthesized by Nakano *et al*\(^{144}\) in its racemic forms. Another spongian diterpene spongialactone A (133) has been reported from *Spongia arabica* of Red Sea\(^{145}\). Its structure was determined by 2D n.m.r. techniques and a series of chemical reactions. The stereochemistry of ring A of (133) was established by \(^1\)H n.O.e. experiments.

Further from *Spongia officinalis* a spongian diterpene 15\(\alpha\), 16\(\alpha\)-diacetoxysspongian (134) was isolated, which was closely related to aplysillin (135), isolated from the dendroceratid sponge *Aplysilla rosea* collected in New Zealand. Aplysillin (135) has an extra acetyl group at C 12\(^{146,147}\).
However, aplysillin was suggested to be a more likely constituent of the dictyoceratid sponges and (135) together with some related antimicrobial diterpenes were isolated from *Spongia officinalis*.\textsuperscript{148}

4.1.2 Diterpenes from Aplysillid Sponges

From the pink encrusting sponge *Aplysilla rosea* collected at shallow depths in waters off Sydney a number of spongian type diterpene aplyroseols and some linear compounds such as ambiofuran (136) were reported. Aplysillin (135)\textsuperscript{139} was not isolated. Ambiofuran was initially isolated as a minor constituent of *Dysidea amblia*,\textsuperscript{149} showing the close association between the two orders.

Other aplysillid sponges have produced related diterpenes derived from a spongian precursor (137). From the bright yellow encrusting sponge *Aplysilla sulphurea* (order dendroceratida) obtained at
depths up to 30 m in waters of Eastern Australia, aplysulphurin (138) was obtained.\textsuperscript{150}

The aplysulphurin skeleton is expected to be formed from the spongian precursor (137) by cleavage of ring B. (Scheme 4.1). The absolute configuration at C 1' of (138) was found to be $R$ and this supports the proposed biogenesis.

\textbf{Scheme 4.1. Formation of aplysulphurin from the spongian precursor}
Two aromatic norditerpenes macfarlandin A (139) and B (140) with structures related to alysulphurin were obtained from the dorid nudibranch *Chromodoris macfarlandi*, which is a shell-less marine mollusc.\(^\text{151}\) Members of this genus are known to feed on sponges from which they obtain their defensive chemicals.\(^\text{152}\) From another dorid nudibranch *Chromodoris norrisi* an unusual rearranged diterpene norrisolide (141) was obtained. This compound was also found to be a minor constituent of the *Dendrilla* sp.\(^\text{153}\)

It was proposed that the norrisane skeleton (142) was derived from spongian skeleton (9) by opening of ring C and contraction of ring B.

Other related diterpenes biosynthesised from the spongian precursor are the dendrillolides A, B and C, obtained from a *Dendrilla* sp., and expected to be formed from (137) by migration of 5,10 bond and opening of ring C.\(^\text{154}\) Separation of macfarlandins C, D
and E from the nudibranch *Chromodoris macfarlandi* and comparison of their spectral data indicated that the structures of the dendrillolides A and B were incorrect.\textsuperscript{155} Faulkner *et al* reisolated the dendrillolides A and B together with dendrillolides D and E and related compounds.\textsuperscript{156} The correct structure of dendrillolide A (143) has been determined, but the structure of dendrillolide B is still unknown. Macfarlandin E (144) was reisolated from the sponge *Aplysilla polyrhaphis* as a major metabolite together with norrisolide (141).\textsuperscript{157} Macfarlandin E has been also reported from a dictyoceratid sponge of the genus *Dysidea*.\textsuperscript{158}

![Chemical structures](143) (144)

Norditerpenes are known to occur in some aplysillid sponges and nudibranchs. A degraded diterpene glaciolide (145) was isolated from a nudibranch *Cadlina luteomarginata* as well as from the sponge *Aplysilla glacialis*.\textsuperscript{159} The nudibranchs were found feeding on the sponge and are known to obtain their secondary metabolites from the sponges, and concentrate them in glands on their dorsum, where they are used to prevent predation.\textsuperscript{152,160}
Other degraded diterpenes have been isolated from *Aplysilla* sp. collected in waters off Jervis Bay, New South Wales,\textsuperscript{161} and from the sponge *Aplysilla glacialis* and specimens of the nudibranch *Cadlina luteomarginta* found feeding on the encrusting sponge.\textsuperscript{162} The skin chemistry of *C. luteomarginta* is known to vary from site to site reflecting the variability in species composition and secondary metabolite content of its sponge diet.

Moreover, isolation of ichthyotoxic metabolites luteorosin (146), 12-\textit{epi}-aplysillin (147) and (148) together with macfarlandin A (139) from the nudibranch *Chromodoris luteorosea*,\textsuperscript{163} found feeding on the sponge *Spongionella gracialis* confirms the taxonomical relationship between the spongiidae family and dendroceratid sponges. These compounds may be obtained from the sponge *Spongionella gracialis* as related compounds have been isolated from this sponge.
In search of further novel diterpenes from aplysillid sponges, two dendroceratid sponges *Aplysilla* var *Pallida*\(^ {164} \) and *Aplysilla violacea* were collected in the waters of Sydney in April 1989 by Dr. Stephen Toth (University of Sydney) and analysed for their metabolite contents.
4.2 Results and Discussion

4.2.1 Aplysilla var Pallida

4.2.1.1 Aplysulphurin (138)

An aplysillid sponge (*Aplysilla var Pallida*) was extracted using ethanol (95%), and the ethanol extract partitioned between ethyl acetate and water. The ethyl acetate soluble portion was purified by silica flash column chromatography to give five fractions. The major u.v. active fraction was subjected to further column chromatography, preparative thin layer chromatography and high performance liquid chromatography. A u.v. active spot, pink on spraying with H$_2$SO$_4$/vanillin that went mustard when heated, was identified as the known compound aplysulphurin (138), obtained in a yield of 0.33%. In the $^1$H n.m.r. spectrum, there were four aromatic protons ($\delta$ 7.0-7.7), a quartet at $\delta$ 4.40 and twenty three protons in the region $\delta$ 0.8-2.5 including an acetyl group at $\delta$ 2.16, a methyl doublet at $\delta$ 1.71 and three other methyl singlets. In the infrared spectrum there were two carbonyl absorptions at 1710 and 1746 cm$^{-1}$, the corresponding signals in the $^{13}$C n.m.r. were at 170.4 and 171.6 ppm. Also the $^{13}$C n.m.r. further contained 6 aromatic carbon signals and 14 other signals consistent with the molecular formula C$_{22}$H$_{28}$O$_5$. In the chemical ionization mass spectrum (isobutane) the [M+H$^+$] ion was at m/z 373. Comparison of the above data with that of the literature suggested the major compound to be aplysulphurin (138).
4.2.1.2 Minor Metabolites of *Aplysilla var Pallida*

*Compound (149)*

A minor fraction obtained from the h.p.l.c. (15% ethyl acetate in hexane) in a yield of 0.09%, appeared to be a mixture of two compounds from t.l.c. The two compounds were separated by preparative thin layer chromatography using dichloromethane (plate run four times) and the two bands extracted.

$^1$H n.m.r. showed that the more polar fraction was a sterol fraction and this was not further identified. The other compound (149) obtained in a yield of 0.004%, from the $^1$H n.m.r. appeared to be related to aplysulphurin (138) with major differences in the aromatic region. There were no aromatic signals in the $\delta$ 7.0 region, these being replaced by $\delta$ 6.00 and $\delta$ 6.18 doublets. Also there was an additional broad triplet at $\delta$ 3.22 and a multiplet at $\delta$ 2.52. The $^1$H n.m.r. still had a quartet at $\delta$ 4.22, an acetyl signal at $\delta$ 2.10 and four methyl signals with one of them being a doublet as in (138). The $^{13}$C n.m.r. spectrum indicates the presence of 22 carbons. The h.r.e.i.m.s. gave the molecular formula $C_{20}H_{28}O_3$ for $M^+$-AcOH ion (m/z 316) indicating the molecular formula for the compound to be
C_{22}H_{32}O_5. That is the same carbon skeleton as aplysulphurin with four additional protons, and this was consistent with the absence of aromatic protons in the $^1$H n.m.r. In the i.r. spectra, the carbonyl absorption at 1730 cm$^{-1}$ indicated δ-lactone as in aplysulphurin.

Using the $^1$H decoupling experiments, it was shown that the δ 3.21 proton was coupled to the proton signal at δ 6.00 (J 6.1 Hz), assigned to H 3, as well as to the δ 2.52 multiplet. The δ 2.52 proton was further coupled to δ 6.18 (d, J 2.4 Hz) assigned to H1. Therefore the δ 3.21 and δ 2.52 signals were assigned to H 3a and H 7a respectively. Comparison of the data of our compound with that of tetrahydrosulphurin-1 (149),$^{165}$ indicated that these were most likely the same compounds. Tetrahydrosulphurin-1 (149) has been isolated from an aplysillid sponge Darwinella oxeata together with aplysulphurin and tetrahydrosulphurin-2 (150) and -3 (151).$^{165}$

![Chemical Structures](image)

In the $^1$H n.m.r data reported for tetrahydrosulphurin-1, H 3a was at δ 3.12 (m) and H 7a at δ 3.10 (m). For our compound (149) H 3a was at δ 3.21 (m) and H 7a at δ 2.52 (m). However, it was not possible for our compound to have the structure of tetrahydrosulphurin-2 (150), since in (150) there would be no proton at H 3a, thus the H 3 signal (δ 6.00, d) should appear as a
singlet. But from decoupling experiments it was shown that the H 3a (δ 3.21) signal in compound (149) was coupled to signals at δ 2.52 (H 7a) and δ 6.00 (d, J 6.1 Hz, H 3). Moreover, if the alternative structure (151) for tetrahydrosulphurin-3 had been obtained, the 1H n.m.r. would show a methyl singlet at H3C 2'. As our compound showed a methyl doublet at δ 1.42 (J 7.4 Hz) together with a quartet at δ 4.22 (J 7.4 Hz) for H 1', structure (151) is also not possible.

Some errors in the 1H n.m.r. data originally reported by Karuso et al.\textsuperscript{165} for tetrahydrosulphurin-1 were recently pointed out by Tischler et al., who reisolated tetrahydrosulphurin-1 (149) from a nudibranch Cadlina luteomarginta found feeding on the sponge Aplysilla glacialis.\textsuperscript{162} In the corrected literature data H 3a was at δ 3.22 (m) and H 7a at δ 2.52 (m) which matches with our data for compound (149) ( H 3a at δ 3.21 (m) and H 7a at δ 2.52 (m)), thus compound (149) and tetrahydrosulphurin-1 are same. Related compounds cadlinolide A (152) & B (153)\textsuperscript{162} have been isolated from the sponge Aplysilla glacialis, with cadlinolide A (151) present as well in the skin extract of the nudibranch (C. luteomarginta). Tetrahydrosulphurin-1 (149) was found only in the skin extract of the nudibranch, suggesting in this case (149) is more likely a product of the nudibranch, formed from cadlinolide A (152) by reduction to alcohol (Cadlinolide B) and acetylation. This possibility is currently being explored by Tischler et al.
The spectral fragmentation pattern is as shown in Scheme 4.2.

Scheme 4.2 Fragmentation of ions m/z 316, 272 and 243 for (149) in e.i.m.s.

The stereochemistry of tetrahydrosulphurin-1 at C1', C 1", C 1 and C 3 were assigned by analogy with that of aplysulphurin (138) and the stereochemistry at C 3a and C 7a was assigned from biogenetic considerations, and this was confirmed from single crystal X-ray determination studies.166
Compound (154)

Compound (154) was eluted from the h.p.l.c. column using 15% ethyl acetate in hexane, obtained in a yield of 0.004%. From the $^1$H n.m.r. spectra, compound (154) appeared to be closely related to aplysulphurin. It had two aromatic protons showing $o$-coupling to each other ($J$ 8.3 Hz), as in aplysulphurin (138). It was clear that there were no acetoxy groups, due to the absence of methyl signals in the region of $\delta$ 2.0. The infrared spectrum still showed two carbonyl absorptions at 1757 and 1732 cm$^{-1}$. The u.v. spectrum contained aromatic chromophore absorptions ($\lambda_{\text{max}}$ 243, 285 and 294 nm). In the chemical ionization mass spectra, the (M+H$^+$) ion was at m/z 345 for the molecular formula C$_{21}$H$_{28}$O$_4$, implying one carbon and one oxygen less than aplysulphurin. Further, the $^1$H n.m.r had an OCH$_3$ singlet at $\delta$ 3.68 and a two proton singlet (CH$_2$) at $\delta$ 5.21. The $\delta$ 4.60 quartet and the methyl doublet were still present suggesting Cl' as in aplysulphurin (138) or tetrahydrosulphurin-1 (149). The above data were in agreement with the literature data for membranolide, thus compound (154) was assigned the membranolide structure (154). Membranolide and a related compound 9,11-dihydrogracilin (155) have been obtained from a marine sponge Dendrilla membranosa.$^{167}$ It has been suggested that these compounds could be the defensive chemicals of this sponge, as this sponge has not been observed to be eaten, though it lacks spicules.$^{168}$
4.2.2 *Aplysilla* *Violacea*

*Compounds (156) and (157)*

The deep purple sponge (*Aplysilla violacea*) was extracted in the same way as *Var pallida* and partitioned into ethyl acetate. The ethyl acetate soluble fraction was evaporated to give the crude extract (957 mg). Repeated flash chromatography followed by preparative thin layer chromatography gave two fairly pure compounds (156) and (157) (single spots on t.l.c.) in a ratio of 3:1. From the $^1$H n.m.r. (156) and (157) appeared to be fairly similar and to be diterpenoids. The major difference was the extra OAc group present in (157) and thus an additional downfield proton at $\delta$ 5.79, with some differences in the multiplicity of other upfield signals. Also a $\delta$ 6.14 singlet in (156) was deshielded to $\delta$ 6.49 in (157). Detailed spectroscopic analysis was carried out only on (156). The $^1$H n.m.r. spectrum of compound (156) integrated for thirty two protons in the region $\delta$ 1.0-2.0, 6 protons at $\delta$ 2.3-3.0 and four deshielded signals in the region $\delta$ 4.5-6.5, for protons next to oxygen or on a double bond. The $^{13}$C n.m.r. spectrum has twenty two carbon signals with the two carbonyl carbons assigned to an
acetoxy group and a lactone group at 167.7 ppm and 169.5 ppm. There were two carbon signals at 153.1 and 114.7 ppm, assigned to olefinic carbons. Two other deshielded carbons at 100.7 and 100.9 were possibly carbons attached to oxygen. The other carbon signals all were in the region 20-60 ppm, for the methyl, methylene, and methine carbons. The $^{13}$C n.m.r and the proton data for compound (156) matched with that of aplyviolene, thus (156) was most likely aplyviolene as this compound was also previously found to be a major compound of this sponge.\textsuperscript{169,170} Previously a compound dendrillolide A was assigned to have the same structure as aplyviolene (156), but since then its structure assignment has been corrected.\textsuperscript{155,156} Thus compound (157) was assigned to be aplyviolacene (also known as macfarlandin E).\textsuperscript{158} Both aplyviolene (156) and aplyviolacene (157) have recently been reisolated from the encrusting sponge \textit{Chelonaplysilla violacea} and their complete spectroscopic data has been obtained.\textsuperscript{170}

\begin{center}
\includegraphics[width=0.5\textwidth]{image.png}
\end{center}

Aplyviolene and aplyviolacene (macfarlandin E) have also been reported by Faulkner and Bobzin\textsuperscript{157} from a marine sponge \textit{Aplysilla polyrhaphis} collected in the Gulf of California. The nudibranch \textit{Chromodoris norris} collected from the same location as \textit{A. polyrhaphis} had aplyviolacene (157) and three other sponge diterpenes in approximately the same ratio as that present in the
sponge, indicating that these compounds were of dietary origin, and there was no preference for any particular metabolite.

**Compound (158)**

A polar band ($R_f$ 0.14, CH$_2$Cl$_2$) from the preparative t.l.c. plate showed four spots on t.l.c. (3:1 CH$_2$Cl$_2$/EtOAc). Purification by h.p.l.c. using 15% ethyl acetate in hexane resulted in three compounds present in less than 1 mg each and (158) in a yield of 0.004%. The $^1$H n.m.r. spectra showed a broad signal at $\delta$ 2.78 which was exchangeable with D$_2$O, indicating presence of a hydroxyl signal. The facile loss of H$_2$O in the mass spectrum was supported by M$^+$/H$_2$O (Found M$^+$/H$_2$O 316.2037. C$_{20}$H$_{28}$O$_3$ requires 316.3028), corresponding to an acetyl group less than aplyviolene (156). The $^1$H n.m.r. spectrum of (158) was similar to that of (156), except that the $\delta$ 2.10 methyl group was missing, further indicating lack of OAc group. In the i.r. spectrum the signal for the hydroxyl group was present ($\nu_{\text{max}}$ 3400 cm$^{-1}$). In the $^1$H n.m.r. the D$_2$O shake also caused sharpening of a broad singlet at $\delta$ 5.61, indicating the $\delta$ 5.61 proton and the hydroxyl signal ($\delta$ 2.78) are on the same carbon. Further chemical correlation was made through $^1$H-$^1$H decoupling experiments. Irradiation of the $\delta$ 6.06 doublet affected the multiplet at $\delta$ 3.00, while irradiation of the $\delta$ 2.69 double doublet changed the $\delta$ 2.89 doublet to a singlet ($J$ 18.4 Hz) and sharpened the broad singlet at $\delta$ 2.22. Comparison of this data with that of (156) and cheloviolute B isolated by Poiner$^{171}$ suggested fragment (159).
The rest of the $^1$H n.m.r data for (158) was similar to that of (156), therefore the structure of (158) was assigned as shown (158). Compound (158) has previously been isolated by Poiner$^{171}$ from *Chelonaplysilla violacea*, named as cheloviolene B. However, there was insufficient material to run a $^{13}$C n.m.r. spectra for final comparison.

Further chemical correlation could not be carried out as the sample of (158) decomposed during an attempt to acetylate it.
4.3 Summary and Conclusion

A number of diterpenes eg. macfarlandin E (aplyviolacene), cadlinolides A and B have been obtained from both the sponge source and dorid nudibranch (mollusks). It has been confirmed now that the nudibranchs obtain these compounds by feeding on the dendroceratid sponges. Though most of the terpenoid compounds are fairly astringent to taste, the mollusks must have evolved special features to feed on these unpalatable sponges and in turn it gives them protection against predators as these mollusks are shell-less.\textsuperscript{152} At the same time the presence of these terpenoid compounds in high amounts in the sponges has allowed these spicule-less sponges to protect themselves from a number of predators. Some of these diterpenes have been observed to have anti-microbial activity, macfarlandin E (aplyviolacene) was marginally active against \textit{Vibro anguillarum} and \textit{Beneckea haneyi} (100ug/disk).\textsuperscript{155} Membranolide and 9,11-dihydrogracillin A showed antibiotic activity against \textit{Bacillus subtilis} (100ug/disk) and \textit{Staphylococcus aureus}.\textsuperscript{167} The antibacterial and antimicrobial activity possibly supplements the bad taste of these compounds.

The terpenoid compounds of the dendroceratid and the dictyoceratid sponges are related in that they appear to be derived from a common spongian precursor. The isolation of two new diterpenes (160) and (161) with spongian skeleton from \textit{Dictyodendrilla carvenosa}, confirms this.\textsuperscript{172}
Both the dendroceratid sponges investigated in this study are apparently rich in diterpenoid compounds. Although aplysulphurin and tetrahydrosulphurin-1 were previously isolated together, membranolide was isolated only from the sponge *Dendrilla membranosa*. Hence this is the first time these three compounds were isolated from the same sponge.
4.4 Experimental

4.4.1 Instruments used

Experimental details are as for Cacospongia and Psammaplysilla sp. sponges.

4.4.2 Method

4.4.2.1 Sponge Collection and Extraction

An aplysillid (Aplysilla var pallida) sponge (identified by Dr. S. Toth of Sydney University, collection no. 6-4-89-1-1) (wet wt. 125 g, dry wt. 25 g) and Aplysilla violacea (identified by S. Toth, identification no. 22-1-89-1-2) (wet wt. 125 g, dry wt. 25 g) were collected at Bare Island, N.S.W., between Jan.-Apr., 1989, cut into small pieces and extracted separately with ethanol (95%) (3x200 ml). The ethanol extracts were filtered and evaporated in vacuo to give an aqueous residues. The aqueous solution was partitioned between ethyl acetate (3x100 ml) and water (100 ml). The organic portion was separated, dried over anhydrous sodium sulphate and evaporated to dryness to give crude extracts of Aplysilla var pallida (915 mg) (3.7%) and A. violacea (957 mg, 3.8%).

4.4.2.2 Column Chromatography of Crude Extract

The crude extracts were separately purified by flash column chromatography. Two columns (15x2 cm) were packed with silica, topped with sand (0.5 cm) and flushed with hexane/chloroform (1:1). Crude extract dissolved in minimum volume of solvent was
applied to the top of sand and eluted with hexane/chloroform (1:1), followed by chloroform, chloroform/ethyl acetate (3:1), chloroform/ethyl acetate (1:1), ethyl acetate and finally methanol. Fractions (20 ml) were collected from each column and combined as appropriate after t.l.c.

4.4.2.3 *Aplysilla var Pallida* Metabolites

After t.l.c. (hexane/chloroform (2:3)) the *Aplysilla var Pallida* fractions were grouped into 5 major fractions (A-E). Of these only fraction B (0.45g, 1.8%) appeared to contain u.v. active compounds of interest. Therefore fraction B was subjected to another flash chromatography using a column (15x1 cm) eluted with hexane/CHCl$_3$ (1:1, 1:5, 1:7, 1:9) followed by CHCl$_3$, EtOAc and MeOH. Fractions (15 ml) were again collected and grouped together after t.l.c. into another 5 fractions (A-E). From the $^1$H n.m.r. fractions B (0.13g, 0.52%) and C (0.19g, 0.76%) appeared to be similar with fraction B much cleaner. From t.l.c. (CH$_2$Cl$_2$) fraction B was almost only one major compound (138) while fraction C appeared to have the major compound (138) and small amounts of other compounds, (149) and (154).

*Isolation of Aplysulphurin (138)*

Fraction B was purified by preparative t.l.c. using silica and eluted with hexane/dichloromethane (1:3). Two very broad bands were observed from u.v. and by spraying the edge of the plate with H$_2$SO$_4$/vanillin. The first band (A) (0.0044 g) was not interesting from $^1$H n.m.r., while band B (0.062g) contained the major
compound (138). Therefore purification of band B by another preperative t.l.c. using hexane/dichloromethane (2:3), plate run twice, resulted in four bands (A-D) of which band B was again the major compound (0.043g, 0.172%) alysulphurin (138). A second batch of alysulphurin (0.034g, 0.14%) was obtained from purification of 100mg of fraction C by preparative t.l.c. in dichloromethane. $\lambda_{max}$ (CH$_2$Cl$_2$) 215 (ε 2353), 231 (4047), 266 nm (880). $\nu_{max}$ 2850, 1759, 1746, 1710, 1370, 1240, 1226, 1075, 950 cm$^{-1}$. Mass spectrum (Cl): m/z 373 (M+H+, 26%), 371 ((4), 314 (19), 313 (100), 286 (8), 285 (10), 257 (7), 201 (3), 189 (6), 173 (2), 128 (5), 81 (4), 69 (6), 61 (25%). $^1$H n.m.r. (CDCl$_3$) $\delta$ 0.51 (3H, s, CH$_3$), 0.95 (3H, s, CH$_3$), 0.21 (3H, s, CH$_3$), 1.25 (2H, m), 1.4-1.7 (4H, m), 1.71 (3H, d, J 7.3 Hz), 1.96 (1H, bd, J 13.9 Hz), 2.16 (3H, s, CH$_3$), 2.27 (1H, bd J 13.9 Hz), 4.40 (1H, q, J 7.3 Hz), 7.06 (1H, d J 1.7 Hz), 7.26 (1H, d, J 1.7Hz), 7.35 (1H, d, J 8.2 Hz), 7.50 (1H, d, J 8.2 Hz). $^{13}$C n.m.r. 17.4, 19.6, 21.3, 276, 31.7, 32.7, 32.7, 32.9, 38.8, 39.3, 39.6, 41.7, 50.9, 100.2, 101.7, 122.5, 129.4, 131.7, 133.5, 138.0, 149.1, 170.4, 171.6 ppm.

Separation of Minor metabolites (149) and (154)

10 mg of fraction C (from the second column) was purified by h.p.l.c. using ethyl acetate (15%) in hexane. Aplysulphurin (4.32 mg, 0.017%) was eluted as a major peak. A second fraction eluting after 5.4 min. (R.I. x 16, flow rate 4 ml/min.) was shown by t.l.c. (dichloromethane) to contain two compounds. Separation by prep. t.l.c. using CH$_2$Cl$_2$, running the plate four times resulted in a sterol fraction (0.83 mg, 0.003%) and compound (149) 0.89 mg, 0.004%) (Found: (M$^+$-OAc) 316.2037. C$_{20}$H$_{28}$O$_3$ requires M$^+$-OAc; 316.2028).
V_max (CHCl_3) 1759, 1730, 1610, 1370, 1070, 1045, 970 cm⁻¹. Mass spectrum: m/z 316 (M-OAc, 14%), 272 (14), 243 (10), 206 (9), 179 (5), 177 (7), 161 (9), 147 (20), 133 (16), 125 (33), 119 (18), 109 (27), 105 (18), 95 (19), 69 (70), 55 (38), 43 (100%). ^1H n.m.r (CDCl_3) δ 0.78 (3H, s, CH₃), 0.91 (3H, s, CH₃), 1.13 (3H, s, CH₃), 1.42 (3H, d, J 7.4 Hz), 0.7-2.0 (9H, m), 2.06 (1H, m), 2.08 (3H, s, OAc), 2.3 (1H, m), 2.52 (1H, m), 3.21 (1H, bt), 4.22 (1H, q, J 7.4 Hz), 6.00 (1H, d, J 6.1 Hz), 6.18 (1H, d, J 2.4 Hz).

Two other compounds were eluted in yields of 0.010% and 0.004%. The first was identified to be a sterol from the ^1H n.m.r. data and not analysed further, while the second was identified to be the known compound membranolide (154) (0.98 mg, 0.004%).

λ_max 210 (ε 713), 243 (1745), 285 (1007), 294 nm (969). V_max (CHCl_3) 1757, 1732, 1614 cm⁻¹. Mass spectrum: (Cl) m/z: 345 (M+H⁺, 68%), 314 (10), 131 (100), 297 (7), 284 (3), 269 (5), 247 (12), 229 (14), 219 (5), 213 (8), 200 (6), 189 (11), 187 (5), 141 (6), 109 (8), 107 (11), 106 (17), 91 (10), 69 (12%). ^1H n.m.r. (CDCl_3) δ 0.46 (3H, s, CH₃), 0.93 (3H, s, CH₃), 1.37 (3H, s, CH₃), 1.74 (3H, d, J 7.1 Hz), 0.8-2.3 (9H, m), 3.68 (3H, s, OCH₃), 4.60 (1H, q, J 7.1 Hz), 5.21 (2H, s), 7.29 (1H, d, J 8.3 Hz), 7.82 (1H, d, J 8.3 Hz).

4.4.2.4 Aplysilla Violacea Metabolites

After t.l.c., the fractions were grouped into 5 major fractions (A-E). From the ^1H n.m.r. only fractions A-C (752 mg, 3.0%) contained signals showing compounds related to spongian. Therefore fractions A-C were combined and purified by another flash column chromatography using silica. Samples (20 ml) were collected and
after t.l.c. (dichloromethane) combined into six major fractions (1-6). Of these the first three (33 mg, 0.0132%) contained mostly fats and sterols from the $^1$H n.m.r., and these were not analysed further. Fractions 4 & 5 (640 mg, 2.55%) appeared to have spongian type compounds while fraction 6 was not interesting (19 mg, 0.076%).

Fraction 4 (100 mg) was purified by preparative t.l.c. using dichloromethane, 5 bands (A-E) were apparent on spraying one edge of the plate with $\text{H}_2\text{SO}_4$/vanillin. Each band was separated and from t.l.c. (dichloromethane) and $^1$H n.m.r., band A (2.5 mg 0.010%) was rubbish. Compounds in bands B, C and D were not pure, hence combined and subjected to another preparative t.l.c. Again four bands (1-4) appeared. Band 1 was the known compound aplyviolacene (157) (6.1 mg, 0.024%) obtained as a clear oil. $^1$H n.m.r $\delta$ (CDCl$_3$) 0.94 (3H, s, CH$_3$), 1.00 (3H, s, CH$_3$), 1.08 (3H, s, CH$_3$), 2.10 (3H, s, CH$_3$), 2.21 (3H, s, CH$_3$), $\delta$1.0-2.0 (10H, m), 2.42 (1H, bdd, J 11.9, 5.0 Hz), 2.65 (1H, dd, J 3.8, 2.3 Hz), 2.72 (1H, d, J 8.8 Hz), 2.87 (1H, t, J 5.0, 3.8 Hz), 4.67 (1H, J 1.8 Hz), 4.89 (1H, J 1.8 Hz), 5.72 (1H, d, J 2.5 Hz), 5.79 (1H, d, J 5.0 Hz), 6.49 (1H, s).

Band 2 (3.2 mg) was not pure and was not analysed further. Band C (21.2 mg, 0.085%) was identified as aplyviolene (156). $v_{\text{max}}$(CHCl$_3$) 2950, 1770, 1755, 1381, 1230, 1170, 1040, 950 cm$^{-1}$. $^1$H n.m.r. $\delta$ 0.92 (3H, s, CH$_3$), 0.97 (3H, S, CH$_3$), 2.38 (1H, m), 2.42 (1H, t, J 2.8, 2.6 Hz), 2.62 (1H, m), 2.67 (1H, bd, J 19.5 Hz), 2.69 (1H, d, J 9.1Hz), 2.88 (1H, dd, J 19.5, 5.6 Hz), 4.59 (1H, d, J 2.2 Hz), 4.84 (1H, d, t, J 2.2 Hz), 5.70 (1H, dd, J 2.6 Hz), 6.14 (1H, s). $^{13}$C n.m.r. (CDCl$_3$) $\delta$ 21.1 (CH$_3$), 23.8 (C17), 25.9 (C18), 26.8 (C 6 or 2), 28.2 (C 2 or 6), 33.1 (C 12), 34.3 (C 19), 36.0 (C 4), 37.5 (C 1 or 3), 37.9 (C 3 or C 1), 38.1 (C 13), 38.5 (C 7), 45.5 (C 8), 49.2 (C 14), 54.1 (C 5), 57.7 (C 9), 100.7 (C
15 or 16), 100.9 (C 15 or 16), 114.7 (C 20), 153.1 (C 10), 167.7 (C 11), 169.5 (OCOCH₃).

Band E (6.1 mg, 0.024%) from the first prep. t.l.c. plate showed a mixture of 4 compounds on t.l.c. (3:1 DCM/EtOAc). These were purified by h.p.l.c. using 15% ethyl acetate in hexane. One major peak and three minor peaks were eluted, the minor peaks were all less than 1 mg each, hence not analysed further. The major peak (158) was eluted after 16 min (1.0 mg, 0.004%). (Found: M⁺ 316.2037. C₂₀H₂₈O₃ requires 316.2038 for M⁺-H₂O). ν max (CHCl₃) 3400, 1775, 1245 cm⁻¹. Mass spectrum m/z: 316 (M⁺-H₂O, 1%), 301 (1), 245 (17), 233 (2), 231 (1), 229 (1), 215 (3), 213 (3), 193 (2), 191 (7), 166 (11), 135 (20), 137 (27), 121 (21), 109 (29), 107 (27), 97 (25), 95 (48), 93 (27), 81 (65), 69 (94), 57 (89), 41 (100%). ¹H n.m.r (CDCl₃) δ 0.75 (3H, s, CH₃), 0.93 (3H, s, CH₃), 0.99 (3H, s, CH₃), δ 1.0-2.0 (8H, m), 2.22 (1H, bs), 2.35 (1H, m), 2.63 (1H, d, J 9.2 Hz), 2.69 (1H, dd, J 18.4, 2.8 Hz), 2.78 (1H, bs), 2.89 (1H, dd, J 18.4, 11.6 Hz), 3.00 (1H, m), 4.58 (1H, d, J 2.8 Hz), 4.81 (1H, d, J 2.8 Hz), 5.61 (1H, s), 6.06 (1H, d, J 5.6 Hz).
CHAPTER 5

BIOCHEMICAL STUDIES ON CACOSPONGIA AND PSAMMAPLYSILLA SPECIES
5.1 Introduction

Marine sponge biochemistry has been particularly of interest due to the increasing number of bioactive compounds isolated from sponges. Since sponges are sedentary filter feeders, they are relatively unselective in their particle feeding, pumping large volumes of water under low pressure through their tissue. Their diet is generally made up of particles of size 50 μm or less, comprising of bacteria, diatoms, fungi and dinoflagellates, and organic carbon (sugars, amino acids and their polymers).173

Biosynthetic studies using labelled precursors have been successful in determination of the biosynthetic pathways of marine organisms. Previously radiolabelled precursors were normally dissolved in sea water,174 however liposome encapsulation has been shown to improve incorporation of precursors in sponges.20

Some of the problems associated with earlier biosynthetic studies were:

i) Keeping organisms for biosynthetic study under stress free conditions for long periods.

ii) The symbiotic association between microalgae, bacteria and sponges indicates that the transfer of nutrients between them was likely to be complex.

The development of improved underwater breathing equipment has been beneficial because of the increased ease with which many organisms can be collected and supplied to the laboratory in a fresher condition.
Biosynthetic studies using [1-\textsuperscript{14}C] acetate determined that the carotenoids found in the \textit{Amphimedon} sp. were mostly of cyanobacterial origin, while aryl carotenoid (162) was likely to be produced by the sponge cells from the symbiont carotenoid zeaxanthin.\textsuperscript{88}

A number of groups have successfully demonstrated biosynthetic study of marine fatty acids and sterols.\textsuperscript{1,83} These techniques have been applied less successfully to study of terpene biosynthesis. Rinehart has shown that the amino acid derived metabolites are synthesized from acetate,\textsuperscript{20} whereas sponges are incapable of \textit{de novo} synthesis of terpenes from acetate.\textsuperscript{36} This was confirmed by M. Garson, who studied the biosynthesis of the novel diterpene isonitrile diisocyanoadociane (59) by a marine sponge of the genus \textit{Amphimedon}.\textsuperscript{175} It was shown that the sponge incorporated label from sodium [\textsuperscript{14}C] cyanide into the isonitrile carbons but under identical incubation conditions failed to incorporate [2-\textsuperscript{14}C] acetate for the synthesis of this terpene.

Biosynthetic studies with purified cell tyes represents an attractive alternative, as it enables location of a particular compound in a sponge without most of the problems associated with working with whole animal tissue. However it does not provide information about
where the compound is synthesized. Density gradient centrifugation is now being widely used to separate dissociated sponge cells into their constituent cell types. The first application to a biomedically interesting sponge was described in a paper on *Aplysina fistularis*. The bromotyrosine derived aerothionin (124) and homoaerothionin (125) were shown to be associated with spherulous cells located near the exhalent canals of the sponge. Moreover X-ray microanalysis was used to show that the spherulous cells were held in a collagenous envelope around spawned oocytes. This data supports the hypothesis that aerothionin and homoaerothionin play a defensive role in the sponge. Subsequently Muller *et al* investigated the cellular location of the cytotoxic compound avarol, isolated from *Dysidea avara*. Their results are suspect as *D. avara* does not contain spherulous cells (P. Bergquist, personal comm. to M. J. Garson), as was suggested by Muller *et al*. Culture of the bacterial symbionts (*Alicaligenes* sp.) associated with the sponge did not produce avarol. Therefore it was concluded that avarol was a sponge rather than bacterial-derived metabolite.

The major terpene diisocyanoadociane (59) from *Amphimedon* sp. was suggested to be generated from sponge cell-mediated modification of a symbiont-produced precursor as *de novo* terpene synthesis was not detected. Cell separation and membrane fractionation studies undertaken by Garson *et al* indicated that diisocyanoadociane (59) was associated with the sponge cell membrane, possibly substituting for the sterols in the *Amphimedon* sp. It was found that (59) was associated with more than one cell-type and thus appears to play a structural role.
In the marine sponge *Aplysina fistularis* the long chain fatty acids and unconventional sterols were found to be localized in the spherulous cells of the sponge. This was done by isolating the spherulous cells from the variety of cell types by density gradient centrifugation and sucrose or percoll density gradient ultracentrifugation. The spherulous cells contained multiple lipophilic spherules (2-7 μm), few mitochondria and small amounts of endoplasmic reticulum. Further, in the marine sponge *Pseudaxinyssa* sp. the distribution of lipids and sterols in cell types was studied by cellularly dissecting the sponge into fractions enriched in each major cell type present in the sponge. The cells could be separated by size as well as function, because the surface cells were much smaller than the internal "symbiont-infested archeocytes." The surface cells also contained higher concentration of VLFA's and lower concentration of sterols than the internally located archeocytes, because the function of the surface cell in insulation from the marine environment is different from the internal archeocytes.

Thus literature precedents suggested that it would be feasible to investigate the cellular chemistry of *Psammaplysilla* sp. and *Cacospongia* sp. using density gradient centrifugation. We chose to work initially on *Psammaplysilla* sp. because the work on *Aplysina fistularis* discussed above had demonstrated a specific cellular location for bromotyrosine metabolites. The hypothesis that we chose to test was that the bromotyrosine metabolites aerothionin, homoaerothionin and aeroplysinin-1 (96) would not be uniformly distributed across the ficoll fractions, produced by centrifugation.
If this work was successful, it was then proposed to work with *Cacospongia* sp. which contained terpenes.
5.2 Results and Discussion

5.2.1 *Psammaplysilla* species

*Psammaplysilla* sp. (identified by John Hooper, QLD museum, collection no. 21-8-88-2-1) (103.5 g, wet wt.) was cleaned of debris and chopped into small pieces. The cells were allowed to dissociate using proteinase enzyme (40 mg) and collagenase (55 mg) in calcium-magnesium free artificial sea water (CMF-ASW) and filtered. The residual material (RE) was left aside for chemical analysis. Centrifugation of the filtrate containing the dissociated cells separated the supernatant from the sponge pellet (SP). The sponge pellet had three layers, the inner and the outer layers were grey while the middle layer was greenish yellow in colour. The pellet was mixed with a small volume of CMF-ASW and applied to the top of prepared ficoll gradients in marked tubes. The tubes were balanced and centrifuged. The accumulation of cells between the layers was as shown in Fig 5.1 The supernatant was further centrifuged to give the bacterial pellet (BP).

The individual bands of cells were removed and placed into tubes labelled A-H. The fractions were washed with CMF-ASW, extracted with ethanol, partitioned between ethyl acetate (3x10 ml) and water (10 ml), and evaporated to give crude fractions (A-H). The metabolite content of each fraction was analysed by t.l.c. (dichloromethane/ethyl acetate 3:1) and $^1$H n.m.r. (Table 5.1).
The bromo compounds were concentrated in fractions E-H i.e. in more denser ficoll gradients (19-26%), while sterols were present in all fractions, with fats and sterols more concentrated in less denser ficoll gradients. Thus like *Aplysina fistularis*, the *Psammaplysilla* sp. concentrates bromo compounds in the denser cells (possibly spherulous cells; limited resources meant that electron microscopy could not be undertaken), and aerothionin and homoaerothionin in the *Psammaplysilla* sp. are likely to play similar roles to these compounds in *A. fistularis*. 
**Table 5.1.** Distribution of the bromo compounds, fats and sterols in *Psammaplysilla* sp. As interpreted from $^1$H n.m.r. and t.l.c. results.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fats</th>
<th>Sterols</th>
<th>Aeroplysinin-1 AT/HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>***</td>
<td>-</td>
<td>-</td>
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<tr>
<td>B</td>
<td>***</td>
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<td>C</td>
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<td>D</td>
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<td>E</td>
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<td>F</td>
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<td>H</td>
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</tr>
<tr>
<td>RE</td>
<td>*</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>BP</td>
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<td>**</td>
<td>*</td>
</tr>
<tr>
<td>SP</td>
<td>**</td>
<td>**</td>
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</tr>
</tbody>
</table>

* - represents amount of metabolites

AT/HT - aerothionin/homoaerothionin

A number of functional as well as ecological roles have been suggested for the antibacterial agents, aerothionin and homoaerothionin in *Aplysina fistularis*. The spherulous cells of *Aplysina fistularis* degenerate and release their contents into the mesohyl matrix, as well as releases the entire spherulous cells into the sea water via exhalant canals. The secretion of antibacterial agents into the intracellular matrix may serve to clump bacteria, increasing retention of food items at the choanocyte chambers as
well as control population of symbiotic bacteria in the mesohyl. Release of antibacterial agents into the surrounding water indicates an ecological role, which was later studied by Thompson et al.\textsuperscript{179}

5.2.2 \textit{Cacospongia} species

\textit{Cacospongia} sp. (15.8 g, wet wt.) was chopped into small pieces and the cells were dissociated and centrifuged (Fig. 5.2). Pellets from each fraction was kept aside for electron microscopic analysis.

![Diagram of cell separation](image)

\textbf{Fig. 5.2 Separation and distribution of cells in \textit{Cacospongia} sp.}

Percentages represent the concentration of ficoll in each individual layer.
The distribution of cells between the layers was as seen in Fig. 5.2, with the middle fractions (D, E & F) having the most cells. The fractions were separated, washed and extracted with ethanol (95%), partitioned between ethyl acetate (3x10 ml) and water. The ethyl acetate layer was dried over anhydrous sodium sulphate, evaporated and the fractions were analysed by t.l.c. (dichloromethane/ethyl acetate 3:1) and $^1$H n.m.r. From the $^1$H n.m.r. spectra bromo compounds, scalaranes and sterols could be identified. The results are summarised in Table 5.2.

The scalarane fraction from the residual extract was purified by flash chromatography and h.p.l.c. (1-2% ethyl acetate in hexane). The two major scalaranes were identified as the known desacetyl-12-epideoxoscalarin (27) and 12-episcalaradial (41) by comparison with literature data.$^{180}$
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fats</th>
<th>Sterols</th>
<th>Bromo-cpd</th>
<th>Scalarane</th>
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</thead>
<tbody>
<tr>
<td>A</td>
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<td>RE</td>
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</tr>
</tbody>
</table>

* represents amount of compounds
- represents absence of compounds

The $^1$H n.m.r. data suggests that the fatty acid methyl esters (characterised by strong OMe peak) were present in all fractions with fraction H having the most fats. The scalaranes were concentrated in fractions D-F that is the middle fractions. The "light cell" pellet (BP) was shown by electron microscopy to contain large numbers of bacteria (Fig 5.3 (c)). There were no scalaranes present in the light cells indicating scalaranes are more likely to be of sponge origin. Bacteria do not usually contain sterols, therefore the presence of sterols associated with the light cell fractions (BP in
table 5.2) more likely indicates the presence of sponge cells, which did not survive the fixation process. The $^1$H n.m.r. data further suggests that ficoll fractions A-I all contain bromoterpenes, thus the cell separation was not successful and the results are not as expected.

Some of the factors contributing to the lack of success of this experiment could be:

a) Cell lysis occurs with the use of proteinase enzyme.

b) Use of ficoll for gradients could have damaged any intact sponge cells. Phagocytosis of ficoll particles may have altered cell density, thus leading to broader bands of cells at the interface. The electron micrographs (done by Chris Battershill of Australian Institute of Marine Science) for ficoll fractions A, B and D (Fig. 5.3 (d)-(f)) reveals remnants of rounded sponge cells, that is cell debris, plus bacteria. Ficoll fraction B contained some sponge cells which could tentatively be identified as remnants of spherulous cells. The cell types in fractions A and E could not be described. Fractions F-I did not yield usable electron micrographs. This may be due to poor fixation combined with the presence of cellular debris and sand which damaged the cells during centrifugation.

Some electron microscopy on intact tissue samples of *Cacospongia* sp. was carried out for comparison with that of the ficoll fractions. A sample of tissue was carefully dissected into darker surface tissue (= pinacoderm) and into interior tissue (= matrix or choanosome). The pinacodermal tissue (Fig. 5.3 (b)) revealed large quantities of bacteria, but there was no sign of intact cells or even cell debris. Matrix tissue (Fig. 5.3 (a)) contained bacteria and collagen bundles
together with occasional cell debris. The bacteria were primarily of one morphological type and were usually rounded. Neither tissue type contained cyanobacteria (= blue-green algae). As these samples had not been subjected to ficoll fractionation, it would appear that the problem lay in poor fixation. Identical fixation protocols have been used successfully in our group's work on *Amphimedon* sp. and *Dysidea herbacea* (M. J. Garson, unpublished work), therefore *Cacospongia* sp. is clearly a difficult candidate for cell separation experiments. A possible explanation for the poor fixation may be that the chemicals present in the *Cacospongia* sp. samples have in some manner interfered with the fixation process. Furthermore, it may be that the sponge cells were squeezed out of the tissue sample during EM sampling. The question thus remains as to whether the *Cacospongia* bromoterpenes are of dietary, symbiotic or sponge origin. From the results obtained for avarol, diisocyanoadociane, and the absence of scalaranes in the BP of *Cacospongia* cell separation experiment implies that the terpenes will usually be of sponge origin. However the high bacterial content of *Cacospongia* sp. allows us to speculate that the bromo metabolites could be of bacterial origin. This is supported on the grounds that a number of brominated metabolites have been isolated from marine bacteria. Fenical and co-workers isolated a bromoquinone, marinone (163) from an actinomycete from Bodega Bay.\(^{181}\)

\[
\begin{align*}
\text{HO} & \quad \text{Br} \\
\text{O} & \quad \text{O} \\
\text{H} & \quad \text{H} \\
\text{OH} & \quad \text{H} \\
\end{align*}
\]

(163)
Bromophenols have also been obtained from bacteria cultured from *Dysidea* sp. by Elyakov et al.\textsuperscript{182} Bromophenols were detected in the bacterial isolates by GCMS, however, n.m.r. was not done to confirm that the compounds were indeed bromophenols, therefore these results must be viewed as preliminary until more evidence is presented.

Some circumstantial evidence for the role of the secondary metabolites in *Cacospongia* sp. can be obtained. Crude extracts of *Cacospongia* sp. gave very weak antibiotic activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, but no antitumour or antiviral activity. The antibiotic properties would probably be associated with the scalaranes as these compounds are found to be biologically active, possessing anti-inflammatory, cytotoxic and ichthyotoxic properties.\textsuperscript{56,183,184} This was confirmed by testing 12-epideoxoscalarin, which gave weak antimicrobial activity against *B. subtilis*. Though the bromoterpenes have structures related to the cymopols (5) & (6) (Chapter 1), they still differ in that they lack the polar hydroxy groups present on the aromatic moiety of cymopols, and these are the likely groups contributing to the antimicrobial activity in the cymopols. The spiro-fused metabolites of *Cacospongia* have similar structure to that of dienone (164) from an annelid\textsuperscript{185} and griseofulvin,\textsuperscript{186} and thus might be expected to have similar antimicrobial properties. However, our spiro-fused metabolites again lacks the polar aromatic substituents present in (164) and griseofulvin.
The secondary metabolites produced by sponges have been known to contribute to the well being of the sponge, ecologically through release of the biologically active metabolites into the sea water, chemically protecting the sponge against other organisms. The scalaranes are known to play a role in the chemical defence of the sponge. A second functional role could be the sponge terpenes play a structural role by substituting for sterols in the sponge cell membrane. Sterols are known to be components of sponge cell membranes, therefore are expected to be present in the sponge in a reasonable quantity. However, the low sterol content of *Cacospongia* sp. together with absence of unconventional sterols, and cholesterol making up bulk (60%) of the sterol fraction, leads to the suggestion that the unusual terpenes in *Cacospongia* sp. may function as sterol mimics as in the *Amphimedon* sp. The bromo metabolites have flat structures, fulfilling the minimum requirement for sterol substitutes to have a flattened, rigid ring structure.
Figure 5.3 (a-b) Electron micrographs of (a) matrix tissue and (b) pinacodermal tissue
Figure 5.3 (c-d) Electron micrographs of (c) light cells and (d) ficoll fraction A.
Figure 5.3 (e-f) Electron micrographs of ficoll fractions B and D
5.3 Summary and Conclusion

The biochemical studies on *Psammaplysilla* sp. and *Cacospongia* sp. led us to the conclusion that certain sponges are more difficult to work with than others. The *Psammaplysilla* sp. and the *Cacospongia* sp were studied using the same experimental procedure. While the verongid sponge gave results which were in line with that previously obtained from *Aplysina fistularis*, the *Cacospongia* sp gave negative results. Since the same experimental procedure was successful with *Psammaplysilla* sp., we can be fairly certain that the major error was not the way the experiment was carried out. This *Cacospongia* sp. was suggested to be unusual because thinly encrusting sponges do not usually have so much bacteria (Clive Williamson, personal communication to M. J. Garson).

A repeat of the experiment with a modified procedure may give the desired results. Use of a different fixative or paraformaldehyde used in conjunction to the fixative may enhance pellet fixation. An alternative to ficoll is also required, as sugar may be taken up by any live sponge cells, resulting in bursting of the sponge cell. An alternative dissociation method without use of proteinase, preferably a mechanical dissociation may be preferable. The technique of "Fluorescence Activated Cell Sorting" has been recently used to separate out sponge from cyanobacterial cells in the tropical sponge *Dysidea herbacea* (M. Unson, D. J. Faulkner and M. J. Garson, unpublished results). It may be possible to adapt this technique for use with *Cacospongia* sp., thus preventing cell lysis.
5.4 Experimental

5.4.1 Sponge Collection

*Psammaplysilla* sp. was collected from the Docks, Jervis Bay or from Pig Island, Wollongong at depths of 10-20 m. *Cacospongia* sp. was collected at the Nuns Pool, North Wollongong, N.S.W. at low tides between August and December 1989. The sponges were maintained in aerated seawater 1-3 days at 25° prior to use.

5.4.2 Materials and Methods

Cell separation on *Psammaplysilla* sp and the *Cacospongia* sp were carried out separately. For each cell separation four litres of calcium-magnesium-free artificial sea water (CMF-ASW) was prepared as follows:

For one litre
- 27.0 g sodium chloride
- 1.0 g sodium sulphate
- 0.8 g potassium chloride
- 0.18 g sodium bicarbonate

The mixture was placed in a 1 litre flask and diluted with glass distilled water to 1 litre. The pH of the solution was adjusted to 7.4 with either sodium hydroxide or hydrochloric acid (using pH meter). The CMF-ASW was refrigerated overnight.

Ficoll solutions were prepared on the day of the cell separation using Ficoll 400 (Pharmacia, Uppsala, Sweden) as follows:
To 50 ml CMF-ASW in 200 ml beakers, Ficoll 400 was added
15.0 g to give 26% solution
12.5 g to give 22% solution
10.0 g to give 19% solution
7.5 g to give 14% solution
5.0 g to give 10% solution
2.5 g to give 5% solution

The centrifuge (Hettich Universal Rotor 1323, Tuttlingen, Germany) was switched on at least 15 minutes prior to use to allow for temperature stabilization.

5.4.2.1 *Psammaplysilla* species

The wet sponge (103.5 g) was soaked in several rinses of aerated CMF-ASW to remove natural sea water. It was then chopped into small pieces (0.5 cm) using a sharp scalpel to prevent squeezing of the tissues. The diced sponge was placed in a minimum quantity (300 ml) of moderately aerated CMF-ASW that contained 40 mg proteinase enzyme K (Sigma Chemical Co., St. Louis, MO) and 55 mg collagenase. The tissue was left at room temperature until the water became cloudy (90 min.) with dissociated cells.

Ficoll gradients were added in the order 26%, 22%, 19%, 14%, 10% and 5% in pre-marked centrifuge tubes (each layer 6.5 ml) and stored on ice. After 90 min. the dissociated cells were filtered by forcing through a cheesecloth (using hand gloves). The residual skeletal material (labelled RE) was kept for chemical analysis. The filtrate containing the dissociated cells was centrifuged using the
GSA head (2400 rpm, 600 g) for 5 min. at 10°. The supernatant was poured off to obtain the sponge pellet at the bottom of the tube (labelled SP). The supernatant was further centrifuged at 13000 rpm (20000 g, using SS34 head) for 15 min. at 10° to give bacterial pellet (BP).

The sponge pellet was mixed with 35-40 ml CMF-ASW to give a slightly dense solution, which was applied (10 ml) to the top of each centrifuge tube containing ficoll gradients. The tubes were weighed in pairs and CMF-ASW was added to balance the tubes. Centrifugation at 2400 rpm (600 g) using HB-4 head gave the accumulation of cells between the Ficoll layers according to density. Each fraction (A-H) (Fig. 5.1) was carefully removed and placed separately in a labelled centrifuge tube (50 ml) containing CMF-ASW (10 ml). The tubes were filled to the top with CMF-ASW, weighed and centrifuged using HB-4 head (2400 rpm, 600 g) for 5 min. at 10° to remove ficoll from the cells. The supernatant was decanted to leave the sponge cell pellet at the bottom of the flask.

5.4.2.2 Cacospongia species

Cell separation on Cacospongia sp (wet wt. 15.8 g) was carried out using 20 mg proteinase enzyme K in the same way as for Psammaplysilla sp. The gradients (30%, 25%, 20%, 15%, 10% and 5%) were prepared using ficoll 400. The distribution of cells between the gradients was as seen in Fig. 5.2. Small samples of all pellets were fixed for electron microscopic analysis.
5.4.2.3 Chemical Analysis

Each fraction was left overnight in ethanol (95%) (20 ml). The ethanol extract was filtered and evaporated to give crude cell extracts. Each extract was partitioned between ethyl acetate (3x10 ml) and water (10 ml), dried (anhydrous sodium sulphate) and evaporated to obtain crude fractions (A-H) for *Psammaplysilla* sp. and (A-I) for *Cacospongia* sp. The cell fractions were weighed, checked, first by t.l.c. (3:1 DCM/ethyl acetate) (observed under u.v. light and by spraying with a solution of 1% vanillin in conc. sulphuric acid), and then $^1$H n.m.r. (Jeol GMX400) in deuterated chloroform with internal chloroform as standard.
APPENDIX 1

DETAILS OF NMR TECHNIQUES USED
A1.1 The Nuclear Overhauser Effect (1D n.m.r. method)

The spectra were obtained on a Jeol GX400 spectrometer using:
- pulse delay 20 sec.
- pulse angle 90°
- no. of scans 400

The Nuclear Overhauser effect allows the estimation of the internuclear distances. N.O.E is described by Derome as arising from alterations of populations of a nucleus which causes relaxation of the nucleus.\(^1\) In conducting N.O.E experiments, the population difference across some transitions are eliminated by irradiating them with a weak r.f. field, while observing the signals from others. If the intensity of a resonance at equilibrium is \(I_0\) and the intensity observed while saturating some other related resonance is \(I\), then N.O.E can be defined as

\[
N_i(s) = I - I_0/I_0
\]

where \(N_i(s)\) is the N.O.E at nucleus \(i\) when nucleus \(s\) is saturated.

The pathways for relaxation in N.O.E. can be explained by considering two -1/2 spin nuclei \(i\) and \(s\) with the same gyromagnetic ratio (\(\gamma\)) but different chemical shifts. If they are in the same molecule but not J-coupled, the energy levels of the nuclei in the states \(\alpha\alpha, \alpha\beta, \beta\alpha\) and \(\beta\beta\) can be described by figure A.1.
Fig. A.1 Energy levels of populations of a homonuclear two-spin system at equilibrium.

The transitions of each nucleus are almost equal in energy, thus the states $\alpha\beta$ and $\beta\alpha$ are nearly degenerate. Also in absence of J-coupling the two transitions of nucleus $i$ have same energy as do those of $s$. The lower state $\alpha\alpha$ will have an excess of nuclei $(N+\delta)$, while the state $\beta\beta$ will be deficient by an equal amount $(N-\delta)$. The population difference between the states will be defined by:

$$\Delta M=0 \text{ transition } \beta\alpha-\alpha\beta \{ 0$$

$$\Delta M=2 \text{ transition } \alpha\alpha-\beta\beta \{ 2\delta$$

If we saturate both transitions and then observe signals due to $i$ (Fig. A.2)
The new population difference will be:

\[
\begin{align*}
\text{i transitions} & \quad \alpha\alpha - \alpha\beta \quad \beta\alpha - \beta\beta \quad \delta \\
\text{s transitions} & \quad \alpha\alpha - \alpha\beta \quad \alpha\beta - \beta\beta \quad 0 \\
\Delta M=0 \quad \text{transition} & \quad \beta\alpha - \alpha\beta \quad \delta \\
\Delta M=2 \quad \text{transition} & \quad \alpha\alpha - \beta\beta \quad \delta
\end{align*}
\]

The population difference across each i transition at thermal equilibrium was \(\delta\) and is still the case therefore saturating s does not affect intensity of i. The population difference between \(\alpha\beta\) and \(\beta\alpha\) becomes \(\delta\), whereas at equilibrium was 0. Thus \(W_0\) (rate constant for the process) acts so as to transfer population from state \(\beta\alpha\) to \(\alpha\beta\) (Fig. A.3.) to try and restore population difference to zero (as at equilibrium). That is, increasing the population of the top of one i transition and decreasing the bottom of another i transition (\(\delta > 0\)). This results in decrease in intensity of signals due to i, hence negative N.O.E. at i due to s.
The population difference between $\alpha\alpha - \beta\beta$ becomes $\delta$, whereas at equilibrium was $2\delta$. Therefore $W_2$ acts to restore population difference to $2\delta$ by transfer of population from $\beta\beta$ to $\alpha\alpha$, thereby decreasing the population of the top of one $i$ transition and increasing the population of the bottom of other $i$ transition, giving positive N.O.E. at $i$ due to $s$.

![Diagram of initial direction of cross-relaxation to establish equilibrium](Diagram)

**Fig. A.3. Initial direction of cross-relaxation to establish equilibrium.**

The N.O.E. on its own is not of much use but the N.O.E. difference spectroscopy is widely used in structure determination. Fig. A.4 outlines the experimental scheme for the N.O.E. difference method.

![Experimental scheme for N.O.E. difference method](Diagram)

**Fig. A.4. DIFFERENCE SPECTRUM = A-B.**
A1.2. 2D NMR Techniques

The spectra were recorded on a Bruker AMX500 n.m.r. spectrometer using a 5 mm inverse $^1$H/broad band probe ($90_\text{H} = 7 \mu\text{sec}$, $90_\text{C} = 11.4 \mu\text{sec}$). For the 2D n.m.r. spectra 512 $t_1$ increments were accumulated into 2048 data points, with 32 scans per $t_1$ increment in case of HMQC spectra and 16 scans per increment in HMBC spectra.

Use of 2D n.m.r. techniques have provided a powerful tool for structure elucidation of natural products. However most 2D n.m.r. methods have poor sensitivity therefore larger amounts of sample are needed. More sensitive $^1$H signal is used to provide indirect information on the $^{13}$C and $^{15}$N nuclei, hence generates better sensitivity. This is called $^1$H detected heteronuclear 2D n.m.r. spectroscopy (inverse experiment). The heteronuclear shift correlation includes two types:

i) $^1$H detected heteronuclear multiple Quantum coherence (HMQC) which provides scalar ($J_{x\text{H}}$) coupling information.

ii) $^1$H detected heteronuclear multiple bond connectivity (HMBC) gives long range coupling ($J_{x\text{H}}$) information.

The $J$ values are not obtained in these experiments.
A1.2.1 $^1$H Detected Heteronuclear Multiple Quantum Coherence.

This experiment was optimized for $J_{^1H-^12C} = 135$ Hz, interpulse delay $= 3$ sec., BIRD relaxation delay time: 600 ms. The sample was not spinning during the experiment.

The HMQC experiment creates and manipulates the heteronuclear multiple-Quantum Coherence then reconverts it to detectable single-quantum proton coherence. To obtain a good HMQC the proton signals associated with $^1H-^12C$ molecules need to be suppressed. The pulse sequence as described by Bax and Subramanian\(^{189}\) (Fig. A.5) uses a Bilinear Rotational Decoupling (BIRD) pulse which requires small number of accumulations to suppress the $^1H-^12C$ peak. Using a delay of 0.3-0.6 seconds after the BIRD pulse, the $^1H-^12C$ spins are allowed to relax to a point where they do not have a $Z$-magnetization component. After the $^1H-^12C$ signal is suppressed, a 90 degree proton ($^1H$) pulse is followed by an interpulse preparation time ($\Delta$), then a $^13C$ 90 degree pulse produces the desired heteronuclear multiple quantum coherence. Both zero- and double-quantum coherence are created which evolve during the evolution time ($t_1$). The final $^13C$ pulse in the sequence (Fig. A.5) reconverts heteronuclear multiple quantum coherence to observable proton single-quantum coherence. HMQC provides direct proton-carbon heteronuclear connectivities through the proton-proton connectivity information relayed between the directly bound proton and its vicinal neighbours.
Fig. A.5 HMQC pulse sequence as described by Bax and Subramanian.189

A1.2.2. Long-Range Proton-Detected Heteronuclear Chemical Shift Correlation (HMBC)

Optimized for $J_{LRH-C} = 6 \text{ Hz} = \Delta_{LR}$, short range coupling suppressed by $\Delta = 3.5 \text{ msec}$, interpulse delay $= 5 \text{ sec.}$, sample was not spinning during the experiment.

Due to the presence of two markedly different components of heteronuclear multiple quantum coherence the BIRD pulse at the beginning of the experiment is excluded. The pulse sequence (Fig. A.6) is as described by Bax and Summers.190 The HMBC pulse sequence consists of a $^1H$ 90 degree pulse followed by interpulse preparation time $\Delta (=1/2(J_{CH}))$, then a $^{13}C$ 90 degree pulse that creates a heteronuclear multiple quantum coherence for the one-bond components of magnetization. After a further preparation
time $\Delta_{LR}$ the second 90 degree carbon pulse is applied. This converts the long range proton-carbon couplings into heteronuclear multiple quantum coherence which evolves during the evolution time $t_1$. At the end of the evolution period heteronuclear multiple quantum coherence is reconverted to observable proton single-quantum coherence which is then detected. HMBC provides indirect connectivity information involving coupling between two-or three-bonds. The proton carbon correlations give useful information about quaternary carbons and hetero atoms, thus providing a means to link structural fragments together.

Fig. A.6 HMBC pulse sequence as described by Bax and Summers.
Figure A.7 400 MHz $^1$H n.m.r. spectrum of (64). Solutions in CDCl$_3$; $\delta$ values in ppm, referenced at $\delta$ 7.25 for CHCl$_3$. 
Figure A.8 400 MHz $^1$H n.m.r. spectrum of (65). Solutions in CDCl$_3$; δ values in ppm, referenced at δ 7.25 for CHCl$_3$. 
Figure A.9 400 MHz $^1$H n.m.r. spectrum of (66). Solutions in CDCl$_3$; $\delta$ values in ppm, referenced at $\delta$ 7.25 for CHCl$_3$
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