A marine chemical ecology study of the sea hare, *Bursatella leachii* in South Africa

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In order to succeed, your desire for success should be greater than your fear of failure

Bill Cosby

Abstract

The large cosmopolitan sea hare *Bursatella leachii* is a common resident in Eastern Cape river mouths during summer and late autumn where they congregate in beds of *Zostera capensis* to breed. In this thesis, the previously known toxic formamide marine secondary metabolite (-)-bursatellin (**2.2**), which may deter predators of South African specimens of the globally distributed sea hare *Bursatella leachii,* was isolated and identified (Chapter 2). There have been no previous chemical ecology studies of *B. leachii* and the latter half of this thesis is devoted to chemical ecology studies of this organism. Interestingly, the isolation of the (-)-diastereomer of **2.2** from specimens of *B. leachii* collected from the Kariega River mouth (near Kenton-on-Sea) suggests that the South African specimens of this species are similar to specimens collected from Puerto Rico and from the Mediterranean Sea. Two different chromatographic techniques for isolating **2.2** were compared in order to maximize the amount of **2.2** isolated from the Kariega River mouth sea hares. The doubling of selected resonances observed in both the ^{1}H and ^{13}C NMR spectra of the bursatellin isolated in this study suggest one of three possibilities; either firstly, the presence of closely related compound(s), secondly, the presence of diastereomers or thirdly the presence of rotamers. Through NMR kinetic studies, we were able to establish that the presence of rotamers was very unlikely due to no change in the relative ratio (3:1) of the ^{1}H NMR signals with an increase in temperature. Although the attempted synthesis of the acetate derivative (**2.28**), as a means of separating a diastereomeric mixture was successful, the chromatographic separation of the proposed acetylated diastereomers was not successful. Preparation of the camphanate ester derivatives (*e.g.* **2.30**) proved to be unsuccessful. Five *B. leachii* specimens were dissected, their organs separated and individually extracted with methanol. The methanol extracts were individually chromatographed on HP-20 media, and the distribution of bursatellin determined by isolation and NMR. It was evident from this investigation that the distribution of **2.2** within individual *B. leachii* specimens was found to be highest within the *B. leachii* ink gland. The lower amounts of **2.2** contained in the digestive system, relative to other organs, was hypothesized to occur because **2.2** is sequestered from the diet of the sea hare and efficiently moved from the gut to various organs around the body where it is stored. The absence of **2.2** from the skin was surprising and may be a result of a smaller mass of skin relative to other organs coupled with the limitations of the chromatographic separation techniques employed. Surprisingly, no bursatellin was found within juvenile sea hares.

Chapter three discusses the isolation of ilimaquinone (**3.1**) and pelorol (**3.19**) from the sponge *Hippospongia metachroma* and the structure elucidation of each compound using computer modeling to illustrate the conformation. It was deemed necessary to isolate these well known and abundant bioactive marine natural products from a sponge as standard compounds in the bioassays given the paucity of **2.2** available for this study. Chapter four describes the assays used to test the biological activity of the bursatellin **2.2** compared to the generally bioactive ilimaquinone and the structurally related and

commercially available broad spectrum antibiotic chloramphenicol. *B. leachii*, a shell-less marine mollusc inhabits a variety of intertidal habitats and, therefore, is exposed to several different predators, yet does not appear to have any specific predators. Potential predators of this sea hare in the Kariega Estuary could be fish and amphipods which are found in close proximity to these sea hares. Results of the assays showed that at roughly natural concentrations, (calculated from the isolated chromatographic yield) feeding was deterred by the fish and amphipods, which implied that **2.2** may confer a defensive role within the organism. The relatively high concentration present within the ink gland of *B. leachii* may support this hypothesis. Surprisingly, given its structural similarity to chloramphenicol, **2.3** did not show any antimicrobial action against five of the six bacterial strains against which it was screened [chloramphenicol inhibited the growth of all the bacterial strains at very low concentrations (0.25 mg/mL)]. Bursatellin was found to be only active against *Staphylococus aureus* at high concentrations *ca.* 2 mg/mL when compared to chloramphenicol. Neither bursatellin nor chloramphenicol showed anti-fungal activity.

Although this study suggests that the sea hares may use chemical defences in addition to opaline ink to defend themselves, they also live within the seagrass *Z. capensis,* which possibly provides the sea hare with a cryptic form of physical defence against several predators that are unable to swim freely within the weed beds in the littoral zone of the estuary.

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List of symbols and abbreviations

1D	one dimensional
2D	two dimensional
$[\alpha]_D$	specific rotation
° C	degrees Celsius
Å	Angstrom
Ac	acetyl
aq	aqueous
au	atomic unit
Boc	*N*- tert-Butyloxycarbonyl
br	broad
calcd	calculated
$CHCl_3$	chloroform
CH_2Cl_2	dichloromethane
d	doublet
dd	doublet of doublets
DCC	dicylohexylcarbodiimine
DCM	dichloromethane
DEPT	distortionless enhancement of polarization transfer
DMAP	4-dimethylaminopyridine
DMSO	dimethylsulfoxide
EIMS	electron impact mass spectrometry
eq	molar equivalent
Et_3N	triethylamine

EtOAc	ethyl acetate
gCOSY	gradient ^{1}H-^{1}H homonuclear correlation spectroscopy
gHMBC	gradient heteronuclear multiple bond correlation
gHSQC	gradient heteronuclear single quantum coherence
h	hours
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HREIMS	high resolution electron impact mass spectrometry
int.	integration
IR	infrared
K	kelvin
LAH	lithium aluminium hydride
lit.	literature
m	multiplet
mg	milligram
mL	millilitre
MeOH	methanol
MHz	megahertz
min	minutes
mmol	millimole
mol	mole
MS	mass spectrometry
mult.	Multiplicity
n	number

$NaNO_2$	sodium nitrite
nm	nanometers
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser enhancement spectroscopy
Pd/C	palladium on carbon
PdO_2	palladium oxide
ppm	parts per million
q	quartet
R.T.	room temperature
s	singlet
SCUBA	self containing underwater breathing apparatus
sp	species
t	triplet
THF	tetrahydofuran
TLC	thin layer chromatography

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Chemical ecology

Ecology can be described as the study of the interactions between organisms and their natural environment.[1] The environment itself can be divided into biotic and abiotic components.[2,3] The biotic system consists of primary producers, consumers as well as detritivores, while the abiotic system includes environmental factors *e.g.* light, temperature, water, geographical, geological and climatological influences.[4]

Community ecology looks at the interaction between species inhabiting a defined area and includes the number, type of species and their relative abundance. Species diversity is defined by both species richness and evenness and increases with environmental complexity.[5] The community's structure is influenced by interactions between species.[4] We need to study interactions between species in order to understand different communities and establish their role in food webs.[6]

There are four general types of interactions found within a community *viz.* mutualism,[5] commensalism,[7] competition and predation.[8] Mutualism occurs when both species benefit from the interaction.[9] Commensalism occurs when one species benefits while the other is neither advantaged nor disadvantaged.[10,11] In the case of competition, both species are negatively affected while predation, parasitism and herbivory allow for only one of the species to benefit while the other species is disadvantaged.[11,12]

Complex relationships between organism and their environment are seen in ecosystems with both interspecific and intraspecific interactions playing key roles in the structure and dynamics of such a system.[6,13] The nature of these interactions may be co-operation in the case of intraspecific interactions[4,14] whereas symbiosis,[9,15,16] competition,[15,17] parasitism, and predation may occur in an interspecific interaction.[7,18]

The field of chemical ecology is a relatively recent area of study and has only developed over the past several decades. It is an interdisciplinary field incorporating both chemistry and biology and focuses on the role of naturally occurring chemical compounds in mediating interactions between organisms (plants and animals) within a community.[19] Ecological studies of terrestrial organisms have been well researched and many of the studies have shown that the interactions involve chemical mediation in one form or another.[20] Valuable information about plant-herbivore and plant-insect interactions, as well as predator-prey interactions have been obtained from these studies.[21,22] This has led to further research into chemical ecology so as to better understand the relationship between secondary metabolite producing organisms and their surroundings.[23] Currently, chemical ecology largely focuses on terrestrial ecosystems, specifically plant-herbivore interactions.[22,24] As some of the isolated compounds show biological activity, biologists are becoming increasingly interested in determining the role of these highly bioactive compounds in the producing organism[25] along with the ecological interactions of these

compounds with other organisms.[26] Much less attention has been focused on marine chemical ecology compared to its terrestrial counterpart, even though oceans are much older, larger and support almost double the number of phyla.[19,27]

Chemistry plays a vital role in ecological interactions within an organism's environment. Chemical cues strongly influence factors such as mating, courtship, predation and aggregation,[7,18] and chemical cues are an essential form of signaling, especially in the aquatic environment, where both auditory and visual sensors are less efficient.[28] Chemical mediation plays a vital role in shaping community structure, especially in predation and competition both within and between species.[29] Many organisms have developed complex chemical defences which render their tissue unpalatable to their consumers.[28] There is a vast amount of information available, that describe feeding attractants, including primary amino acids, nucleotides, nucleosides organic acids and several quaternary ammonium salts.[30] Conversely, feeding deterrents include compounds such as terpenes, alkaloids and halogenated hydrocarbons which are found within marine plants, microbes and numerous sedentary organisms.[28] These ecological interactions between organisms can be observed through the use of bioassays in the form of bioassay-guided separation, thus identifying some of these compounds involved in this interaction.[19]

Green plants form the foundation of every terrestrial food web which represent an important carbon source and are consumed by numerous organisms like bacteria, fungi, animals and even other plants.[31] Herbivory, therefore, has ecological consequences to the individual plant, population, community and ecosystem, determining the patterns of coexistence of plants as well as nutrient cycling within a system.[32] Although plant chemical ecology dates as far back as the 1950's, only recently has it emerged as an area of research.[2] Stahl, one of the first pioneers of chemical ecology, carried out several feeding experiments on slugs and snails, and observed that plant secondary metabolites played a vital role in plant-insect interactions. He concluded that, as a direct result of selection pressure, the chemical diversity of plants evolved as a defensive mechanism against herbivores.[2,33] Kerner's studies on plant-insect interactions highlighted key mechanical (spines, thorns and trichomes) and chemical defences (bitter compounds, alkaloids and oils) essential for plant survival.[2] Ecological studies on different terrestrial systems have provided information about the ecology, evolution and co-evolution of plants and animals.[34] These studies support the notion that plants produce complex chemicals in response to herbivore feeding on specific parts of their structure. Although the production of these chemicals is energetically costly, it appears that the benefits outweigh the cost, as this strong defensive system prevents their consumers from completely destroying the plants.[12] Although thousands of terrestrial plant secondary metabolites have been isolated and described in the literature, little is known about their ecological roles.[23,26]

The immobility of plants has consequently resulted in natural selection for chemically defended plants which are less palatable to consumers.[15] Several forms of defence are used by plants against a numerous range of predators. The most obvious of these is physical defence,[35] which includes mechanical and structural defences to discourage browsers from feeding on the plants *e.g*. thorns and

spines.[2] To deter herbivores, plants have evolved thicker bark and an outer covering for their seeds and fruit to prevent penetration by insects and other organisms, as well as thickening and lignifications of the cell wall surrounding the vascular bundle to deter larger herbivores.[36] Insects can be deterred by single celled plant hairs known as trichomes which provide protection by covering the entire leaf with hair. Trichomes may also excrete noxious compounds to deter vertebrates or sticky substances to hinder the feeding of insects.[37] Field studies have shown that herbivory induces a positive response in plant regrowth under high nutrient conditions.[38]

Secondary metabolites play a significant role within plants, including attraction of pollinators and defence against herbivores.[39] Within plant chemistry, there are three important defensive classes, namely phenolics,[40] terpenes and alkaloids, although amino acids, protease inhibitors, cyanogenic compounds and toxic proteins also play a role in their defence.[12,25] The most important phenolic compounds in angiosperms and gymnosperms are tannins,[2,41] which reduce the digestibility of plant tissue and are highly concentrated within the vacuoles of leave cells, protecting the leaves from herbivory and disease.[34] Lignin is also a phenolic compound found within the woody cell wall that gives structural support to the plant and forms a barrier against herbivores and other organisms.[42] Terpenes are present within all plants and play a role in both primary and secondary metabolism. Sterols and carotenes are examples of primary metabolites, while monoterpenes, diterpenes and triterpenes are examples of secondary metabolites.[43] Latex, which is found within some plant families, contains toxic terpenoids which are used in defence.[24] Alkaloids have a bitter taste and many are toxic to herbivores. The alkaloids produced are normally specific to species of plants, and produced in small quantities.[33]

With the discovery of large numbers of new compounds from marine organisms, the field of chemical ecology is expanding rapidly.[28] Collaboration between chemists and ecologists in the 1980 s began to explore the types of chemical mediation within the marine systems similar to those studied within the terrestrial realm (Table 1).[39] Marine chemical ecology has focused mainly on small organic molecules from marine organisms, which have developed complex chemical defence mechanisms,[31] including algae, dinoflagellates and invertebrates such as sponges, molluscs, ascidians and cnidarians.[12,23] These organisms are exclusively marine, and are a source of novel secondary metabolites.[44] Several marine microorganisms also produce secondary metabolites which may be of interest to humans.[45] The pharmaceutical industry has benefited greatly from the discovery of secondary metabolites produced by these organisms, for the treatment of several diseases, including cancer and AIDS.[45,46] Other applications of these compounds may be in agriculture, chemical industrial as well as aquaculture.[46] One of the challenges is the isolation of these chemicals from the producing organism as they are produced in trace quantities in these invertebrates.[47] Initial theories suggested that these compounds were chemical waste products of metabolism.[2] It was later realized that these highly complex compounds evolved as a direct result of natural selection and, consequently, are produced as a response of organisms to their environment.[23] With intensified pressure on marine organisms, due to predation and limited space (on

inshore reefs) light and food availability,[23] they have developed a range of physical and chemical defensive mechanisms in order to survive.[25] These secondary metabolites form the basis of ecological specialization and thus shape the community organization and species distribution.[4] Although these organisms have evolved complex chemicals to prevent predation, several of their predators have evolved a tolerance to these chemicals and therefore are able to sequester these compounds for their own defence.[48]

Table 1: Distribution of general classes of compounds found within the terrestrial and marine environment.

Compounds	Terrestrial Environment	Marinel Environment
Alkaloids	Common	Rare
Nitrogenated compounds	Very common	Rare Some terpenoids, acetogenins (mixed biosynthesis) Exceptions: bacteria, phytoplankton and cyanobacteria, as well as hosts such as sponge, tunicate, bryozoa due to symbiosis between the two.
Halogenated compounds	Brominated compounds are rare Chlorinated compounds found within fungi and occasionally higher plants	Very common in both red and green sea weed and sponge. Brominated most common Chlorinated also common
Tannins	Very common Result of shikimic acid pathway	Equivalent is phlorotannins in marine algae

1.2 Marine natural products

Although oceans cover approximately 70% of the earth's surface,[49] the study of marine natural products has only recently commensed.[50] Studies of natural products in the terrestrial environment have shown the importance and complexity of the chemical and ecological mediation that these secondary metabolites play in the terrestrial organisms life in several aspects such as symbiosis, mutualism, chemoreception, most importantly in communication and defence.[7,51] The high levels of biological activity of these natural

products lead to investigations of natural products in the marine environment.[50] The oceans are both physically and chemically a relatively stable environment and thus support high levels of biodiversity.[12,22] With advancement in technology *e.g.* NMR, there has been greater opportunity for the study of marine organisms and their ecology. This has opened the door to the discovery of numerous novel natural products from the sea (Figure 1).[7] Advancements in technology led to an increase in the number of new natural products isolated from marine organisms.[52] Significantly, the increased availability of SCUBA diving equipment allowed scientists and chemists to explore the biological and ecological diversity of this unique environment.[47]

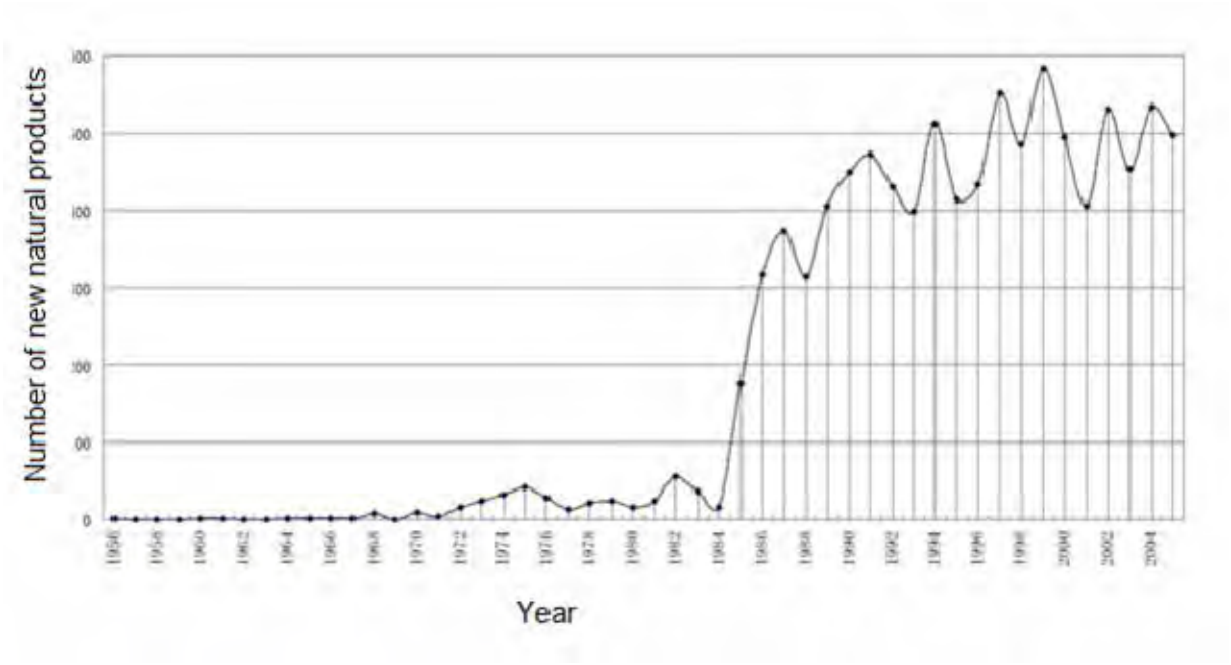
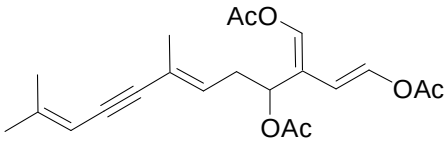


Figure 1: Numbers of new marine natural product structures published in the chemical literature (1986-2004). Reproduced from Hu *et al.* 2011.[52]

The presence of certain secondary metabolites within algae, sponges and corals may act as deterrents associated with the defence of these immobile organisms, [53] and may determine their susceptibility to herbivory by fish and other predators.[54] Those organisms which are more vulnerable to attack are usually defended both physically and chemically, while those less vulnerable to attack are considered to have fewer forms of chemical defence.[35] Although it is thought that those organisms chemically rich are less susceptible, it is not always the case.[55] Predators such as nudibranchs feed on chemically rich prey such as sponges and incorporate this into their own defence mechanisms.[48] The green seaweed, *Chlorodesmis fastigiata,* is fed upon by the rabbitfish, *Siganus argenteus*, but avoided by other rabbit fish like *S. spinus* which feed on alternative green seaweed as well as other herbivorous fish.[56] This chemically defended alga provides a refuge for species such as amphipods, crabs as well as ascoglossan molluscs.[7] It is not entirely clear as to what the presence of some of secondary metabolite

do in terms of deterrence of predators or toxicity within some organisms.[28,57] The green seaweed *Caulerpa racemosa* is readily eaten by numerous herbivorous fish although it produces a sesquiterpenoid metabolite caulerpenyne (**1.1**) in relatively high concentrations (1-2 % of dry mass).[56]



1.1

McClintock *et al.*[19] have carried out a number of chemical ecology studies on Antarctic sponge metabolites and their research remains some of the foremost chemical ecology research performed in polar marine environments. The focus of one aspect of this ongoing programme was to examine the feeding deterrent properties of several solvent (methanol, hexane and chloroform) extracts from eighteen Antarctic marine sponges.[19] The common ecologically relevant predator of all these sponges, a spongivorous Antarctic sea star, *Perknaster fuscus*, was used to determine the biological activity of compounds isolated from the sponges. The sponge extract was dissolved in a minimal volume of solvent and dried on pellets or krill until all solvent was removed. Pellets could be fed to the star fish and feeding observed.[19,58] Alternatively, the sponge extract could be placed on the end of a stirring rod and placed directly in the ambulacral groove in the middle of the arm equidistant from the mouth and arm tip. The star fish was turned over to its ventral surface (Figure 2) and the feeding behaviour responses were used to determine the acceptance and rejection criteria. If the food source was moved towards the mouth and/ or the stomach extruded, this was to register food and was considered acceptance. In a case where the food source was moved away from the mouth or removed from the ambulacral groove, this was considered to constitute rejection over the twenty minute observation period.[53] Results of the study revealed significant tube feet retraction in chloroform and methanol extracts in 75% of the eighteen species tested. Deterrent properties of extracts resulting tube feet retraction were compared with fish feeding assays using reef fish and feeding them on extract containing pellets.[9,19,53]

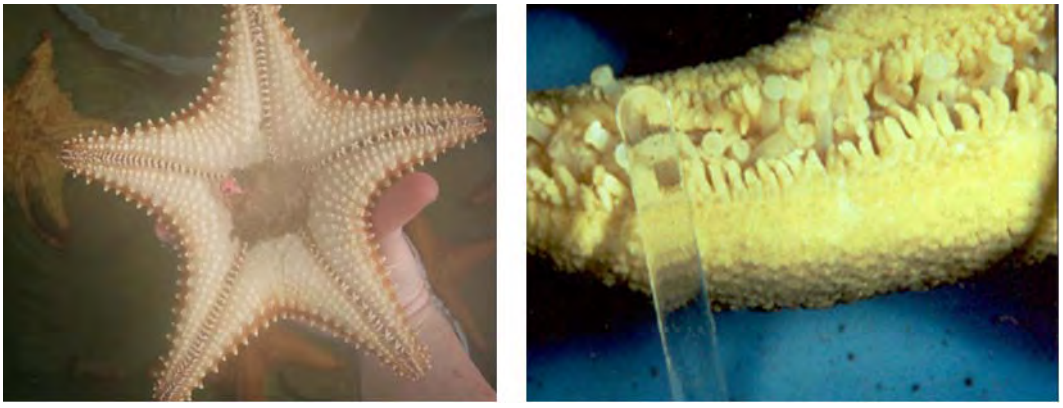
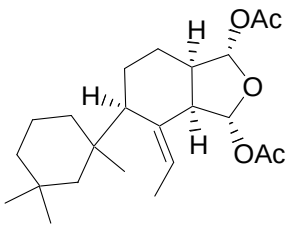


Figure 2: The ventral surface of a sea star (left) and the sea star tube feet bioassay (right).

Further investigation of the chemical constituents of these extracts indicated that secondary metabolites such as 9,11-dihydrogracilin A (**1.2**), dendrillin (**1.3**) and picolinic acid (**1.4**) were present in bioactive extracts from the sponge *Dendrilla membranosa* [19,23] and chemical ecology-based assays involving the sea star *P. fuscus* caused tube feet retraction in response to contact with the compound **1.4**.[19] Further exploration of the bioactive extracts from other Antarctic sponges revealed that the sponge *Kirkpatrickia variolosa* produced a novel group of alkaloids known as variolins (**1.5**), which show antiviral and antitumour properties.[59] The purple pigment of *K.variolosa* was tested in the star fish assay resulting in retraction of the *P. fuscus* tube feet. Cytotoxic discorhabdin pigments (*e.g.* discorhabdin G, **1.6**) isolated from the Antarctic sponge, *Latrunculia apicalis,* also displayed results similar to those described above in the star fish assay causing retraction of *P. fuscus* tube feet.[19]



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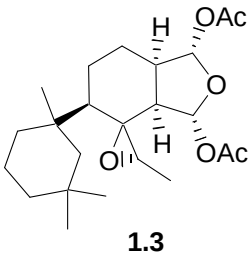

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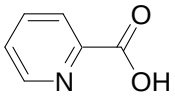

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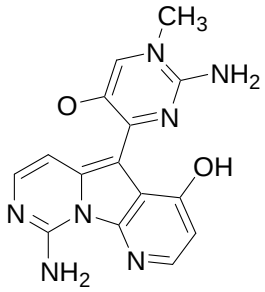

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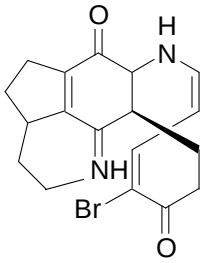


1.6

Although some of the metabolites isolated from these Antarctic sponges may have been evolutionary adaptations, it is most likely that they are involved in defense, possibly as a response to either sympatric predators, competition from other species for space on the Antarctic marine reefs, or as fouling agents to inhibit overgrowth by organisms that may interfere with their filtration of sea water.[19,27,60]

Interestingly, Guilford and coworkers came up with the theory that sponges with colouration are more likely to contain bioactive metabolites,[9] where bright colouration may be viewed as an aposematic strategy to warn predators of the toxic constituents contained in these brightly coloured sponges. Not surprisingly given this over simplification of a very ecologically complex environment, little evidence was found within tropical sponges by Pawlik *et al.*[57] to support this hypothesis, even though the tropical environment has numerous visual predators like fish which seldom feed on marine sponges.[35,61]

Sessile marine invertebrate ascidians form a dominant ecological group of organisms within the Antarctic region.[19,44] Anti-feeding properties were observed in extracts of the colonial ascidian *Distaplia cylindrical* by McClintock *et al.*[62] Comparisons between feeding assays in pieces taken from a whole colony and alginate feeding pellets[62] revealed that the sea star *O.validus* rejected the colony pieces as well as alginate feeding pellets, while the alginate control feeding pellets were accepted. Examination of the acidity of the ascidian tissue showed that the outer tunic had a pH of 1.5 and, not surprisingly, when this tunic was fed to the sea star it invoked a rejection response.[63] Interestingly, McClintock *et al*.[62] found that the tunic of *D. cylindrical* harboured the amphipod *Polycheria Antarctica f. acanthopoda.* Living within domiciles like ascidians, McClintock *et al*.[62] hypothesized that these amphipods were protected from their predators by the ascidian defences.[62] The hypothesis was tested using a common amphipod predator, the omnivorous fish, *N. coriiceps,* which was fed on fresh tunicate pieces. Results of this study consistently showed that the tunicate pieces were rejected by the fish, thereby confirming this hypothesis.[64]

The benthic environment provides a habitat for many thousands of different marine organisms, many of which are still unknown to science.[65] Knowledge of the link between marine biodiversity and novel marine natural products[66] has led to a great interest in the study of sessile and soft-bodied marine organisms over the last three decades.[67] These sessile organisms face challenges including biofouling, predation, limited access to nutrients, interspecific and intraspecific competition for space on hard substrates *e.g.* reefs.[15] Competition for limited resources occurs at both a spatial and temporal level due to limiting factors such as space,[14] temperature, light, food availability and water currents,[49] forcing the organisms to become more complex and adapt to the niches offered by the marine environment in unique ways.[19] In some cases, immobile organisms defend themselves with some form of physical defence such as a shell,[68] while other sessile marine species have evolved chemical defences to reduce predation by other species, assist in prey capture and as reduce settlement and overgrowth by other species.[69]

Sponges, cnidarians, ascidians and other invertebrates which lack physical defence structures often produce biologically active substances which help them defend themselves (Figure 3).[23] These secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms, but may constitute waste from metabolism or chemicals produced for defence, competition or that aid in reproductive success.[9] The most common classes of marine natural products are terpenoids, polyketides, alkaloids, steroids, peptides and phenols.[70]

The complexity of marine secondary metabolites was thought to have been associated with their unique ecological roles, although no experimental evidence has emerged to prove this.[2] One of the most widely studied areas in chemical ecology is that of chemical defences against predation and marine predator-prey interactions have received much attention.[16,18]

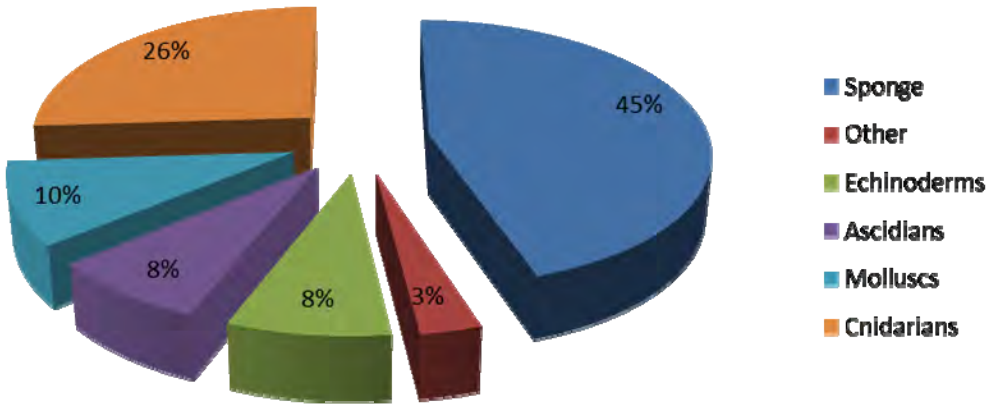


Figure 3: Overview of the relative numbers of marine natural products isolated from the main marine invertebrate phyla. Adapted from Harper *et al.* 2001**.**[71]

1.3 Chemical ecology of molluscs

There are over a hundred thousand species of molluscs, found in freshwater, marine[51,72] and terrestrial habitats. Molluscs of the Class Gastropoda comprise both the marine and aquatic snails and slugs *viz.* pulmonates (terrestrial snail and slugs), prosobranchs (marine snails) and opisthobranchs (sea slugs and sea hares).[73,74] The majority of molluscs have a shell which presents a form of physical defence for these molluscs, while others have adapted efficient swimming mechanisms by which they are able to escape from predators *e.g.* cephalopods and pteropods.[72] The slow moving soft-bodied molluscs *e.g.* sea slugs which lack these forms of defence, have evolved highly complex chemical defences which are of great interest to natural product chemists because of the structural novelty of the bioactive compounds which form part of their chemical defence systems.[75]

Opisthobranch molluscs are a subclass of Gastropoda containing a diverse range of species.[43] These gastropods demonstrate a possible evolutionary shift involving the loss of a shell (significant energy is expended in transporting a relatively heavy shell) from shelled snails to shell-less sea slugs as a result of the drastic reduction of the shell in several subclasses.[43] This unique trait of shell reduction, shown only in this phylum, makes opisthobranchs of great interest in the study of molluscan evolution.[76] The loss or a highly reduced shell has led to many of these organisms evolving defence mechanisms such as cryptic colouration or chemical defences.[48,72] As part of a chemical defence, some molluscs produce unique secondary metabolites such as polyketides, peptides and terpenes,[77] thus offering an attractive source of

novel bioactive natural products.[78] The study of the chemical ecology of opisthobranchs has, therefore, been of great interest to both biologists and chemists, with the emphasis on identifying compounds used in the chemical defences of these organisms.[18] Opisthobranchs appear to feed largely on sponges, specifically those that are chemically defended.[48] The secondary metabolites sequestered by the opisthobranchs from their sponge diet are concentrated and distributed throughout their tissues and used within their own defence system.[51,70] Chemical ecology studies of opisthobranchs have therefore, mainly been focused on nudibranchs. Secondary metabolites found within many nudibranchs have often also been isolated from their sponge diet, thus confirming a direct relationship between dietary selectivity and nudibranch chemical defence.[79] Some nudibranchs possess the ability to modify the sequestered sponge secondary metabolites which they obtain from their diet while other species have been shown to be able to biosynthesize their own defensive secondary metabolites *de novo*. [54,72]

Sea hares, are also soft-bodied marine herbivorous opistobranch molluscs that lack a shell.[54,75] Sea hares defend themselves *via* both passive and active chemical defences.[80] Passive chemical defence involves the production of unpalatable secondary metabolites sequestered from their algal diet which are present in mucus and on their skin, which deters predators from feeding on them,[61] while active defences comprise a mixture of glandular fluids also known as opaline ink, rich in proteins, which is secreted from glands in the mantle and discharged from the siphon.[48] Numerous bioactive secondary metabolites have been obtained from sea hares over the past decades.[81] Sea hares are able to concentrate their dietary metabolites, thereby producing strong chemical deterrents against their predators.[82]

1.3.1 Chemical signaling

Chemical signaling is an essential form of communication that mediates ecological interactions [7] in the ocean and affects the behavior of numerous marine organisms,[6] ranging from bacteria to benthic invertebrates as well as fish.[18,35] These chemically mediated interactions strongly affect population structure, community organization, and ecosystem function,[6] production of chemical cues including, allelochemicals, pheromones, kairomones [18,23] and metamorphic inducers,[83] which determine foraging strategies and habitat choice[7] and competitive interactions including predation.[84] Intraspecific communication is important for the identification of conspecifics, alarm signaling, search for mates and reproductive behaviour,[85] while interspecific communication allows for predators to detect their prey as well as for the prey to detect the presence of predators.[7] The different forms of communication involve allelopathy, attraction, defence, pheromones and antibiosis (Figure 4).[85]

Allelochemicals, more commonly known as chemical cues, play an important role in interspecific communication.[28] They constitute signals in predator-prey relations, host-parasite and herbivore-plant

interactions,[28] and play a role in chemical defence.[83] Recognition, location and selection of prey, host or habitat choice are strongly influenced by both volatile and non-volatile compounds.[86]

Kairomones, unlike pheromones are chemical signals produced by one organism to relay information to another organism not of the same species.[1] Although this signaling assists in the location of food [1,4] and detection of prey as well as association with predators, it proves to be less favourable to the producing organism than to the other organisms that receive this signal, and may allow a predator to locate the organism producing the kairomones.[1] As a result of this, in some cases, these kairomone signals are highly reduced, for instance when detected by a predator, the prey's behaviour will immediately change to defence.[87]

Conversely, pheromones are chemical cues produced by conspecifics. These pheromones may play a role in aggregation behaviour,[30,73] sex attraction [88] and alarm signals.[73,79] These species-specific messages are found within multicomponent mixtures which, although they may be released in very small quantities, are still detected by predators.[15,76]

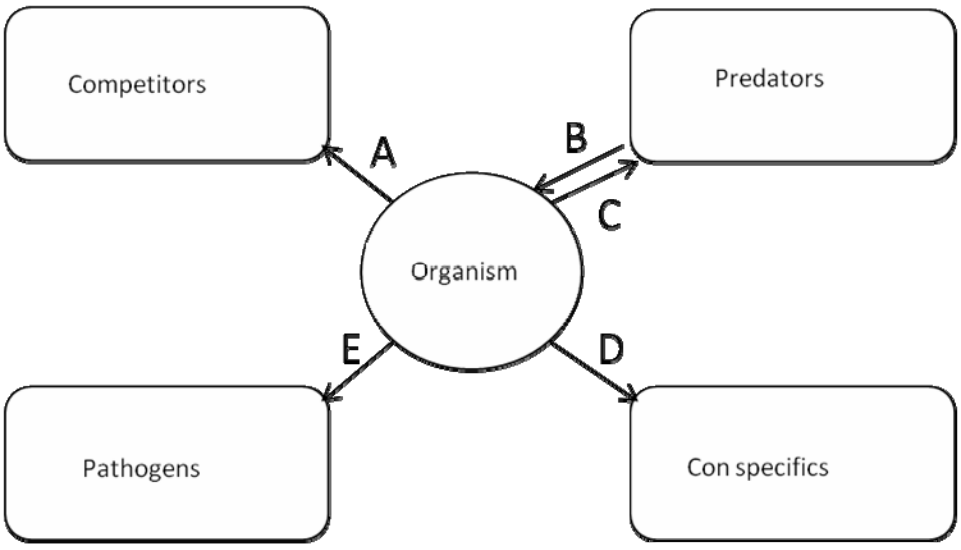


Figure 4: An illustration of chemical ecological interactions between organisms. (A) Allelopathy; (B) Attraction; (C) Defence; (D) Pheromones; (E) Antibiosis. (Modified from Fink 2007).[88]

1.3.2. Pheromone regulation

Pheromones play a critical role in signaling within molluscs, insects, humans and other organisms, especially in mating, clustering and locomotion and feeding.[30,73] Sea hares of the genus *Aplysia* appear to be solitary organisms that aggregate only to breed. Nagle and coworkers have suggested that pheromones released during the egg-laying process sustain aggregating in sea hares.[30] Apparently

pheromones produced by the egg cordons attract more conspecifics to the aggregation site and stimulate widespread mating and egg laying in these hermaphrodite molluscs.[30]

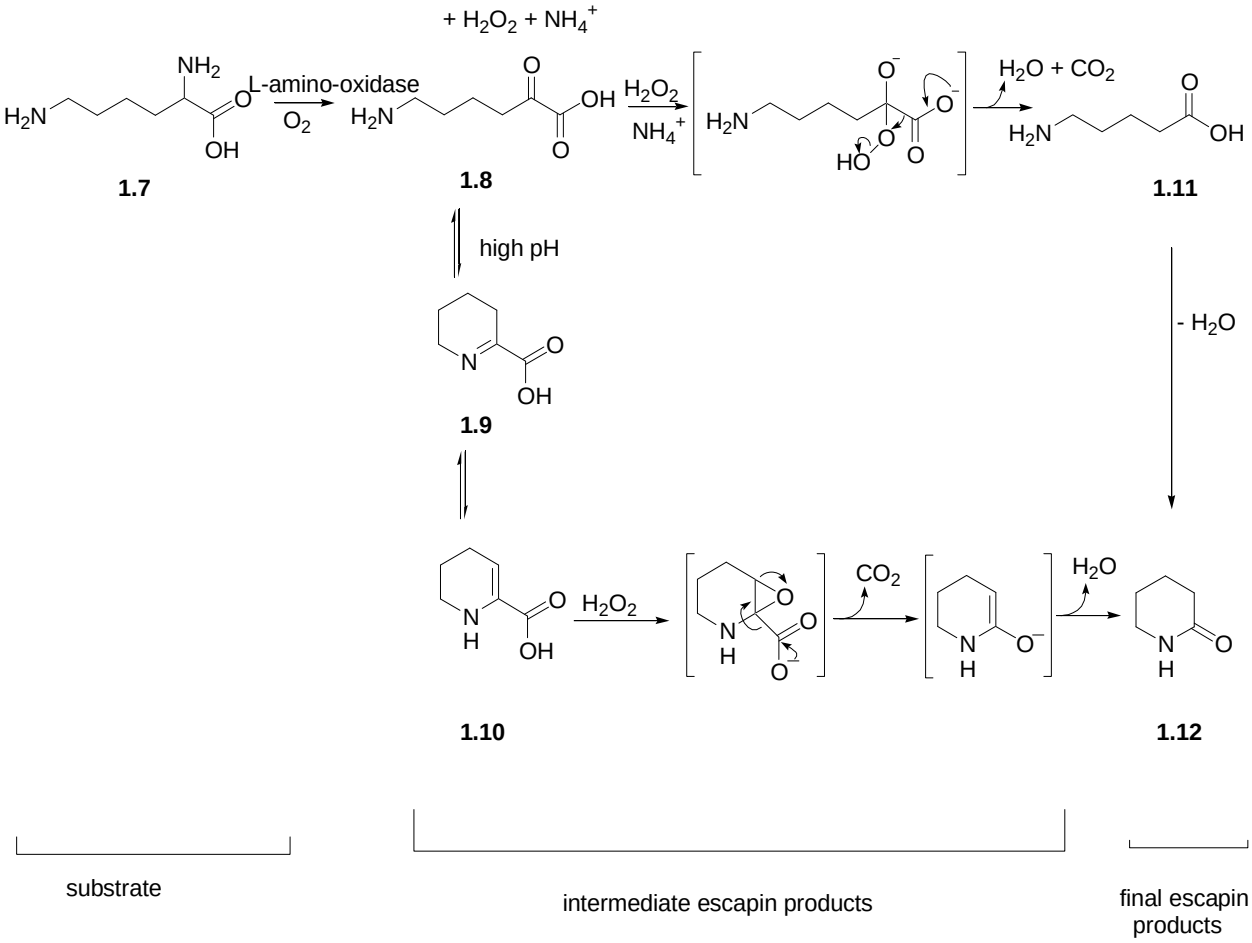
Behavioural comparisons between isolated and aggregated organisms showed isolated *Aplysia* to be less responsive to stimuli than aggregated organisms. Further experiments have revealed that the presence of conspecifics as well as egg cordons increase feeding tendencies as opposed to isolated organisms whose feeding behaviour is reduced by comparison.[73] The induced feeding behaviour of individuals due to aggregation is thought to increase *Aplysia*'s mating efficiency.[73]

1.3.3 Chemical defence in sea hares

The chemical ecological interactions of sea hares and other opisthobranch molluscs have proven to be of great interest to ecologists as they use both chemical and behavioural techniques to defend themselves.[76] Some shell-less molluscs use nematocytic-based defences and chemical defences to protect themselves from predation.[89] Other shell-less molluscs, *e.g*. sea hares produce chemical defences largely thought to have originated from their diets, to protect themselves.[90] These chemicals are incorporated into their own defensive mechanism [91] and may also aid in camouflage, protection of the eggs [92] and act as cues for settlement and metamorphosis.[90,93] Sea hares are usually found on the algal beds where they feed. They are slow moving and swimming organisms yet there are very few known predators of these organisms.[70] Once under attack, these gastropods release a complex purple ink containing opaline from their ink glands , which deters predators [94] and also acts as a chemical cue for conspecifics (Figure 5).[95] The purple colour of the ink is attributed to two secondary metabolites, phycoerythrobilin and aplysioviolin (a violet pigmentation which is characteristic of red algae).[76] These secretions are not produced by all sea hares and are associated with their diet, as illustrated by *Aplysia californica,* which preferentially feeds on red alga [69] and produce such a purple ink secretion. *Aplysia vaccaria*, conversely feed on brown algae but produces no ink.[70]

Figure 5: The release of glandular fluids by *Aplysia californica* when under attack. (Reproduced from Barsby 2006).[47]

The enzyme escapin is also present within the ink gland of sea hares, and has been thought to be associated with defense, as the opaline and ink are secreted simultaneously once the sea hare is under attack (Figure 5).[47,95,96] L-amino oxidase, oxidizes L-lysine (**1.7**) in the opaline gland to form α keto-acid acid (**1.8**) and hydrogen peroxide and an ammonium ion (Scheme 1). Compound **1.10** is also known as an escapin intermediate product.[95,97] An equilibrium mixture is then formed, with which the hydrogen peroxide interacts.[98] This intermediate mixture comprises Δ^1-piperideine-2-carboxylic acid (**1.9**) and Δ^2-piperideine-2-carboxylic acid (**1.10**). The non enzymatic reaction of **1.8**, **1.9** and **1.10** with hydrogen peroxide form the final escapin products, *viz.* δ-aminovaleric acid (**1.11**) and δ-valerolactam (**1.12**), in the mantle. The latter two compounds have been shown to provide chemical defence against *A. californica* predators *e.g.* the California spiny lobster.[95]



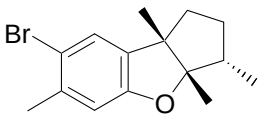
Scheme 1: The biosynthetisis of escapin (δ-aminovaleric acid **1.11** and δ-valerolactam **1.12**) from L-lysine **1.7** in *Aplysia* species.

1.4. Sequestered natural products from sea hares

The brominated sesquiterpenes aplysin (**1.13**), aplysinol (**1.14**) and debromoaplysin (**1.15**) were isolated from the opisthobranch *Aplysia kurodai,*[99] of which aplysin was the first halogenated marine sesquiterpene to be isolated from opisthobranchs.[100] Chemical conversions of algal metabolites may occur in the digestive glands. An example of this is laurinterol (**1.16**), which undergoes a conversion to form the cyclic ether aplysin.[101] Halogenated compounds from most species of *Aplysia* are thought to be obtained from their diet of red algae, specifically from the genra *Laurencia* [81] and *Plocamium*.[66]

Investigation of the digestive glands of *A. californica*, were shown to contain mainly halogenated monoterpenes and sesquiterpenes such as pacifenol (**1.17**) [102] which is characteristic of the red algae metabolites, specifically from *Laurencia pacificia*, on which they feed.[43] Interestingly, *A*. *vaccaria* feeds on brown algae and therefore concentrates crenulides and other non-halogenated diterpenes.[43]

Storage of specific algal dietary metabolites in the digestive glands has also been observed in *A*. *parvula.*[103,104] Elatol, a very powerful fish deterrent and cytotoxic halogenated metabolite, is selectively stored in the digestive glands.[104] Although the algae contain both elatol (**1.18**) and iso-obtusol (**1.19**),[105] it appears that the elatol is selectively concentrated, possibly due to the fact that iso-obtusol is less active than elatol in pharmacological assays.[104]



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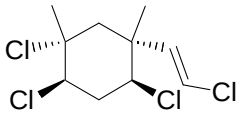

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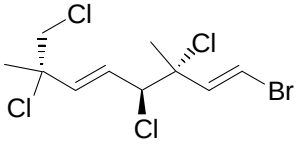

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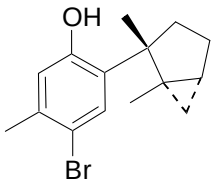

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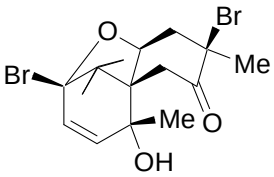

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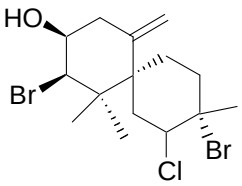

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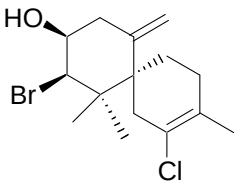

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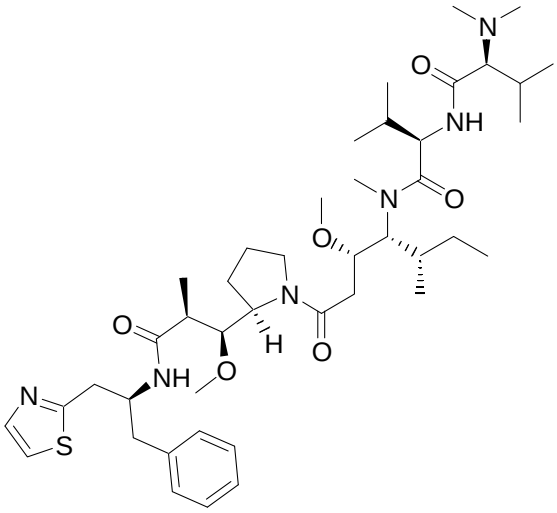
The genus *Dolabella* is well known for its production of bioactive compounds, and of particular interest are dolastatins and peptides from the sea hare *Dolabella auricularl.*[106] Dolastatins are successful cell growth inhibitors and possess antineoplastic properties.[107–109] Dolastatin inhibits tubulin, polymerization which makes this compound potentially important in cancer chemotherapy.[110] Dolastatins 10 (**1.20**) and 15 (**1.21**) have numerous pharmacologically relevant properties such as cytotoxicity and antiproliferative properties and have proven to be very active antitumour compounds. [111] Dolastatin 10, a pentapeptide, was first isolated in 1970 and shown to be the most active compound in the series.[106] It underwent phase I clinical trials in the 1990's and is currently in phase II clinical studies.[106,110] Both vascular and hematological toxicity was observed, although other toxicities were not evident.[106] Studies into the structure activity relationships around the dolastatin scaffold lead to the discovery of soblidotin (**1.22**) (auristatin PE; TZT-1027) a synthetic derivative developed as an antibody-drug conjugate,[112] which maintained its anticancer property bu reduced the deleterious toxicity observed in the natural product analogues, therefore resulting in a more efficient pharmacologically useful compound [113] which is in stage III clinical trials.[114]

Dolastatin 15 **1.21** is a seven amino acid peptide that was isolated in trace amounts and due to its complexity [115] and water insolubility, led to investigations into analogues of dolatstatin 15 which gave rise to derivatives cemadotin (**1.23**) and synthadotin (**1.24**).[115] Cemadotin is currently in the second phase of clinical trials for antitumoural and antiproliferative activity. Synthadotin is a third generation synthetic analogue of dolastatin which is in phase II clinical trial for the treatment of lung cancer.[106]



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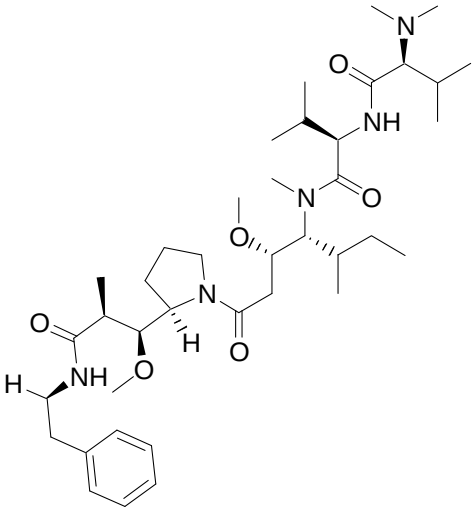

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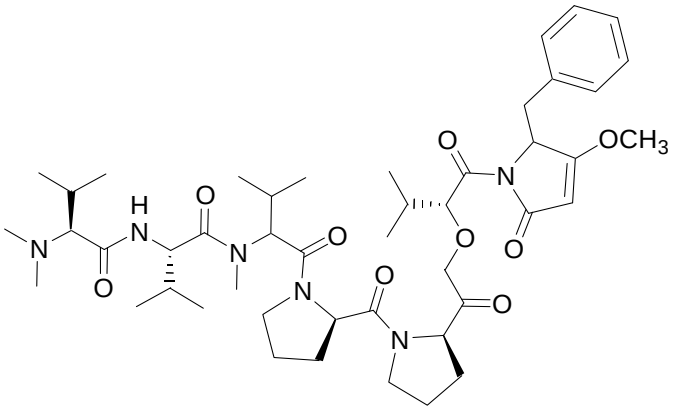

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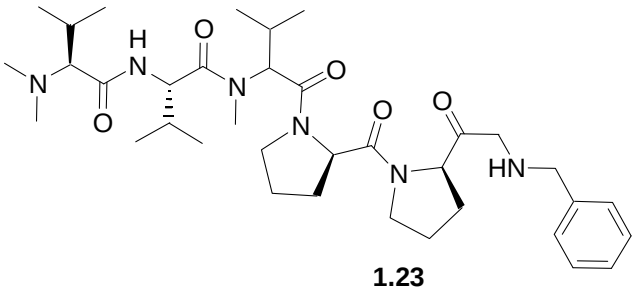

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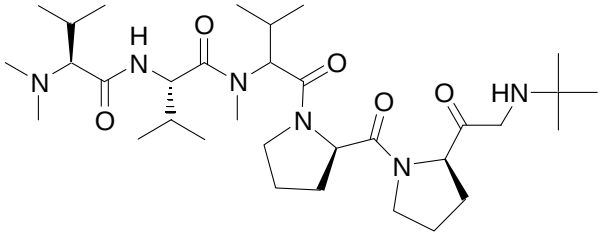

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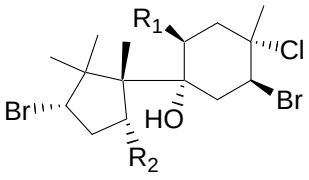
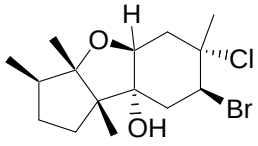
1.4.1 Sequestered natural products from South African sea hares

Several halogenated sesquiterpenes were isolated from the circumtropical sea hare *Aplysia dactylomela, viz.* algoane (**1.25**)**,** 1-deacetoxyalgoane (**1.26**), 1-deacetoxy-8-deoxyalgoane (**1.27**), ibhayinol (**1.28**), nidificene (**1.29**) and prepacifenol epoxide (**1.30**).[116]

McPhail and Davies-Coleman also isolated (3Z)-bromofucin (**1.31**) collected in the Tsitsikamma Marine Reserve on the south coast of South Africa, from the cosmopolitan sea hare *A. parvula*.[117]



	R1	R2
1.25	OAc	OH
1.26	H	OH
1.27	H	H



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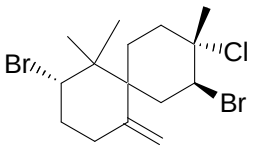

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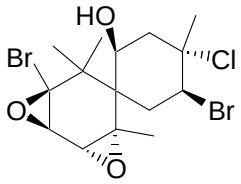

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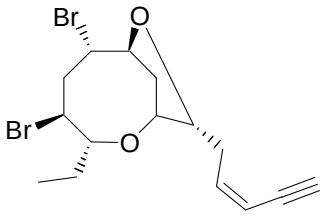

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1.4.2 The biology of the sea hare *Bursatella leachii*

Bursatella leachii is a circumtropical sea hare [118] (Figure 6) belonging to the Order Anaspidea and is a member of the family Aplysiidae.[119,120] Three subspecies of *Bursatella leachii* have been identified, *B. leachii savignyana, B. leachii plei* and *B. leachii leachii*, of which only the latter two are found in South African coastal waters. More commonly known as the ragged sea hare, the characteristic branched papillae and spotted body are observed in all the subspecies with several variations in overall colouration.[82] These sea hares are rarely found in open patches of the seabed, and are found more regularly in shallow intertidal zones of estuaries,[121,122] in close association with the eelgrass, *Nanozostera capensis,* and algal mats, upon which they feed.[79,123] Numerous studies have shown that algae which offer a high nutritional diet to sea hares may also act as a settlement inducer for the larvae of certain species.[79,93]

Little is known about the biology of *B. leachii,* although the family Aplysiidea has been well studied. An annual life cycle is observed within aplysideans, in which the usual aggregation [124] occurs in the breeding season. *B. leachii* is a hermaphrodite and individuals can function as either a sperm recipient or donor during copulation.[30,73] Eggs are laid in large masses and hatch into planktotrophic larvae which grow and develop over approximately a month, after which they settle and metamorphose to adults. Adults spend most of their active hours feeding, copulating and laying eggs.[93,124]

Their periodic occurrence in high numbers [124,125] in estuaries is probably a result of their short life span, generally considered less than a year.[125] Alternatively, their perceived patchy distribution may be the result of the difficulty in locating juveniles located within the thick sea grass and mats where they develop

and grow.[79] Metamorphosis only occurs under certain conditions *e.g.* during seasonal changes and may also be related to water depth and salinity. These factors may delay the settlement of the population during the breeding season.[1,126] Initially the idea of migration of *B. leachii* from the open ocean into estuaries was proposed but it was later rejected, due to lack of supporting evidence.[127]

There are no known predators of these sea hares,[128] although the juveniles and eggs are more likely to be preyed upon, possibly due to their defense system being neither fully developed nor functional.[129] As described earlier, defence in sea hares is achieved either through active or passive defence.[76] *B. leachii* feed on cyanobacteria mats and films as well as algae, and is able to concentrate the secondary metabolites obtained from its diet and incorporate these secondary metabolites into their defence.[70,82] The sea hares release a purple ink similar to that of squid to fend off predators.[94] Interestingly, this secretion has been shown to have anti-HIV activity.[76]

Figure 6: *Bursatella leachii* from Cape Balos. Reproduced from Kriti.[172]

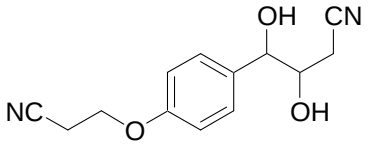
1.5 Aims of this thesis

The aims of the thesis are to identify secondary metabolites which may deter predators of the globally distributed sea hare *Bursatella leachii* which also occurs along the South African coastline. There have been no previous chemical ecology studies of *B. leachii.*

CHAPTER 2
ISOLATION OF BURSATELLIN

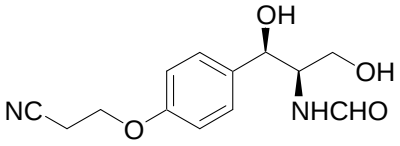
2.1.1 Bursatellin

Bursatellin **(2.1**) was first isolated in 1980, from *B. leachii pleii* collected near Paguera, Puerto Rico.[43,130] Silica gel chromatography of the dichloromethane extracts of the animal's digestive gland revealed the presence of the diol dinitrile bursatellin **2.1**, while an ethanol extract yielded various common aliphatic lipids and steroids.[130] Gopichand and Schmitz proposed the chemical structure bursatellin **2.1** from high resolution mass spectrometry, infra red, ultra violet and nuclear magnetic resonance spectroscopy (NMR) **2.1**.[130]



2.1

Several years later, an Italian group of researchers, studying the sea hares *B. leachii leachii and B. leachii savignyanain* collected from the Mediterranean Sea, obtained similar spectra for a compound appearing to have the structure of the bursatellin **2.1** isolated in 1980 by Gopichand and Schmitz.[131] The Italian researchers led by Guido Cimino, however, noted an additional down field singlet δ_H 7.28 ppm which they attributed to a formamide proton.[131] The same peak was also observed by Gopichard and Schmitz in the original 200MHz NMR spectrum of **2.1** but was erroneously thought to be an artifact by these two researchers and thus ignored. Using NMR data acquired from a 500 MHz NMR spectrometer, Cimino *et al.*[131] clearly established the presence of a formamide in **2.1** which necessitated a revision of the structure of bursatellin from **2.1** to (**2.2**)[131]. The original structure proposed for (-)-bursatellin **2.1** was also later shown to be incorrect by synthesis (Scheme 2)[131,132] and the absolute configuration of **2.2** was also established through this synthesis. Interestingly, the revised structure of bursatellin **2.2** closely resembles the natural product, chloramphenicol, (**2.3**) a broad spectrum antibiotic, [43,131] suggesting a possibly antibiotic ecological role for bursatellin.



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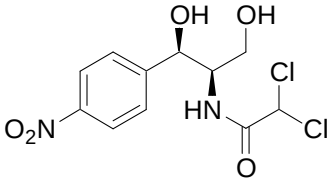

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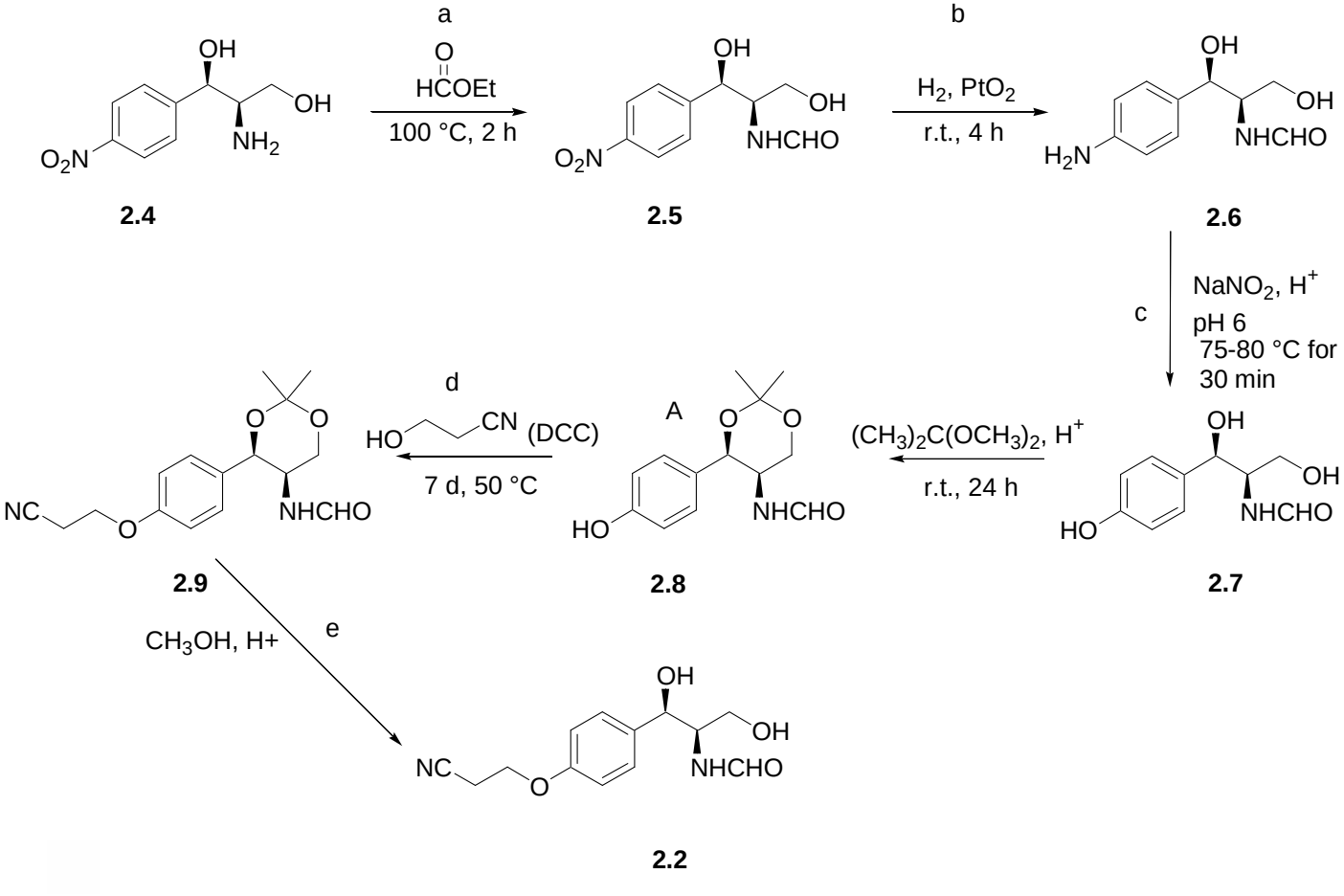
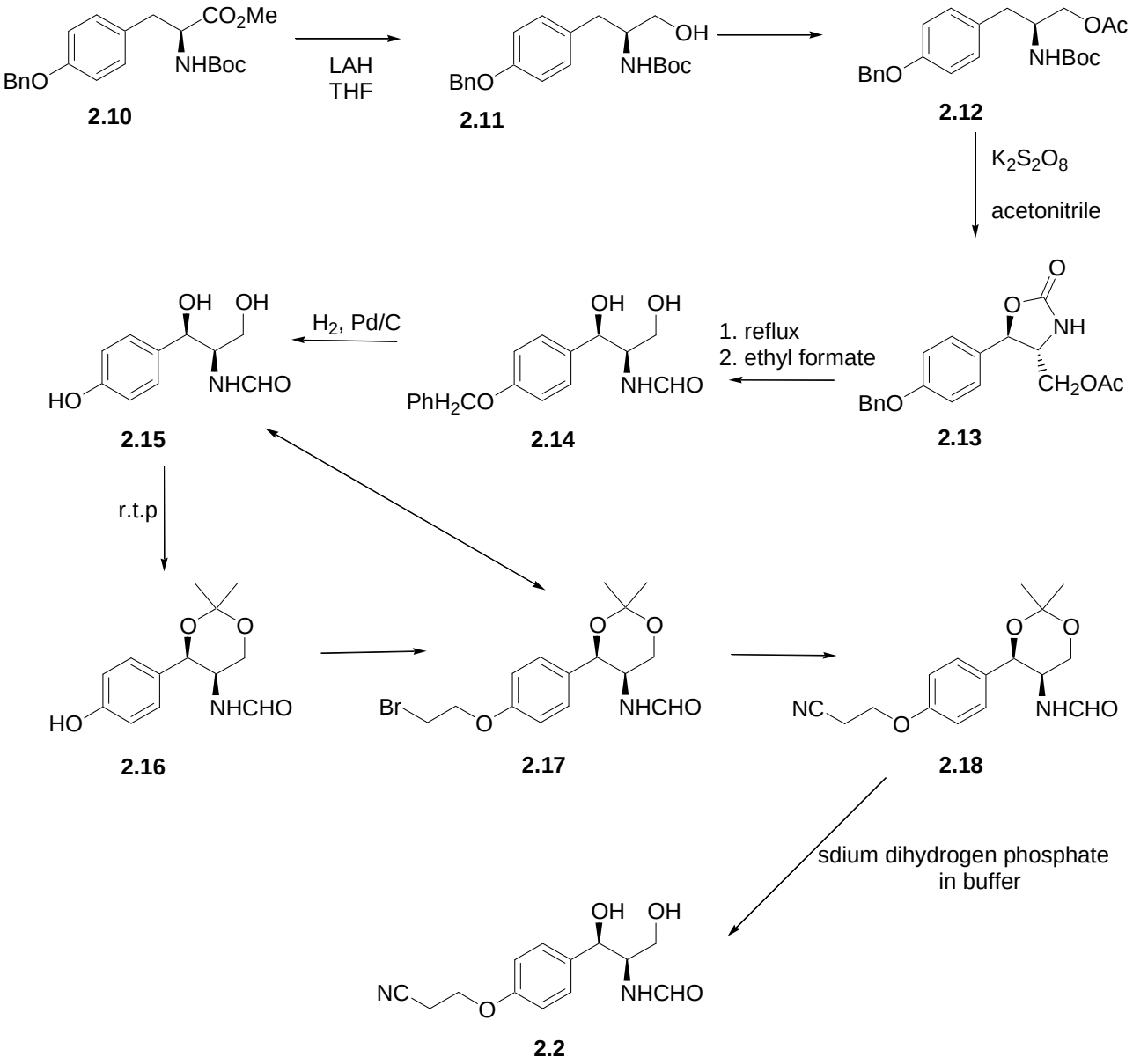
2.1.2 Synthesis of bursatellin



Scheme 2: The synthesis of bursatellin from chloramphenicol *D*-base. The processes involved formylation (A) reduction; (B) diazotization; (C) reduction; (D) deprotection (E).[133]

Racioppi *et al.*[133] synthesized **2.2** (Scheme 2) of bursatellin through the formylation (a) of chloramphenicol *D*-base (**2.4**), a commercially available compound, using ethyl formate (100 °C, 2 h) to yield the formamide (**2.5**) which was then reduced with hydrogen in the presence of palladium, to yield the aromatic amine (**2.6**). $NaNO_2$ in sulphuric acid was added to the amine at pH 6, and the solution heated (75-80 °C) for half an hour. The solution was extracted with n-butanol, and the phenol (**2.7**) was obtained

from silica gel chromatography of the butanol extract. The phenol was then converted into an acetonide (**2.8**). The acetonide was reacted with an equivalence of 3-hydroxproprionitrile and dicyclohexylcarbodiimide to yield the ether (**2.9**) The last step required the deprotection of **2.9** to yield bursatellin which was identical in all respects to the natural product (-) bursatellin (**2.2**).[133]



Scheme 3: Kawamine and coworkers synthesis of bursatellin **2.2.**[132]

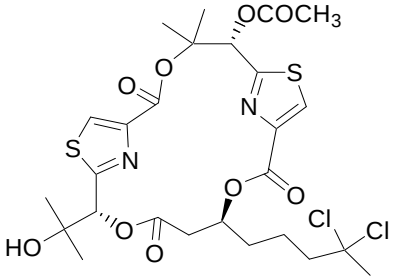
An alternative method used by Kawamine and coworkers [132] to synthesize bursatellin (scheme 3) utilized N-Boc-*O*-benzyltyrosine methyl ester (**2.10**) as the primary starting material, which was reduced to give the alcohol (**2.11**). Acetylation of the alcohol gave (**2.12**), which was then oxidized with potassium thiosulfate to produce the cyclic carbamate (**2.13**). Hydrolysis followed by an N-formylation of **2.13** using ethyl formate yielded the N-formate (**2.14**). The benzyl group was then removed under hydrogen in the presence of Pd/C to yield the phenol (**2.15**). Dimethyoxypropane in acetone was added in the presence of camphorsulfonic acid at room temperature to obtain the acetonide (**2.16**). 1,2-dibromoethane and potassium carbonate in ethanol was added to **2.16**, stirred overnight to produce the bromide (**2.17**). Attempted nucleophilic substitution with sodium cyanide in DMSO was added to the bromide which then

regenerated the phenol **2.15** due to the retro-Michael fragmentation reaction, as a result of the presence of sodium hydroxide within the commercially available sodium cyanide. The cyanide (**2.18**) was finally formed by the addition of sodium biphosphate as a buffer to the reaction mixture. The final step involved hydrolysis of the cyanide to obtain (-)-bursatellin. The synthesis of (-)-bursatellin (**2.2**) was confirmed through comparison of spectroscopic data with those reported in literature.

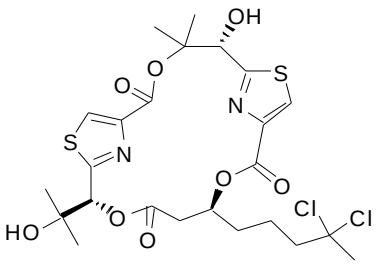
2.1.3 Other natural products isolated from *Bursatella leachii*

Although hectochlorin (**2.19**) was isolated from *Bursatella leachii,*[82] it was originally isolated from the cyanobacterium, *Lynbya majuscula* from Hector Bay, Jamaica and later from Bocas del Toro, Panama. This cyanobacterium form part of the sea hare's diet.[78,82] The planar structure of this compound was determined using NMR and mass spectrometry, with the absolute configuration derived by x-ray crystallography.[134] Bioassays indicated that hectochlorin possesses antifungal, antiproliferative and cytotoxic activity and also inhibits cell growth.[78,112] Hectochlorin has also shown the ability to promote actin polymerization and behaves similarly to jasplakinolide (**2.20**).[135] Deacetylhectochorin (**2.21**) is a derivative of hectochlorin isolated from *B. leachii*, isolated from the purification of hectochlorin by reverse phase HPLC in 0.09 % yield. [82]

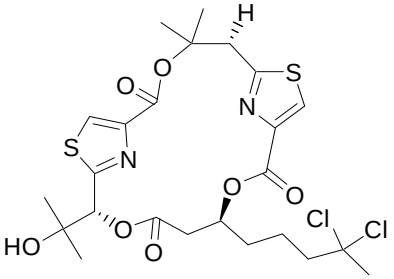
Malyngamides S (**2.22**) and X (**2.23**) were both isolated from *B. leachii* collected off Easter Beach in New Zealand.[136] Malyngamides are a class of lipopeptides isolated from several marine cyanobacteria with *Lyngbya majuscula* being of particular interest as numerous bioactive secondary metabolites have been isolated from this species.[137] Although these secondary metabolites have been isolated from red algae, it is thought that these organisms obtain these metabolites from their diet which are subsequently incorporated into their own defence system.[76] Malyngamide S demonstrates anti-inflammatory and cytotoxic properties,[78] while malyngamide X with its unique skeleton appears to have both antimalarial [136] and antitumour properties.[82]



2.19



2.20



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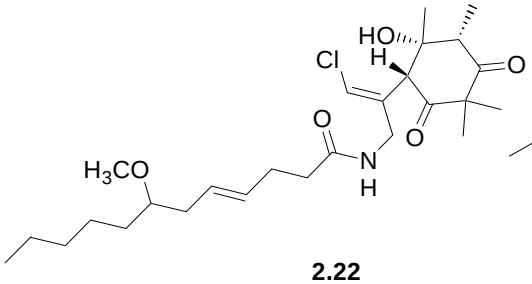
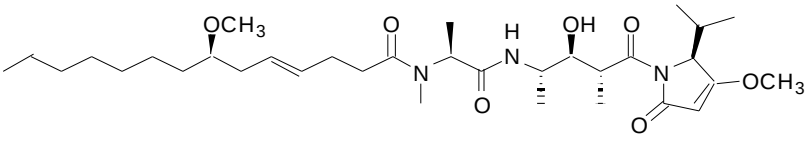


2.22



2.23

2.2 Isolation of bursatellin from South African specimens of *B. leachii*

Bursatella leachii (Class Gastropoda, Subclass Opisthobranch, Order Anapsidea) were collected from the shallow intertidal zone of the Kariega River Estuary (33°41' S; 26°44' E), at Kenton on Sea in the Eastern Cape, South Africa, in April 2011 and July 2012 (Figure 7). Specimens were placed on ice for transportation to the laboratory.

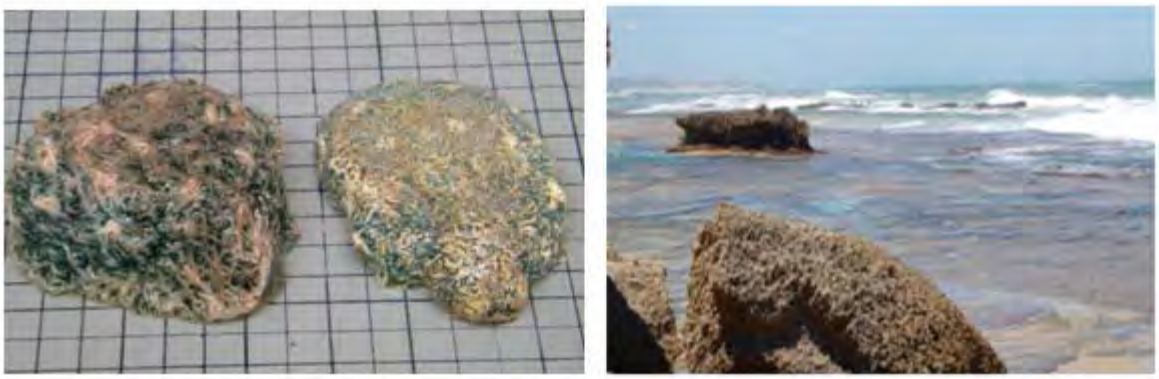
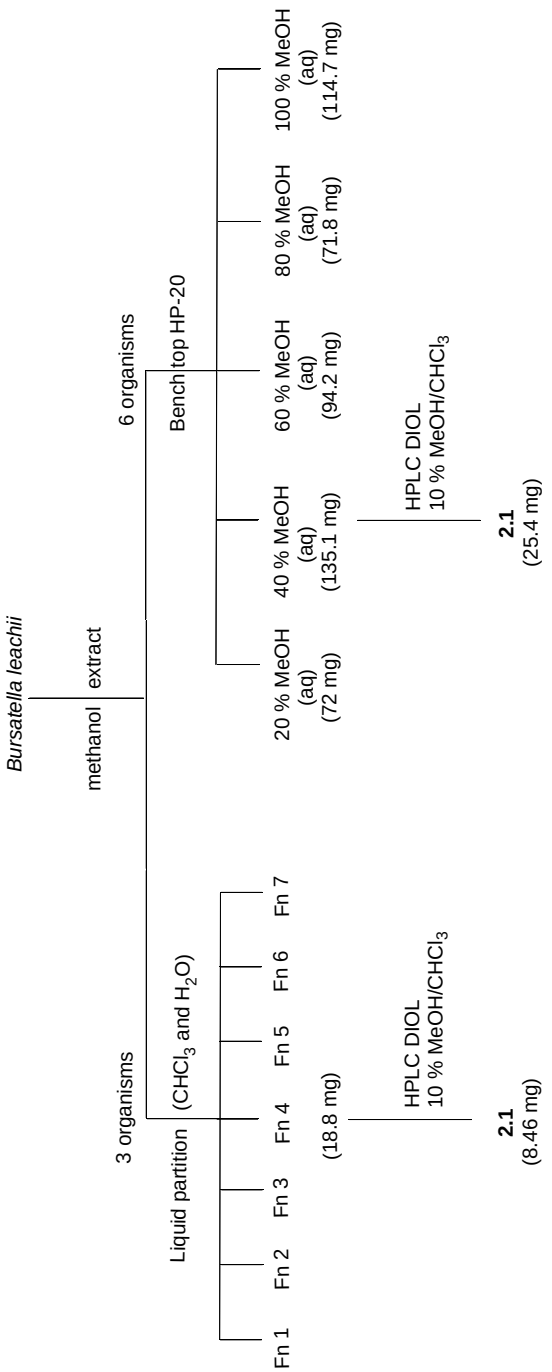


Figure 7: *Bursatella leachii* (left) and from the Kenton intertidal zone (right).

Body masses and sizes of each specimen of *B. leachii* were measured prior to freezing the organisms. Frozen *B. leachii* were subsequently cut up into small pieces of approximately 1 cm^3 and lyophilized. The methanol extract obtained from the lyophilized *B. leachii* was cyclically loaded onto a column of HP-20 resin. Aqueous methanol fractions (20%, 40%, 60%, 80% and 100% methanol) were eluted from the

column (Figure 8). The fractions obtained from the HP-20 column were examined using proton experiments that were acquired on a 600 MHz NMR spectrometer. The ^{1}H spectrum obtained for the 40% methanol fraction was of particular interest because of the presence of characteristic peaks in the aromatic region (δ_H 6.8-8.0 ppm).



Scheme 4: Two chromatographic techniques used to isolate pure bursatellin.

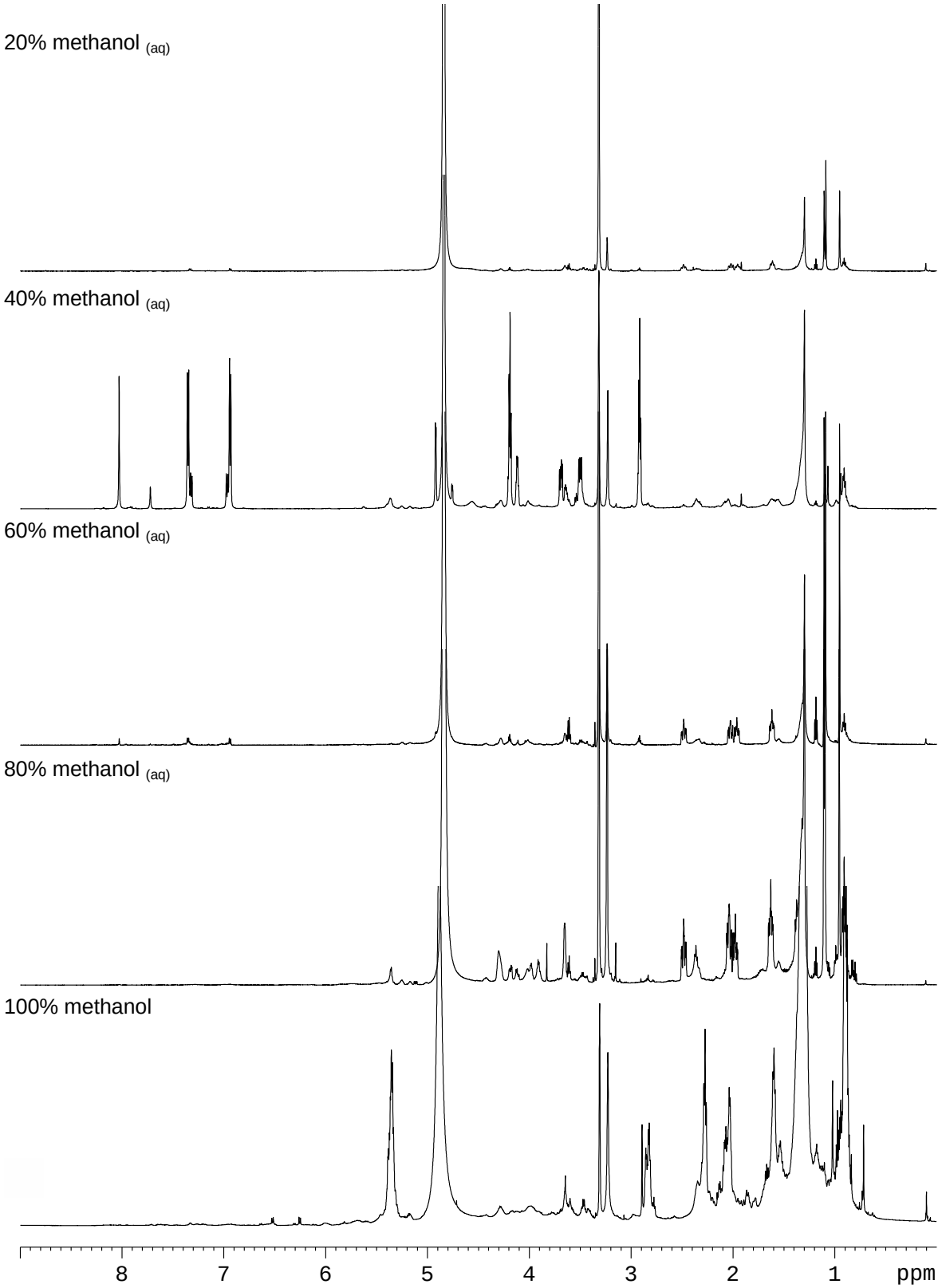


Figure 8: ^{1}H NMR (600 MHz, MeOD) of HP-20 fractions from the chromatography of the *B. leachii* methanol extract.

Interestingly, bursatellin isolated from *B. leachii plei* collected off the Puerto Rican coast was reported to have an optical rotation of $[\alpha]_D$ -8, while that of the *B. leachii savignyana and B. leachii leachii* species from the Mediterranean, yielded bursatellin with an optical rotation of $[\alpha]_D$ +12.[133] Previous syntheses of these compounds imply that the two stereoisomers are antipodal and not diastereomeric.[133] Bursatellin isolated from the *Busatella leachii*, from the Kariega river mouth in this study had an optical rotation of $[\alpha]_D$ -5 suggesting an affinity with the Puerto Rican specimens of *B. leachii*.

Based on the ^{1}H NMR spectrum, the 40 % aqueous fraction was purified on a DIOL® column to yield bursatellin (**2.2**) ($[\alpha]_D$ -5). Comparison of the isolation of bursatellin *via* liquid partitioning of the methanol extract of *B. leachii,* between chloroform and water followed by a DIOL benchtop column, with the HP-20 resin backloading procedure (Scheme 4) used to obtain the original samples of bursatellin, indicated that the HP-20 resin protocol was a more efficient isolation method for bursatellin (8.46 and 25.4 mg respectively of **2.2** from the same starting mass of methanol extract). Analysis of the ^{1}H and ^{13}C NMR spectra (Figure 9) confirmed the presence of bursatellin.

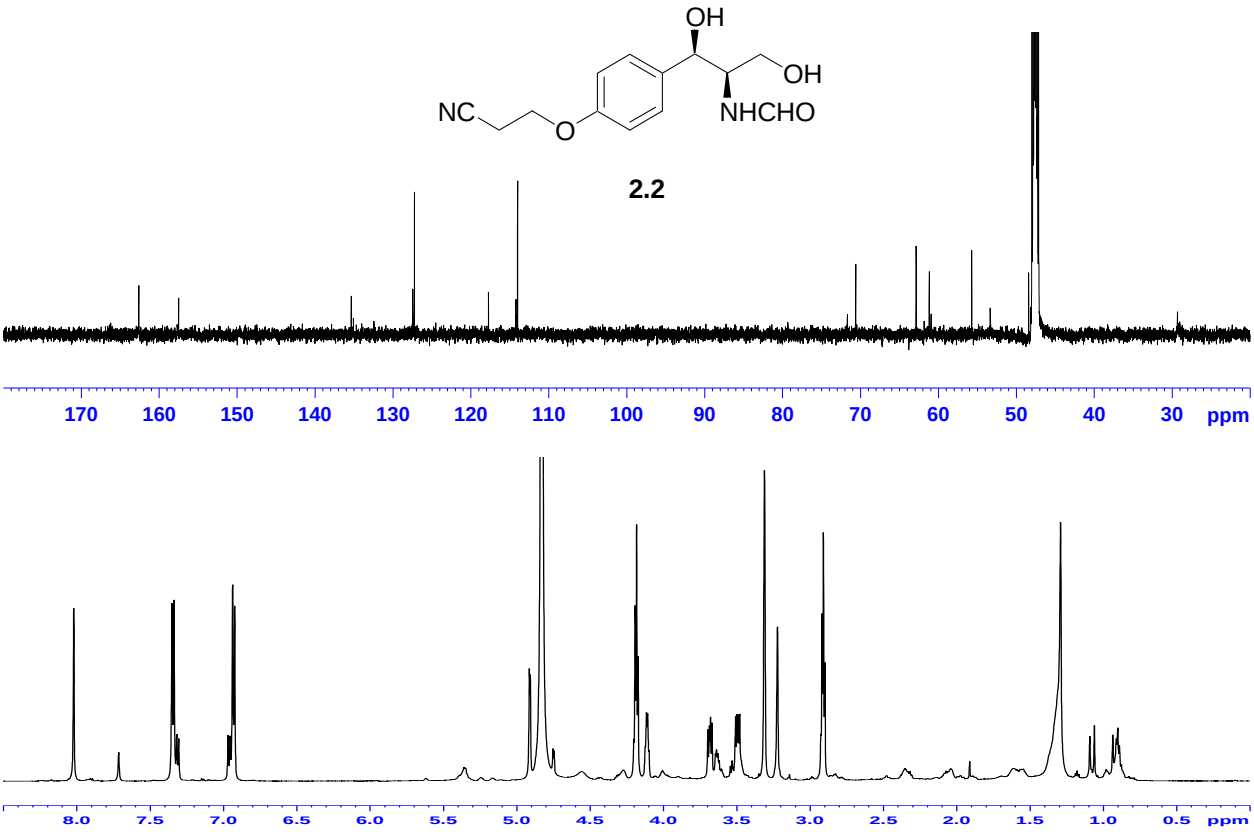


Figure 9: ^{1}H (600 MHz, MeOD) and ^{13}C (150 MHz, MeOD) NMR spectra obtained for bursatellin.

The molecular formula of **2.2** ($C_{13}H_{16}N_2O_4$) was deduced by mass spectrometry and confirmed by NMR and HREIMS [M^++ Na] (287.0923). The IR spectrum of **2.2** contained two broad bands (3414 and 3413

cm^{-1}) consistent with the presence of hydroxyl functionalities as well as a sharp absorbance (1638 cm^{-1}), indicating the presence of carbonyl group as well a broad peak (2230 cm^{-1}) corresponding to a nitrile functionality. The nitrile carbon (δ_C 119.2) and formamide carbonyl carbon (δ_C 164.0) were evident in the ^{13}C NMR spectrum. The four aromatic resonances (two overlapped) in the ^{13}C NMR spectrum (δ_C 158.9, 136.8, 128.7 and 115.4 ppm) and the two deshielded proton signals (H-5 and H-6) in the ^{1}H NMR spectrum (δ_H 7.35 and 6.96 ppm respectively) of **2.2** suggested the presence of a para di-substituted benzene ring system and accounted for four of the seven double bond equivalents required by the molecular formula. The nitrile and the formamide carbonyl accounted for the remaining three double bond equivalents.

The gCOSY spectrum (Figure 10 and Table 2) clearly showed the coupling between the methine proton (H-2, δ_H 4.10) and the oxymethylene protons (2H-1, δ_H 3.50, 3.69) and the oxymethine proton (H-3, δ_H 4.91). Important J^3 HMBC correlations are provided in the structure accompanying Figure 10.

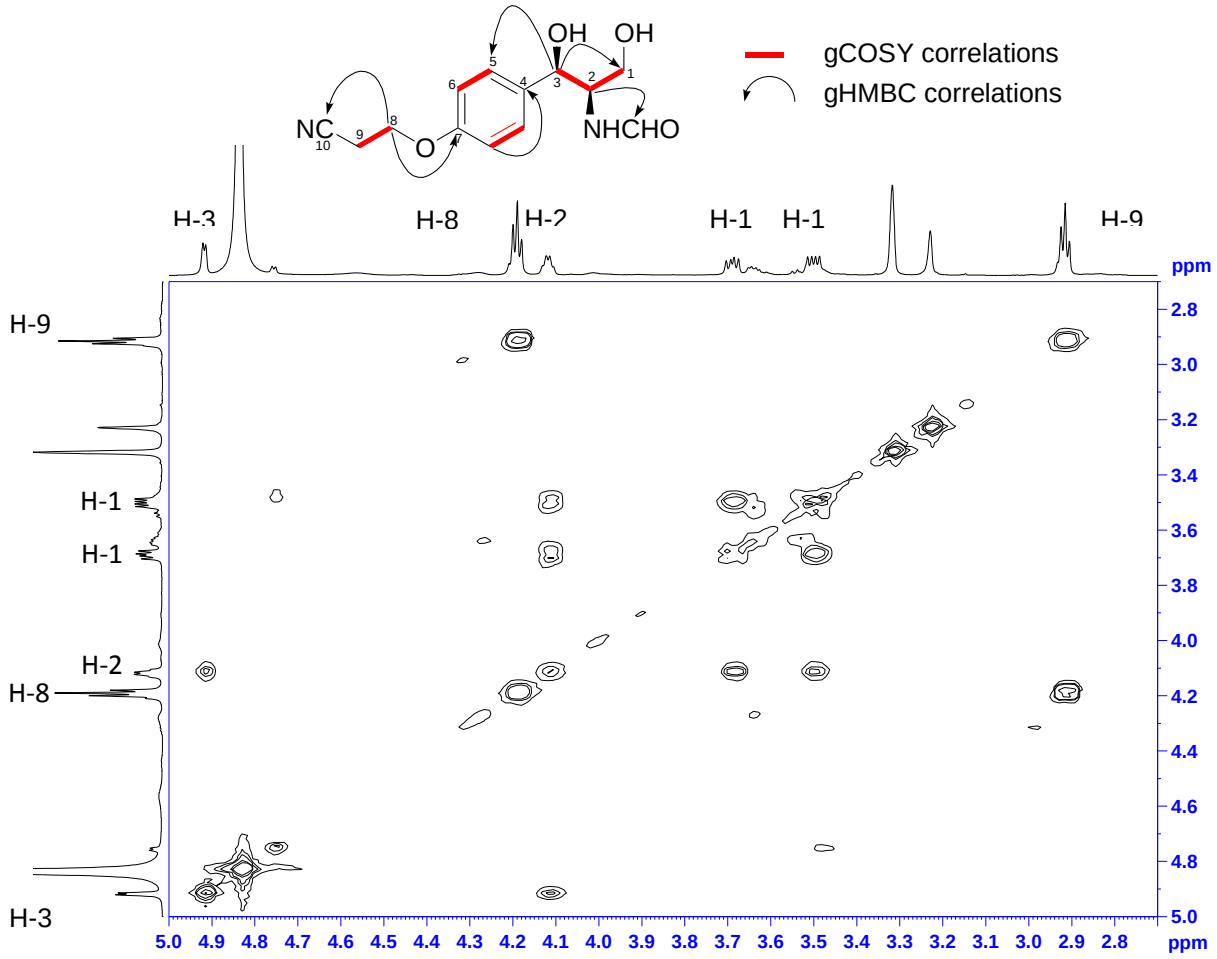


Figure 10: A region (F1 = δ_H 2.7 - 5.0 ppm; F2 = δ_H 2.7- 5.0 ppm) of the gCOSY spectrum (600 MHz, MeOD) of **2.2**. The accompanying structure shows the key gHMBC correlations and gCOSY correlations used to confirm the structure **2.2.**

From the gHMBC spectrum, the methine proton (H-2, δ_H 4.10) correlated to the formamide carbon δ_C 164.0 (δ_H 8.02). The methine (H-3, δ_H 4.91) correlates to both the methylene carbon (C-1, δ_C 62.6) as well as the aromatic carbon (C-5, δ_C 128.7). The methylene proton (H-8, δ_H 4.19) exhibits gHMBC correlations with both the carbon of the nitrile (C-10, δ_C 119.2) as well as the aromatic carbon C-7 (δ_C 158.9). The exchangeable protons of the alcohol and amino groups were not observed in the ^{1}H NMR spectrum.

Table 2: ^{1}H (600 MHz, MeOD), ^{13}C (150 MHz, MeOD), gCOSY and gHMBC NMR data for **2.2**.

	bursatellin			
Carbon	δ_C ppm	δ_H ppm (int., mult., *J*/Hz)	gHMBC	gCOSY
1	62.6	3.50 (1H, dd, 6.4) 3.69 (1H, dd, 6.0)	C-2, C-3	H-2,
2	57.1	4.10 (1H, m)	C-1 C-,3, CHO	H-3
3	72.0	4.91 (1H, d, 4.0)	C-1, C- 2, C-5	H-2
4	136.8	q	C-2, C- 3, C-6	
5	128.7	7.35 (2H, d, 8.6)	C-3	C-6
6	115.4	6.94 (2H, d, 8.5)	C-5	C-5
7	158.9	q	C-5, C-6, C-8	
8	64.3	4.19(2H, t, 6.0)	C-9	H-9
9	19.0	2.92 (2H, t, 6.0)	C-8	C-8
10	119.2	q	C-8, C-9	
CHO	164.0	8.02 (1H, s)	C-2	

Doubling of selected resonances in both the ^{1}H and ^{13}C NMR spectra of the bursatellin isolated in this study suggest one of three possibilities:

(i) Presence of a closely related compound

The bursatellin is not pure and may be contaminated with a small amount of closely related compound(s) (Figure 11). No extra signals, that would be attributed to bursatellin were seen in

the NMR spectra, thus suggesting that this possibility was not the reason for the doubling of the signals.

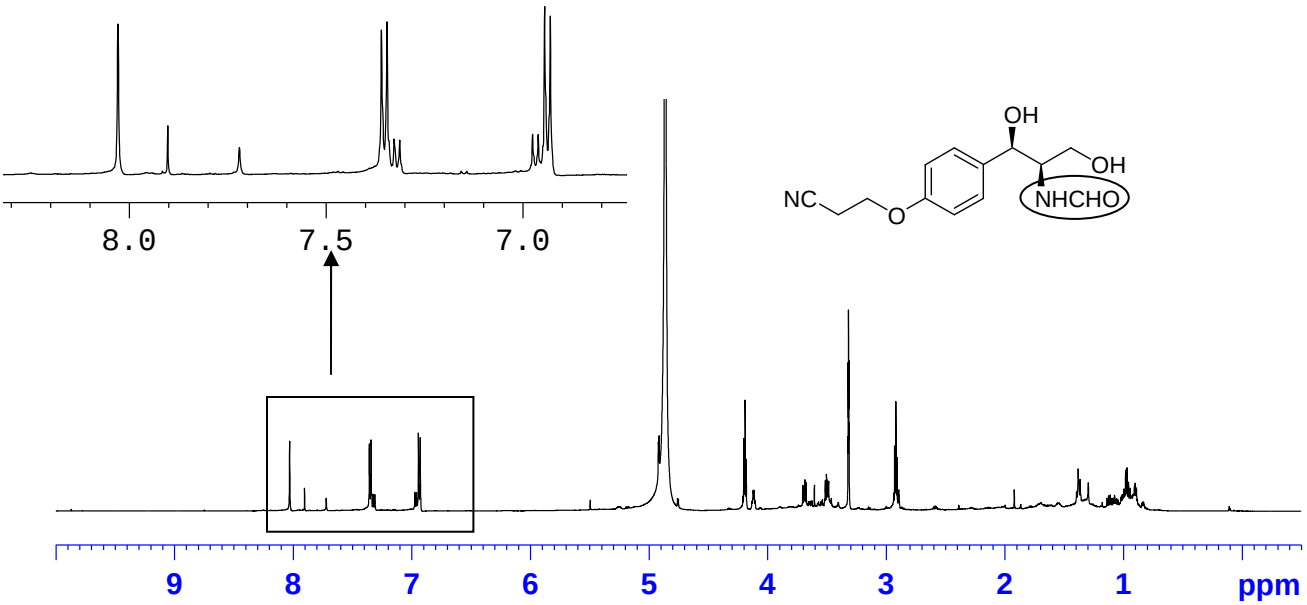
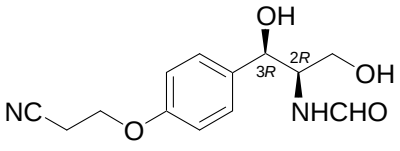


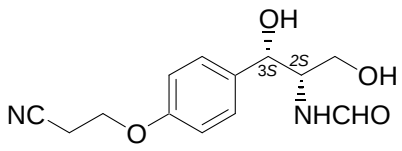
Figure 11: Section of ^{1}H (600 MHz, MeOD) NMR spectrum of bursatellin illustrating the doubling of peaks in the aromatic region (square box) of the spectrum from that of the formamide group (circled).

(ii) Presence of a diastereomers

Bursatellin has two chiral centres and therefore four possible diastereomers may exist (2*R*, 3*R)* (**2.24**), (2*S,* 3*S)* (**2.25**), (2S, 3*R*) (**2.26**) and (2*R*, 3*S*) (**2.27**). Two of these diastereomers (-)-bursatellin (2*R,* 3*R)* **2.24** *and* (+)- bursatellin (2*S,* 3*S*) **2.25** have been isolated from *B. leachii.* The possibility that a third, as yet unidentified, minor diastereomer may exist in the South African specimens of *B. leachii* cannot be discounted.



2.24



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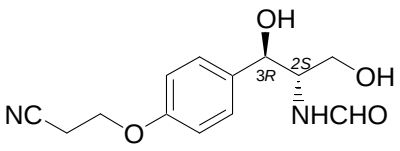
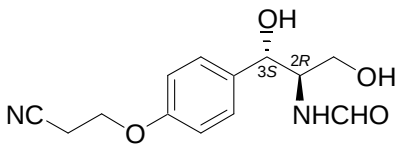


2.26



2.27

Diastereomers have different physical and chemical properties and can usually be separated by chromatography. Exhaustive attempts to isolate a possible minor diastereomer of bursatellin using DIOL chromatography with a variety of solvent systems (hexane/ethyl acetate, methanol/ dichloromethane, methanol/chloroform) as well as reversed phase chromatography (methanol/water) proved unsuccessful. Acetylation of bursatellin to give the diacetate (**2.28**) was carried out in an attempt to reduce the polarity of the possible mixture of diastereomers and make them more amenable to chromatographic separation. Bursatellin was dissolved in pyridine and acetic anhydride and stirred at room temperature for 24 h. The reaction was quenched with MeOH and concentrated *in vacuo* to yield bursatellin acetate **2.28**. Exhaustive attempts to separate the mixture of acetates were unsuccessful. The structure of the diacetate was confirmed from HREIMS with the molecular formula $C_{17}H_{21}N_2O_6$, indicating acetylation of both hydroxyl functionalities. The presence of the methyl signal observed at δ_H 2.08 confirmed the formation of bursatellin acetate **2.28** (Figure 12).

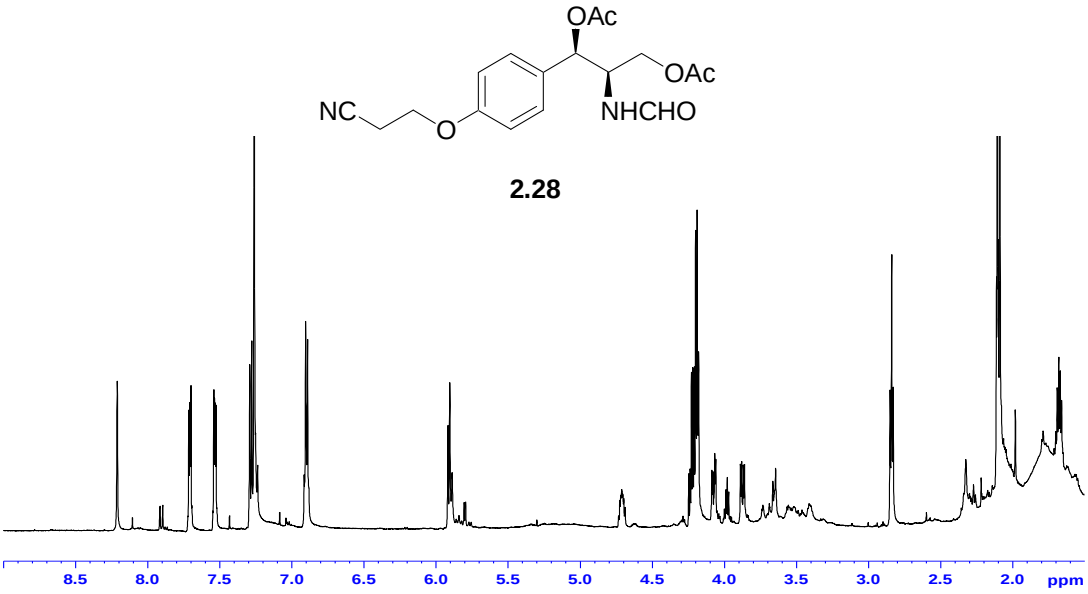


Figure 12: ^{1}H (600 MHz, $CDCl_3$) NMR spectrum obtained for bursatellin acetate **2.28**.

(iii) Presence of rotamers

Restricted rotation of the N-C=O bond in formamides has been observed previously (Figure 13)[131] with the doubling of the resonances attributed to carbons and protons in the vicinity of the N-formyl group (*e.g. N*-formyl-*o*-toluidine and *N,N'-bis*-formyl-*o*-tolidine).[138]

The presence of two isomers of the two compounds in solution shown in Figure 13 was observed with the doubling of signals of the formyl group in the ^{1}H NMR as well as doubling of all carbons

in the ^{13}C NMR. The ratio of the isomers deduced from NMR peak integration was found to be in the ratio of 3:1.[138] In some cases, these *cis* and *trans* isomers can be separated because the single bond rotation is so slow, as found in thioamides. This is due to the fact that resonance gives the single bond some double bond character and therefore slows the rotation down.[138]

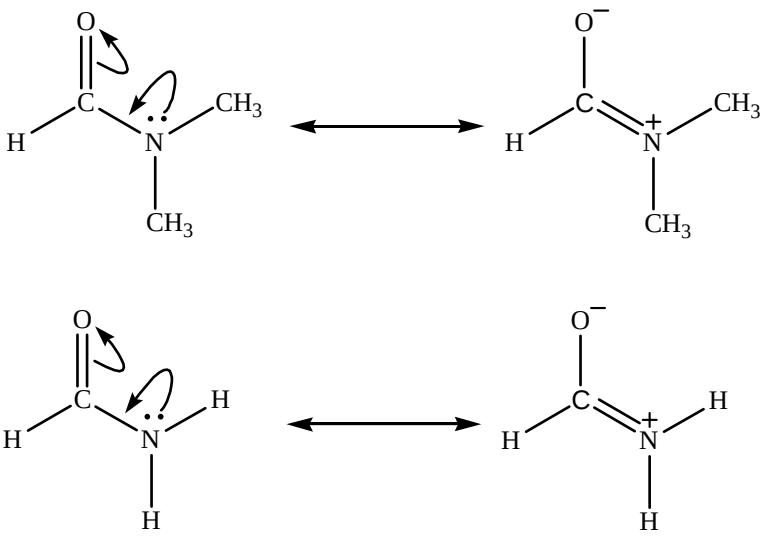


Figure 13: Hindered bond rotation about the C-N bond gives the single bond double bond character in both dimethylformamide and formamide.[138]

Upon further examination, doubling of peaks were observed in both the ^{1}H and ^{13}C spectra of **2.2**. The observation in the ^{13}C NMR spectrum were accounted for, due to the restricted rotation about the N-formyl bond, therefore only carbons close to the N-formyl bond experienced doubling whilst others did not. Similar doubling of peaks also appeared in the ^{1}H spectrum, possibly implying the presence of rotamers, which could be confirmed through kinetic studies.

The temperature of a DMSO-d_6 solution of the HP-20 chromatography sample rich in bursatellin was increased in five degree intervals ranging from the lowest temperature of 10 °C up to 45 °C, whilst acquiring ^{1}H spectra at regular intervals (Figure 14). It was expected that in the presence of rotamers, that with an increase in temperature that the two signals would merge together to form one large peak, as the increase in temperature would supply the activation energy required to overcome the energy barrier associated with the restricted rotation. This was not the case, as there was no change in the relative ratio (3:1) of the signals with the increase in temperature; therefore if indeed rotamers are present, potentially higher temperatures would be required to overcome their rotational energy barrier. Methanol standards were used to calibrate temperatures <25 °C while 10% glycol in DMSO was used to calibrate temperatures above 25 °C, to ensure that the temperatures measured throughout the study were accurate.

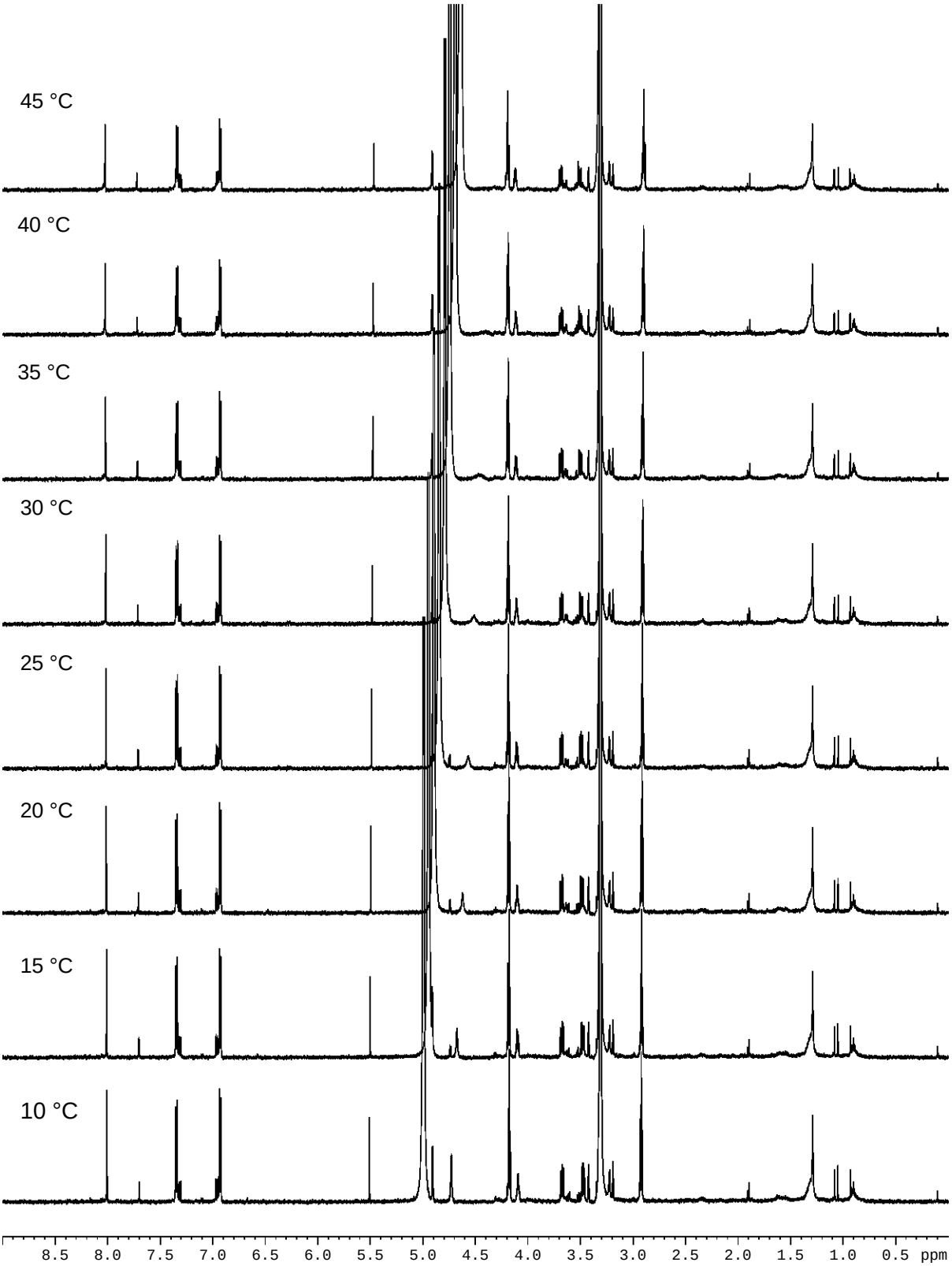


Figure 14: ^{1}H NMR (600MHz, MeOD) spectra of 40% methanol (aq) HP-20 chromatography fraction of *B. leachii* methanol extract heated from 10 °C, to ensure that the temperatures measured throughout the study were accurate.

Molecular modeling of the bursatellin revealed how the stability of the most stable conformer of the enantiomers (2*R*, 3*R)* **2.24** and (2*S*, 3*S)* **2.25** was maintained through hydrogen bonding to the oxygen of the carbonyl group as well as the unusual hydrogen bonding to the nitrogen of the nitrile (Figure 15). The hydrogen bond to the nitrile has a bond length of 2.158 Å which matches the reported values in literature.[139]

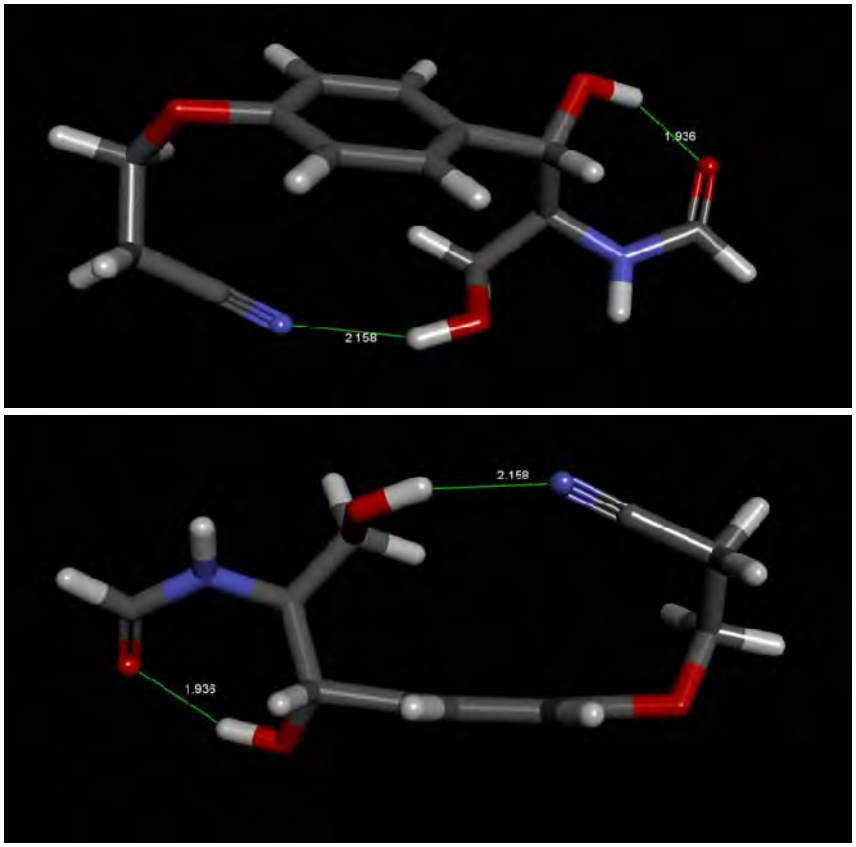


Figure 15: Stick representation of the computer-modelled conformation of bursatellin showing the possible hydrogen bonding that stabilizes the structure.

2.3 Bursatellin distribution within *B. leachii*

2.3.1 Distribution of bursatellin in the juvenile *B. leachii*

Three juvenile *B. leachii* were collected and studied. Juvenile *B. leachii* extract was obtained in the same manner as previously mentioned in the section, with methanol and loaded onto HP-20 resin with the different polarities being eluted in the normal manner. It can be seen from Figure 16 that there was no bursatellin present within the 40% methanol $_{(aq)}$ fraction within the juveniles. Interestingly, the results from the ^{1}H NMR spectrum of the 100% methanol fraction showed evidence of the presence of aromatic metabolites in this fraction.

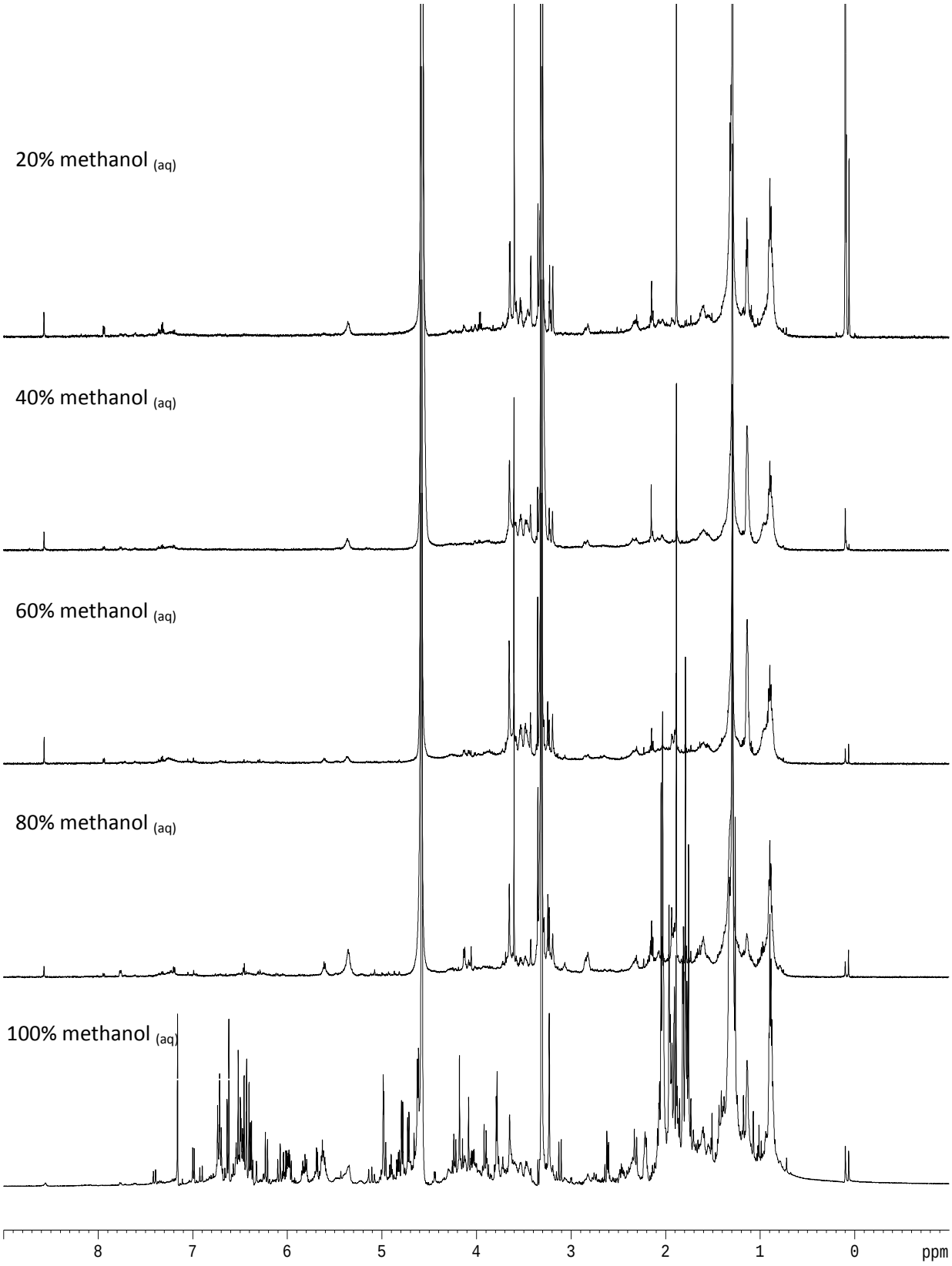


Figure 16: ^{1}H NMR (600MHz, MeOD) spectra of crude HP-20 chromatography fractions from three juvenile *B. leachii.*

2.3.2 Distribution of bursatellin in adult *B. leachii*

In an attempt to establish the distribution of bursatellin in South African adult *B. leachii*, five sea hares were dissected (Figure 17). From the dissection, it is evident that the sea hare has a distinct head with the presence of rhinophores used in the sensory system. The crop connects the head to the stomach. *B.leachii* is a hermaphrodite and therefore, has both male and female reproductive organs. The digestive gland is essential in producing the digestive enzymes, absorbing all the nutrients from the diet as well as excreting the waste products.

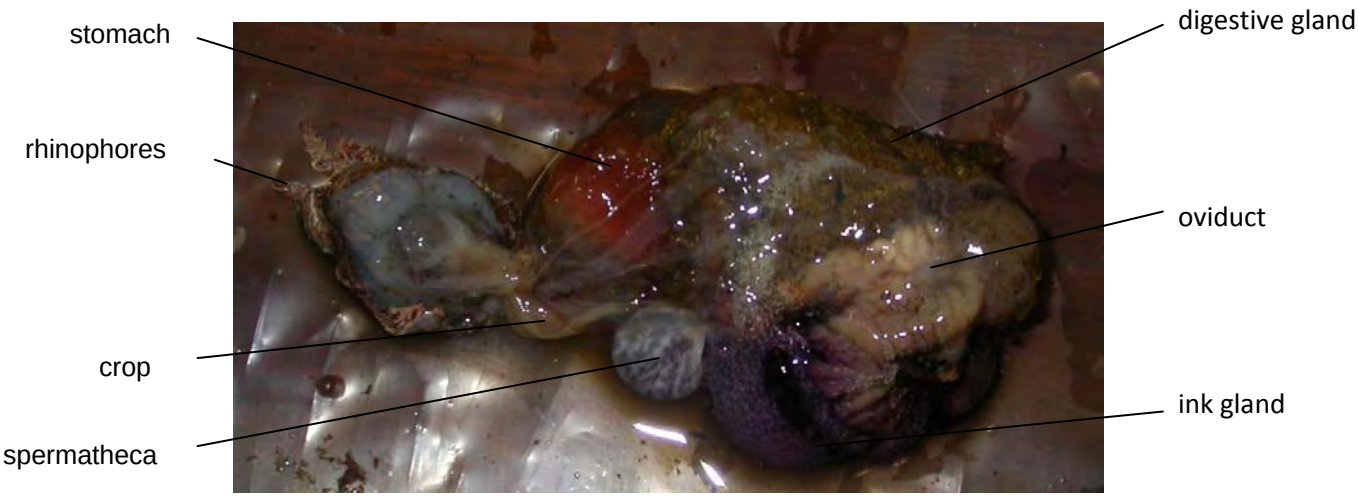


Figure 17: Dissection of a specimen of the sea hare *B. leachii* collected from the Kariega estuary.

The partitioned organs (Figure 18) were soaked in methanol and fractionated with HP-20 resin and analyzed using NMR spectroscopy.

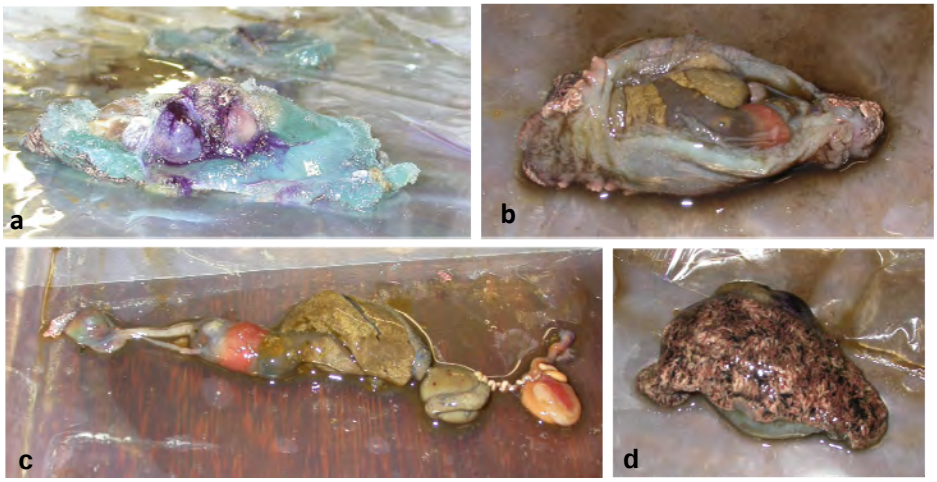


Figure 18: Dissected organs from the sea hare *B. leachii* (a) ink gland, (b) all body parts (c) digestive glands (d) skin.

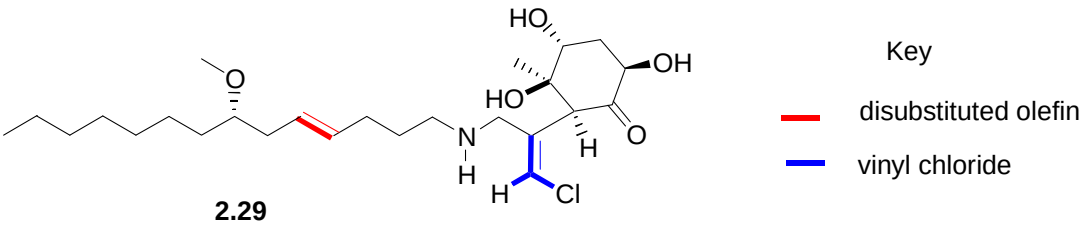
Bursatellin (**2.2**) was found to be present within the 40% methanol $_{(aq)}$ fractions of the ink gland, digestive system as well as all other body organs except the skin. Bursatellin was also found to be present within the 60% methanol $_{(aq)}$ fraction of the ink gland, with reduced amounts of **2.2** found within each organ (Table 3). It is evident from this investigation that highest concentrations of **2.2** were found within the ink gland. The digestive system contained lower amounts of **2.2** compared to the rest of the body, most likely because bursatellin is sequestered from the diet of the sea hare and then stored in various organs around the body. The absence of **2.2** from the skin was surprising and may be a result of the smaller mass of skin relative to other organs *i.e.* bursatellin although present may be in such relatively small amounts that it remained undetected.

Table 3: Distribution of bursatellin isolated through HP-20 resin from five sea hares.

Organ	Bursatellin	Amount (mg)
Ink gland	✓	30.6
Digestive system	✓	9
Skin	X	0
All other organs	✓	26.4

2.4 Other Natural Products from *B. leachii*

Apart from bursatellin, an attempt was made to isolate other natural products *e.g.* malyngamides (**2.29**) from the tissue of *B. leachii*. Malyngamides were originally isolated from the cyanobacteria *Lyngbya* which *B.leachii* regularly feeds on.[43] Malyngamides are easily identified by their lyngbic acid moiety with its variation of between twelve to twenty carbon atoms and the characteristic ^{1}H and ^{13}C signals associated with a vinyl chloride signal (δ_H 6.31 and δ_c 121.1) and disubstituted olefin (δ_H 5.49 and δ_C 128.3 an 130.6).[140]

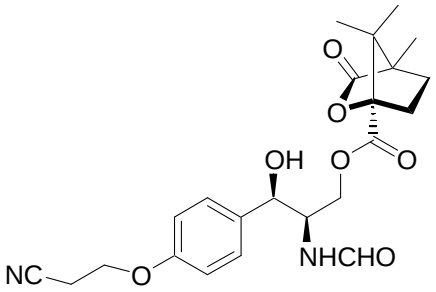


2.29

Unfortunately malyngamides were not deteced in the ^{1}H NMR spectra of the fractions eluted off either a C-18 reversed phase column or HP-20 column.

2.5 Synthesis of the camphanate ester

The chiral compound (1*S*)-camphanic chloride was used, in an attempt to separate the mixture of enantiomers of **2.2**. Esterification was expected to occur at the primary alcohol as it is the most reactive site, although it was not possible to ensure that esterification occurred at this site only.



2.30

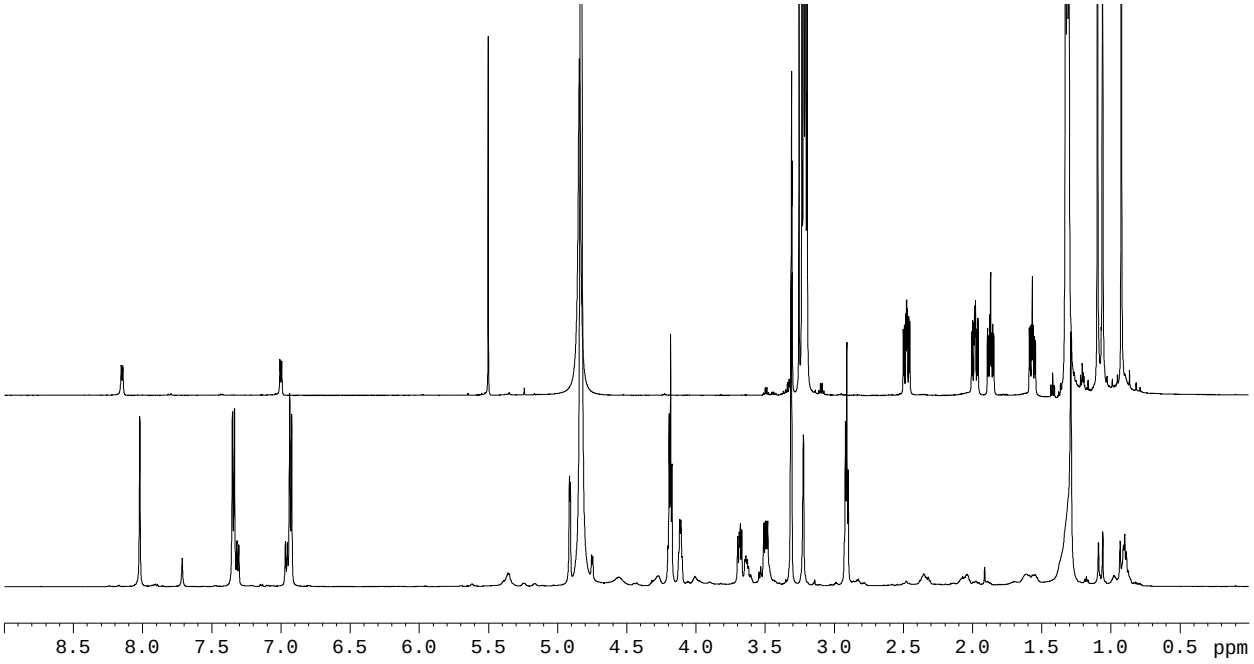


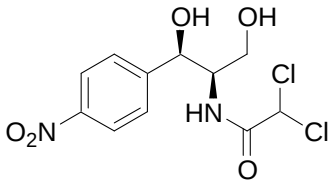
Figure 19: ^{1}H NMR (600MHz, MeOD) spectra of the bursatellin **2.2** (bottom) and bursatellin camphanate ester **2.30** (top).

From the ^{1}H NMR spectra (Figure 19), it is evident that the synthesis was unsuccessful as there appears to be a loss of bursatellin proton signals and the integrations of the two proton peaks in the aromatic region do not equate to those of the camphanic chloride.

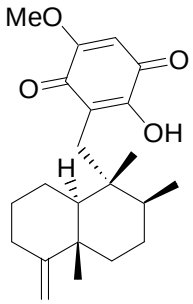
CHAPTER 3
ISOLATION OF ILIMAQUINONE AND PELOROL

3.1 The isolation of ilimaquinone from the sponge *Hippospongia metachroma*

The amount of bursatellin which we had in hand (*ca.* 50 mg) was too small to embark on a series of chemical ecology studies and needed to be conserved until the bioassays were well established. Two options were explored in order to establish the bioassays. Firstly, find a prolific source of a known bioactive natural product, isolate this and use this natural product as a control in the bioassays. Secondly, purchase a commercially available bioactive compound related to **2.2** *e.g.* chloramphenicol (**2.3**), for the same purpose. Both approaches were adopted in this thesis.



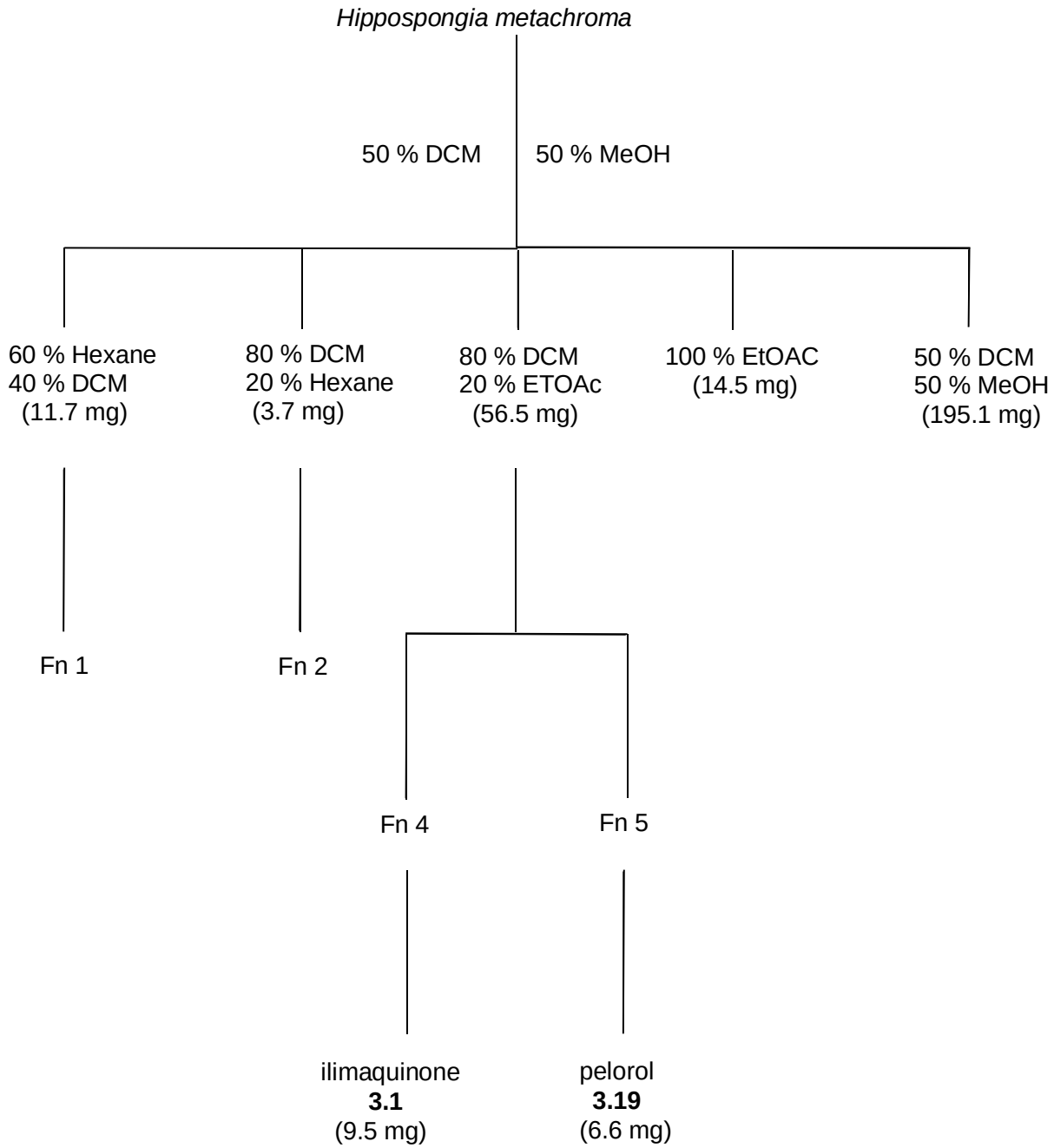
2.3



3.1

While the bursatellin isolation was under way, Dr Sunassee, a post doctoral research fellow in our laboratory, identified the presence of a common bioactive sponge metabolite ilimaquinone (**3.1**) in large amounts in specimens of the sponge *H. metachroma* trawled from the Mascerene Plateau, Western Indian Ocean in December 2010 at a depth of 100 m.

Ilimaquinone was first isolated from the Hawaiian sponge *Hippospongia metachroma*, which was collected off the shores of the Island Lanai.[141] Ilimaquinone was extracted from the sponge in an aqueous methanol/acetone solution, from which the pure ilimaquinone was isolated from silica gel in 4% yield. Crystallization of Ilimaquinone was finally successfully achieved from hexane yielding orange needles.[142] Ilimaquinone is a member of the sesquiterpenoid quinones, which possess a 4.9 friedodrimane sesquiterpene skeleton and a pendant 1,4-benzoquinone moiety.[143] Apart from the sponge *H. metachroma*, ilimaquinone **3.1** has been isolated from the sponges *Fenestraspongia* and *Dactylospongia elegans*, mixed with a closely related metabolite, 5-*epi*-ilimaquinone (**3.2**) within in all of these sponges.[144]



Scheme 5: Isolation of ilimaquinone **3.1** and pelorol **3.19** from the sponge *Hippospongia metachroma.*

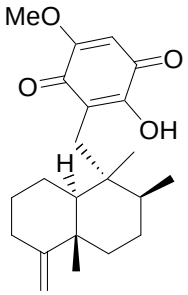
The unique marine natural product **3.1** shows interesting biological activities such as antimicrobial, cytotoxic, anti-HIV, antiviral and anti-inflammatory activities.[145] Ilimaquinone was also shown to protect cells from the toxic effects of diphtheria and ricin toxin.[143] Other biological activities associated with protein trafficking caused microtubule–independent fragmentation of the Golgi apparatus.

Closely related metabolites such as **3.2** and isopongiaquinone (**3.3**) were isolated from an Australian sponge *Fenestraspongia*[144] and *Stelospongia conulata* respectively.[146] A closely related metabolite avarol (**3.4**), with a 4,9-freidodrimane skeleton, has been isolated from the sponge *Dysidea avara*.[147] The sesquiterpenoquinone avarone (**3.5**) which appears to be structurally related to **3.1** showed similar biological activity such as antitumoural, anti-inflammatory and antimicrobial activity.[145] Closely related to **3.5** are arenarone[142] (**3.6**) and slightly differing arenarol (**3.7**).[148] Other closely related metabolites like smenospogine (**3.8**), smenospongidine (**3.9**) smenospongiarine (**3.10**), mamanuthaquinone (**3.11**) arerone (**3.12**) and avenarone (**3.13**) have also been isolated.[142] Dactyloquinones A-E (**3.14-3.18**) also closely resemble the sponge metabolite **3.1**.[149]

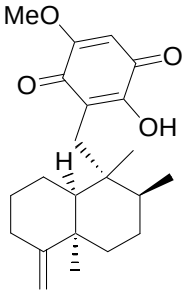
The frozen sponge *H. metachroma* from the Macarene Plateau was cut into smaller pieces and freeze dried. The lyophilized sponge was then steeped in a 50:50 MeOH/DCM solution (3 × 100 mL), sonicated and the solvent filtered off. The MeOH/DCM solution was then reduced to a brown solid (17 g) *in vacuo*. The brown solid was then passed through a DIOL column and fractions were collected based on the colours of the eluting fractions. A bright orange fraction yielded iIlimaquinone **3.1** with pelorol (**3.19**) following shortly afterwards, in a bright yellow fraction.

Ilimaquinone was obtained from the bench top DIOL column in a mixture of closely related metabolites which proved difficult to separate initially. Accordingly, the mixture was dissolved in pyridine and acetic anyhdride and left to stir for 24 h The reaction was then quenched with methanol, and the acetylated mixture was concentrated *in vacuo*. Further purification was carried out on HPLC semi preparative DIOL column using 3:2 hexane/ethyl acetate to yeild ilimaquinone acetate (**3.20**).

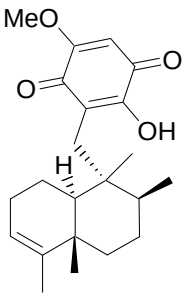
The molecular formula of ilimaquinone **3.1** was determined through HREIMS data as $C_{22}H_{30}O_4$. The IR spectrum of **3.1** suggested the presence of hydroxyl and carbonyl functionalities (ν_{max} 3429 and 1634 cm^{-1} respectively) and was in agreement with literature values.[150] The seven quaternary carbon signals at δ_C 182.3, 182.0, 161, 7, 160.4, 117.3, 43.3 and 40.4 in the ^{13}C NMR spectrum and seven methylenes δ_C 102.0, 36.6, 32.3, 27.9, 33.0, 28.6 and 23.1 in the DEPT 135 spectrum, as well as the three methyl proton signals at δ_H 1.02, 0.95 and 0.82 in the ^1H NMR were also in accordance with the chemical structure of **3.1** (Figure 20). The presence of the methoxy signal (δ_H 3.83) and the exocyclic methylene protons (δ_H 2.44 and 2.51), the quinone proton (δ_H 5.85) as well as the $=CH_2$ (δ_H 4.40 and 4.42) in the ^1H spectrum, were also consistent with that of ilimaquinone.



3.1



3.2



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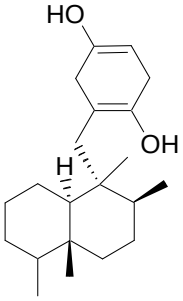

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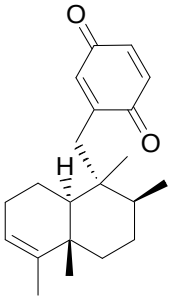
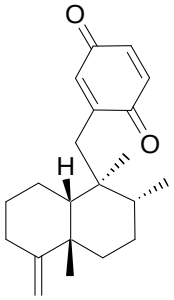
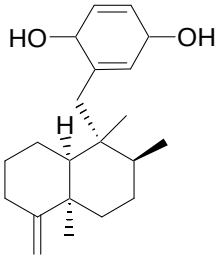


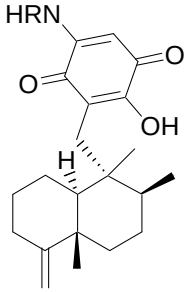
3.5



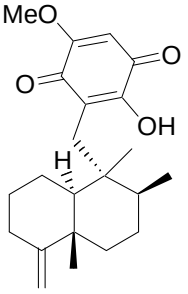
3.6



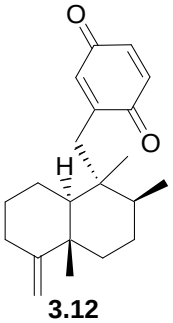
3.7



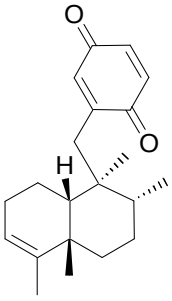
3.8 R:H
3.9 R:CH_2CH_2Ph
3.10 R:*i*-pentyl



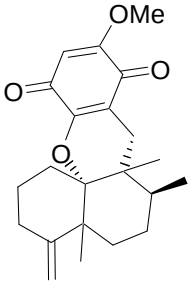
3.11



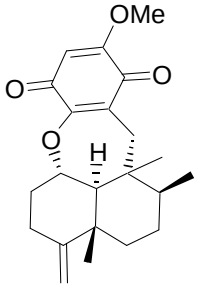
3.12



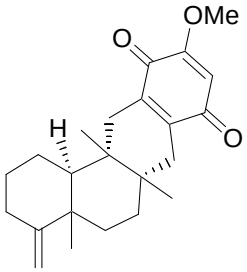
3.13



3.14 5 B-Me
3.15 5 a- Me



3.16



3.17 5 B-Me
3.18 5 a- Me

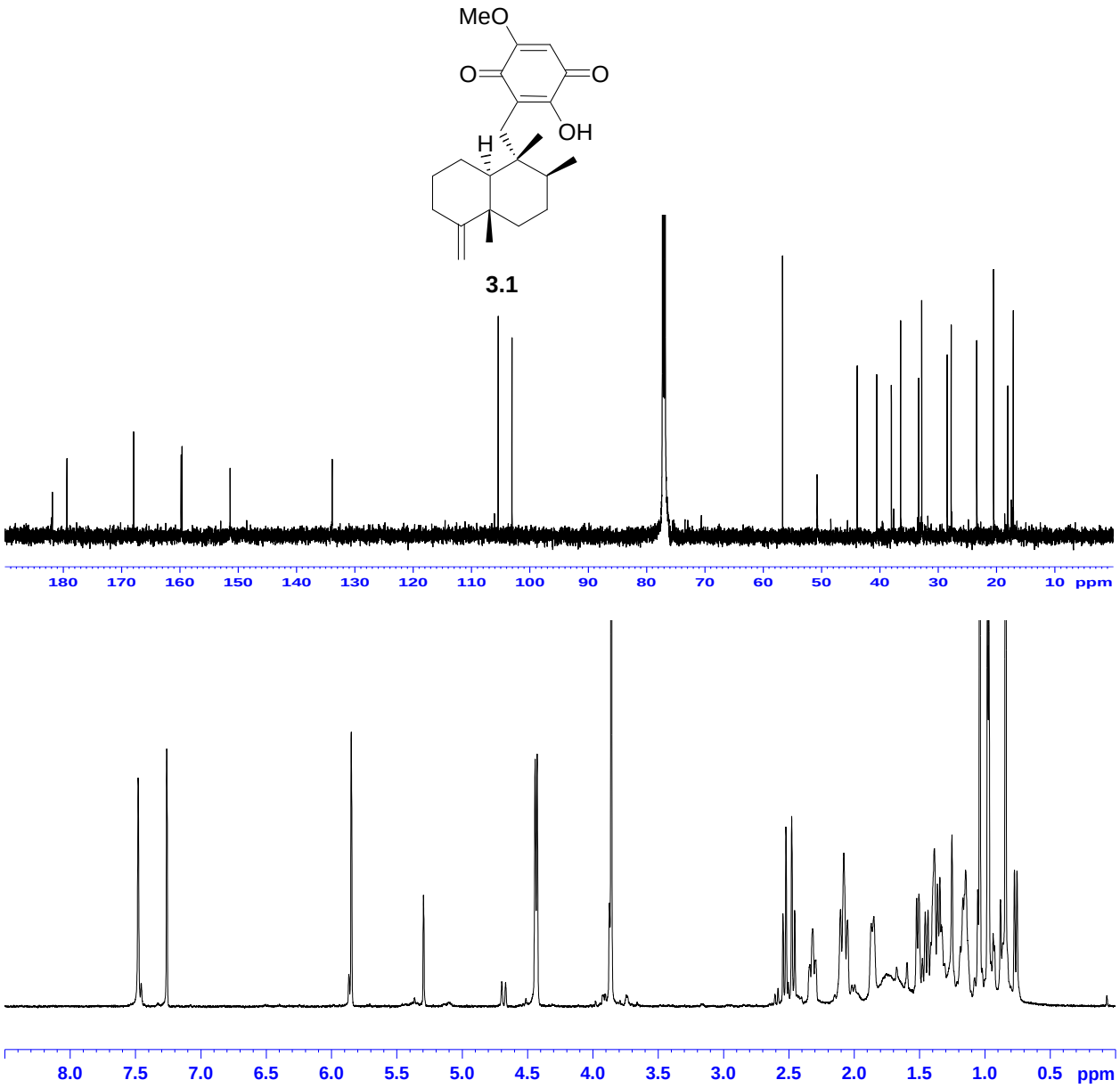


Figure 20: ^{1}H (600 MHz, MeOD) and ^{13}C (150 MHz, MeOD) NMR spectra obtained for ilimaquinone **3.1**.

The specific rotation of the ilimaquinone isolated in this study was consistent with the ilimaquinone isolated previously ($[\alpha]_D$ -22.4, lit.[142] -23.2). An in depth 2D-NMR analysis (Figure 21) was used to assign all proton and carbon signals in ilimaquinone (Table 4).

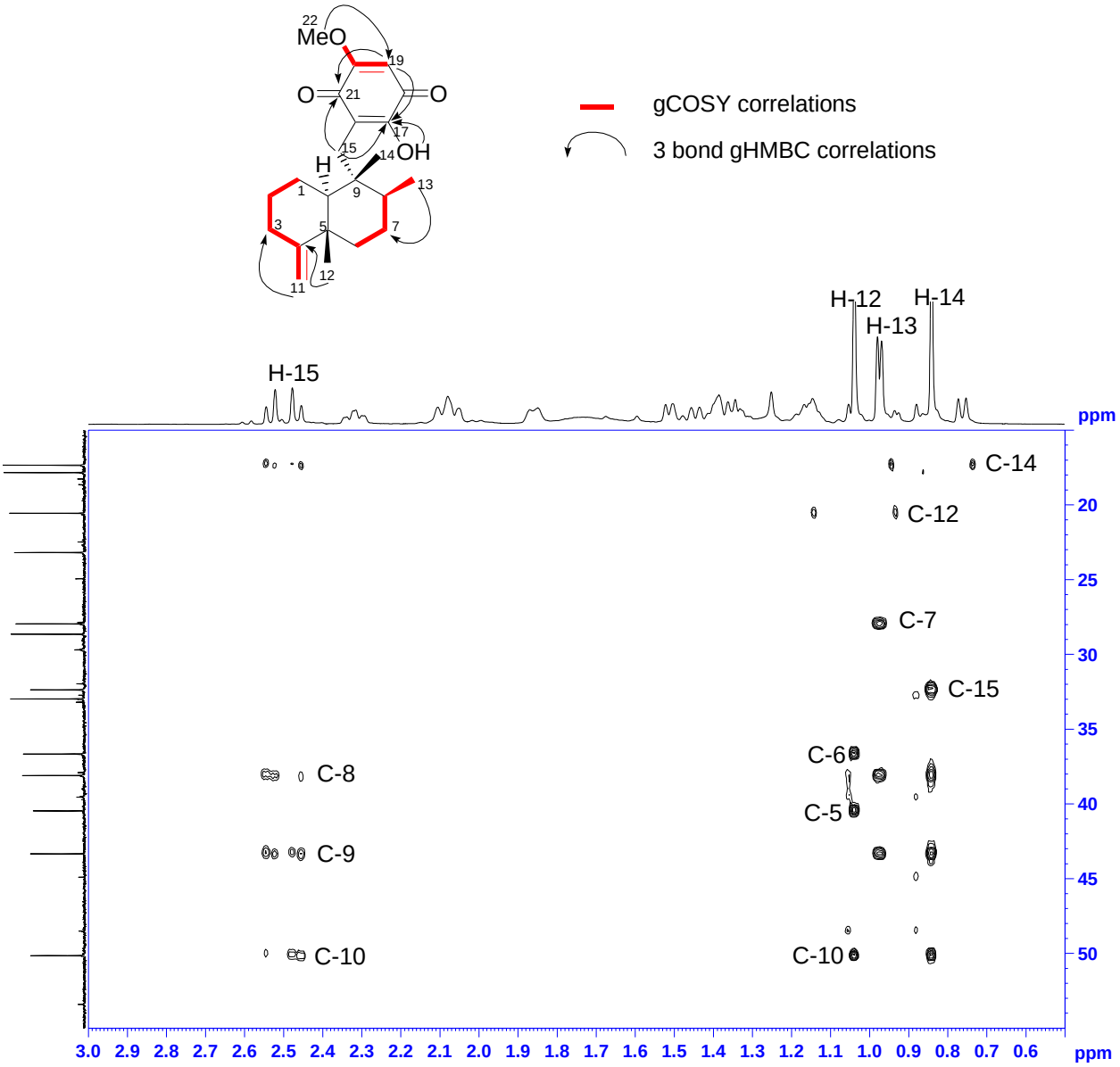


Figure 21: A region (F1 = δ_H 0.5-3.0 ppm; F2 = δ_C 15-55 ppm) of the gHMBC spectrum (600 MHz, $CDCl_3$) of **3.1** illustrating the five bond gHMBC correlations. The accompanying structure shows the key gHMBC and gCOSY correlations used to elucidate the structure of **3.1**.

Correlations observed in a two-dimensional gCOSY NMR experiment (Figure 21 and Table 4) were able to distinguish between methylene protons (H-2, δ_H 1.16) and (H-8, δ_H 1.14) through correlations to neighbouring protons (3H-1, δ_H 1.47, 2.08) (H-10, δ_H 0.76) and (H-13, δ_H 0.95) although the two signals appeared to overlap each other. Correlations from the methine proton (H-8, δ_H 1.14) through to the methyl protons (H-13, δ_H 0.95) were observed, while the methylene proton (H-2, δ_H 1.16) correlated to both methylene protons (2H-1, δ_H 1.47 and 2.08). Methylene protons (2H-6, δ_H 1.34 and 1.50) were confirmed

through correlations to the methylene (H-7, δ_H 1.36) which correlates to the methine and confirmed the ring structure in ilimaquinone. The methine proton (H-19, δ_H 5.83) was clearly isolated on the quinone moiety.

The gHMBC showed the correlations between the hydroxy protons (H-17, δ_H 7.50) of the quinone moiety and C-16, C-18 and C-19. The benzylic protons (2H-15, δ_H 2.44 and 2.51) correlated with C-16, C-17 and C-21, thus linking the quinone moiety to the rest of the molecule. The methoxy protons (H-22, δ_H 3.83) correlated to the isolated methine C-19. This methine proton (H-19, δ_H 5.83) correlated to the carbonyl C-21, confirming the structure of the quinone moiety. Methyl protons (H-14, δ_H 0.82) and (H-13, δ_H 0.95) correlated to both C-8 and C-9 indicating that they attached to neighbouring carbons. The methyl (H-12, δ_H 1.02) correlated to the quaternary carbon (C-4, δ_C 160.4) confirming the structure **3.1**

The methylene protons (2H-3, δ_H 2.06, 2.31) and (2H-6, δ_H 1.34 and 1.50) all correlated to the methyl proton (H-12, δ_H 1.02), while correlations were observed between methine protons H-2, δ_H 1.16) and (H-10, δ_H 0.76) in the NOESY. The NOESY also indicated that there is no correlation between the methine proton (H-10, δ_H 0.76) and the methylene proton (H-12, δ_H 1.02), implying that they were in different planes, which agrees with the above structure **3.1**.

As a final confirmation of the structure **3.1**, the ilimaquinone was acetylated by stirring this compound with pyridine and acetic anhydride (18 h) in the normal manner and purified on reverse phase analytical column 5:1 Hexane/Ethyl acetate. The ^{1}H and ^{13}C NMR (Figure 22) data and HREIMS mass data obtained for the synthetic ilimaquinone acetate **3.20** were identical to those obtained in literature.[142]

Table 4: ^{1}H (600 MHz, MeOD) and ^{13}C (150 MHz, MeOD) NMR spectra obtained for ilimaquinone **3.1**.

	Ilimaquinone			
Carbon	δ_C ppm	δ_H ppm (int., mult., *J*/Hz)	gHMBC	gCOSY
1	23.1	1.47 (1H, dd, 12.5, 3.0) 2.08 (1H, br d, 16.1)		H-2, H-3
2	28.6	1.16 (1H, m) 1.83 (1H, m)		H-1, H-10
3	33.0	2.06 (1H, d, 13.7) 2.31 (1H, td, 13.8, 5.3)		H-1, H-2, H-11
4	160.4	q		
5	40.4	q		
6	36.6	1.34 (1H, m) 1.50 (1H, dd, 9.6, 2.7)		
7	27.9	1.36 (2H, m)		
8	38.1	1.14 (1H, m)		H-13
9	43.3	q		
10	50.1	0.76 (1H, dd, 11.7, 2.0)		
11	102.5	4.42 (1H, s) 4.40 (1H, s)	C-3, C-4, C-5	H-3
12	20.5	1.02 (3H, s)	C-4, C-5, C-6, C-10	
13	17.8	0.95 (3H, d, 6.4)	C-7.C- 8. C-9	H-8
14	17.3	0.82 (3H, s)	C-8, C-9, C-10, C-15	
15	32.3	2.44 (1H, d, 13.7) 2.51 (1H, d, 13.7)	C-16, C-17, C-21	
16	117.3	q		
17	153.3	7.50 (1H, br s, 17-OH)	C-16, C-17, C-18, C-19	
18	182.3	q		
19	102.0	5.83 (1H, s)	C-17, C-20, C-21	H-22
20	161.7	q		
21	182.0	q		
22	56.8	3.83 (3H, s)	C-19, C-20	H-19

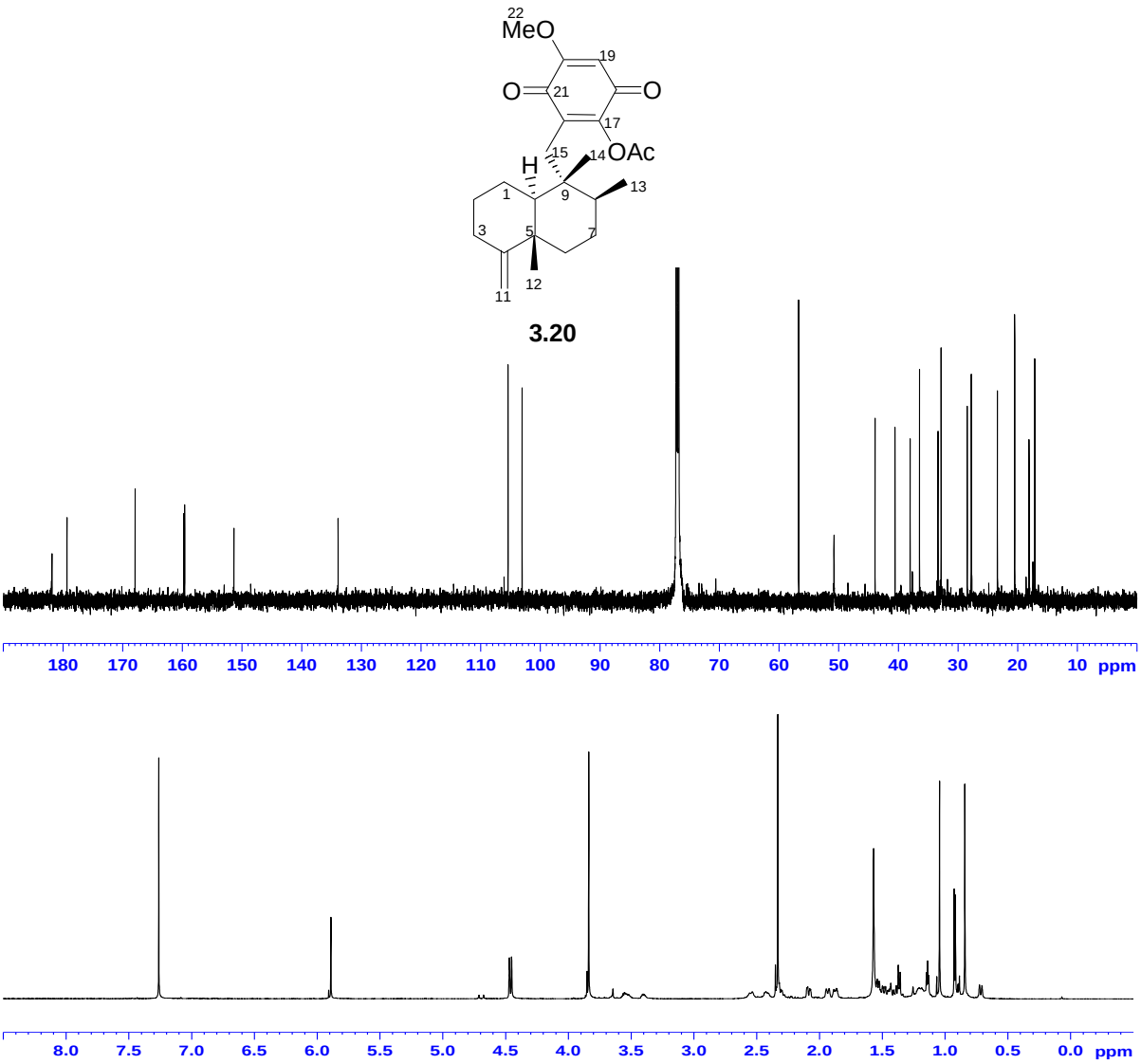


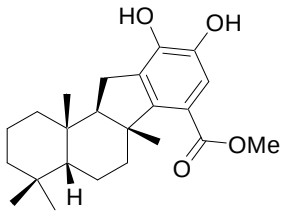
Figure 22: ^{1}H (600 MHz, $CDCl_3$) and ^{13}C (150 MHz, $CDCl_3$) NMR spectra obtained for **3.20**.

The molecular formula of **3.20** established from HREIMS data of $C_{24}H_{32}O_5$, a characteristic band at 1638 cm^{-1} of the carbonyl functionality in the IR spectrum and optical rotation data ($[\alpha]_D^{20}$ -6.2, lit.[150] -8.3), and the presence of the methyl signal (δ_H 2.32) in the ^{1}H NMR spectrum thus confirmed the synthetic product to be ilimaquinone acetate.

3.2 The isolation of pelorol

The molecular formula of pelorol (**3.19**) ($C_{23}H_{32}O_4$) was deduced from ^{13}C and ^{1}H NMR (Figure 23) and confirmed by HREIMS. The IR spectrum contained a broad band (ν_{max} 3415 cm^{-1}) consistent with the

presence of hydroxyl functionalities as well as an IR band in the carbonyl region (ν_{max} 1638 cm^{-1}). Both ^{13}C and ^{1}H NMR spectra indicated the presence of four methyl groups δ_H 1.21, 1.04, 0.85 and 0.83, a methoxy proton δ_H 3.83 as well as benzylic protons δ_H 2.48 and 2.51. The DEPT 135 indicated the presence of five methylene carbons δ_C 42.5, 40.2, 36.4, 19.6 and 18.4. The quaternary carbon signals at δ_C 168.2, 149.5, 143.5, 140.5, 130.0 118.1, 65.2, 37.2 and 33.1 and two methine signals at δ_C 65.2 and 57.1 in the ^{13}C NMR (Figure 25) and DEPT 135 spectra were also consistent with literature. Optical rotation data ($[\alpha]_D^{20}$ -67.8, lit. -69.4) unequivocally confirmed the structure.



3.19

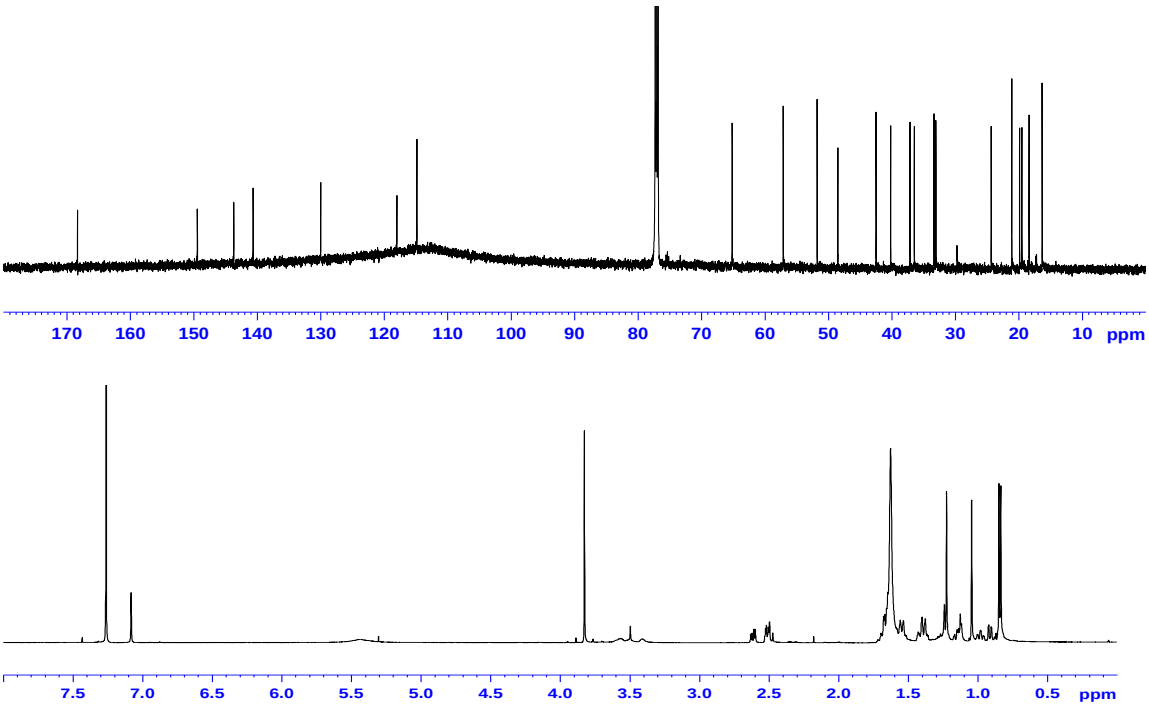


Figure 23: ^{1}H (600 MHz, MeOD) and ^{13}C (150 MHz, MeOD) NMR spectra obtained for pelorol **3.19**.

Correlations wereobserved in a two-dimensional gCOSY NMR experiment (Figure 24 and Table 5) between methine (H-5, δ_H 0.91), and methylenes (2H-6, δ_H 1.64-1.67 and 1.54). Benzylic protons of the pentane ring (2H-15, δ_H 2.48 and 2.51) correlated to the methine proton (H-9, δ_H 1.64-1.68), with a NOESY correlation observed (H-2, δ_H 1.64-1.67) and the methylene protons (2H-1, δ_H 0.98 and 1.54).

The gCOSY showed correlations from the methylene protons (2H-1, δ_H 0.98 and 1.54) to methylene protons (2H-2, δ_H 1.40, 1.64-1.68 and H-3, δ_H 1.14 and 1.40).

gHMBC correlations were observed between (H-11, δ_H 0.83) and C-5, C-4 and C-3, the correlation to C-3 being used to distinguish between overlapping methylene protons of C-3 and C-7. The benzylic protons of the pentane ring (2H-15, δ_H 2.48 and 2.51) correlated to the aromatic carbons C-16, C-17 and C-21 on one side as well as the carbons of the double ring structure C-9, C-10 and C-13.

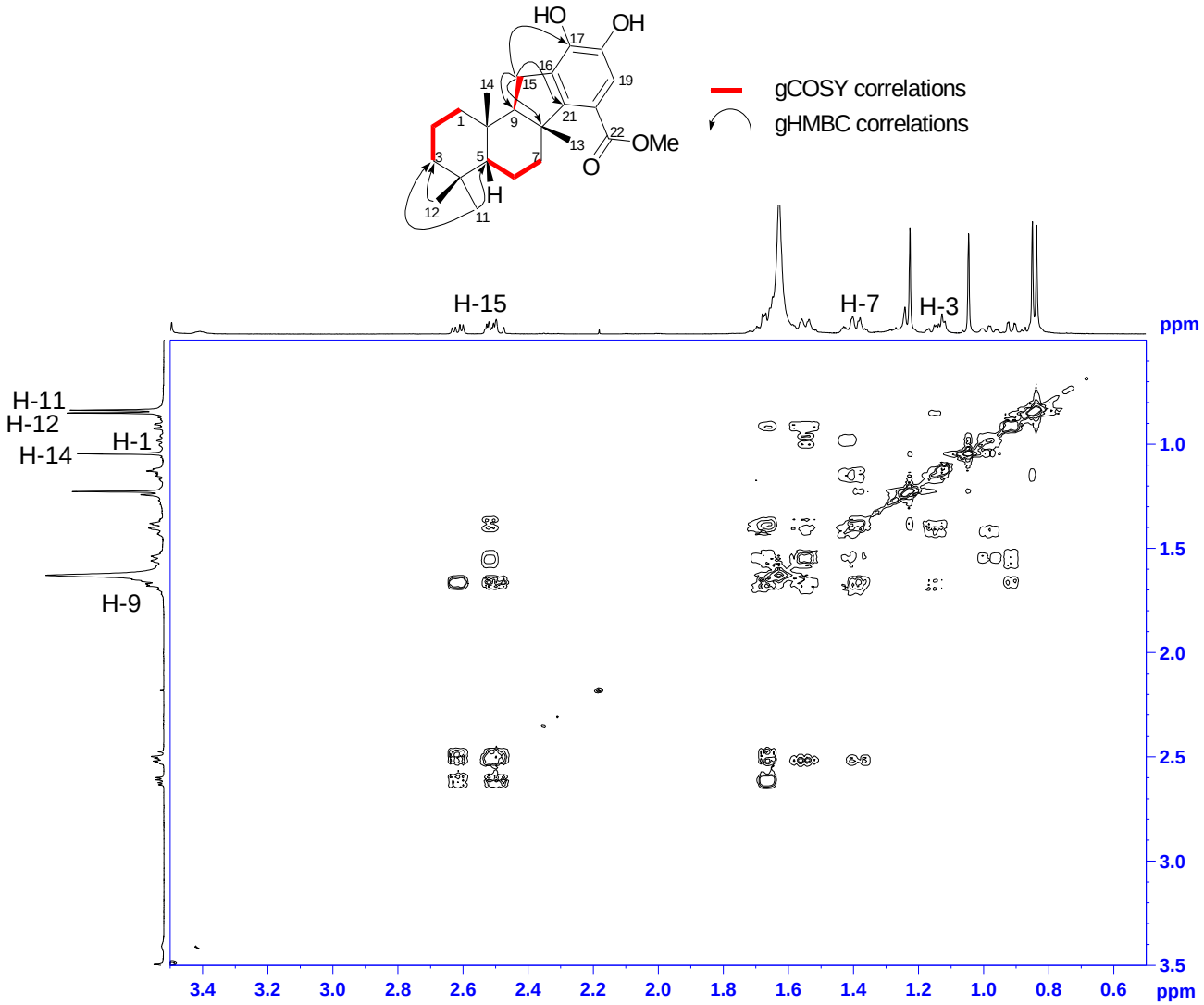


Figure 24: A region (F1 = δ_H 0.5-3.5 ppm) of the NOESY spectrum (600 MHz, $CDCl_3$) obtained for **3.19**. The accompanying structure illustrates the NOESY correlation used to determine the geometry of the isomer.

The four methyl groups (H-11, δ_H 0.83), (H-12, δ_H 0.85), (H-14, δ_H 1.04) and (H-13, δ_H 1.21) were used to assist in the determination of methylenes at positions (2H-7, δ_H 1.38) and (2H-3, δ_H 1.40 and 1.64-1.68). The benzylic protons of C-15 showed a NOESY correlation to two methyl protons (H-13, δ_H 1.21) and (H-

14, δ_H 1.04), which were deduced to be C-13 (δ_C 19.8) and C-14 (δ_C 16.3) respectively. The methyl protons of C-13 (H-13, δ_H 1.21) correlated to those of C-14 (H-14, δ_H 10.4) which correlated to those of C-12, which in turn correlated to those of C-11 (δ_C 33.3) which was more easily observed once the molecule was modeled and the structure further analyzed (Figure 25).

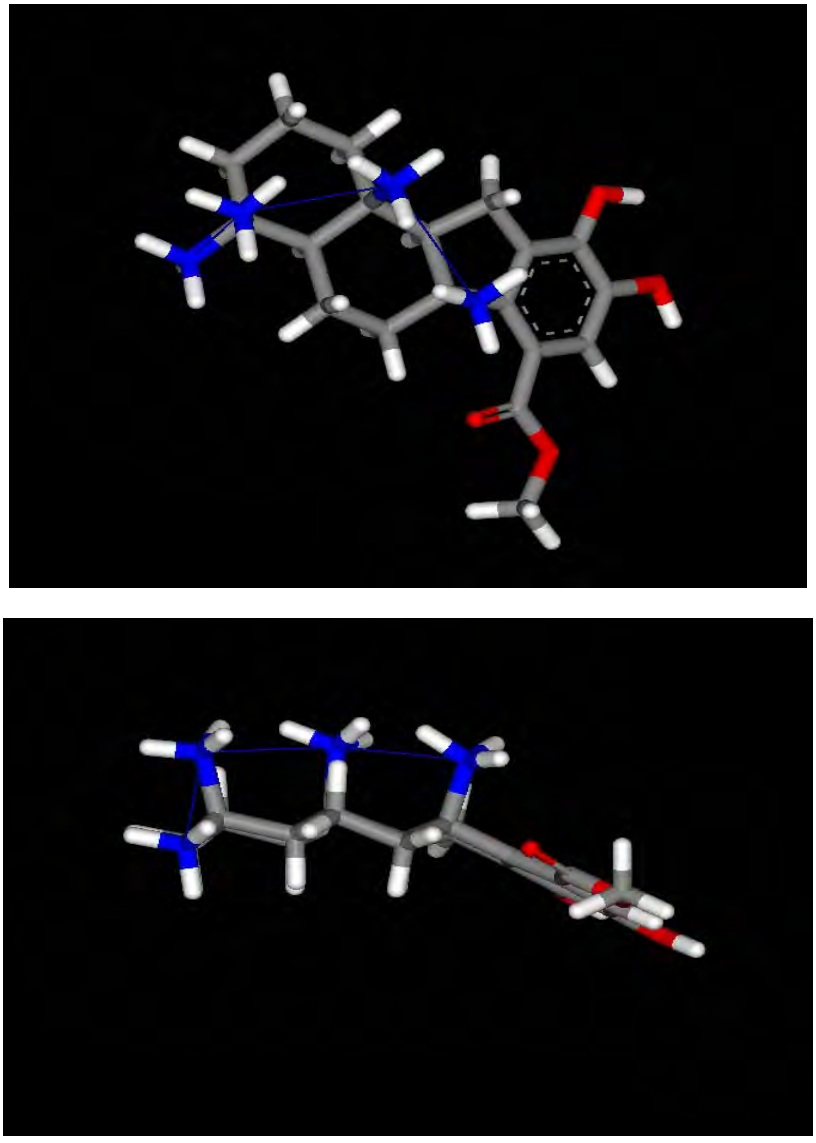


Figure 25: Stick representation of the computer-modelled conformation of pelorol **3.19** showing the NOESY correlations (blue) of protons of the methyl carbons (blue).

Table 5: ^{1}H (600 MHz, MeOD) and ^{13}C (150 MHz, MeOD) NMR spectra obtained for pelorol **3.19**.

Carbon	3.19		Literature [147]	
	δ_C ppm	δ_H ppm (int., mult., *J*/Hz)	δ_C ppm (mult.)	δ_H ppm (int., mult., *J*/Hz)
1	40.2	0.98 (1H, td, 13.2, 3.4) 1.54 (m)	40.1 (t)	0.98 (1H, td, 12.6, 3.0) 1.53 (1H, m)
2	18.4	1.40 (1H, m) 1.64-1.68 (1H, m)	19.5 (t)	1.55 (1H, m) 1.65 (1H, m)
3	42.5	1.14 (1H, td, 13.7, 4.1) 1.40 (1H, m)	42.4 (t)	1.15 (1H, td 13.8, 4.8) 1.38 (1H, m)
4	33.1	q	33.0 (s)	q
5	57.1	0.91 (1H, dd, 12.3, 2.4)	57.1 (d)	0.92 (1H, dd, 12.6, 2.4)
6	19.6	1.54 (1H, m) 1.64-1.68 (m)	18.3 (t)	1.40 (1H, m) 1.68 (1H, m)
7	36.4	1.38 (2H, m)	36.5 (t)	1.38 (2H, m)
8	48.5	q	48.5 (s)	q
9	65.2	1.64- 1.68 (1H, m)	65.1 (d)	1.65 (1H, m)
10	37.2	q	37.1 (s)	q
11	33.3	0.83 (3H, s)	21.1 (q)	0.86 (3H, s)
12	21.1	0.85 (3H, s)	33.3 (q)	0.84 (3H, s)
13	19.8	1.21 (3H, s)	19.8 (q)	1.22 (3H, s)
14	16.3	1.04 (3H, s)	16.3 (q)	1.05 (3H, s)
15	24.3	2.48 (1H, m) 2.51 (1H, dd, 14.3, 6.2)	24.3 (t)	2.48 (1H, m) 2.52 (1H, dd, 14.0, 5.5)
16	130.0	q	130.0 (s)	q
17	143.5	q	143.7 (s)	q
18	140.5	q	140.6 (s)	q
19	114.9	7.08 (1H, s)	114.8 (d)	7.09 (1H, br s)
20	118.1	q	117.8 (s)	q
21	149.5	q	149.3 (s)	q
22	168.2	q	168.6 (s)	q
23	51.7	3.83 (3H, s)	51.8 (q)	3.83 (3H, s)

CHAPTER 4
BIOASSAYS

4. 1 Feeding assay

Although many predators feed on a variety of chemically defended prey, their feeding choices are based on the nutritional value of prey, as well as the presence of stimulants and deterrents. Organisms produce several secondary metabolites, some of which act as chemical deterrents and therefore, have an effect on the feeding preferences of their prey.[150] Although there are no known predators of sea hares, the sea hares posses the ability to defend themselves through a variety of mechanisms. The ink produced by numerous species of sea hares have been described as a form of chemical defence against potential predators, as in the case of *Aplysia californica* and its predator the sea anemone *Anthopleura sola* and the spiny lobster, *Panulirus interrupts*. The ink is associated with sensory disruption, unpalatability and phagomimicry.[151,152]

A major hurdle in setting up ecologically relevant bioassays to explore chemical defence strategies in marine invertebrates is identifying suitable predatory species that are representative of possible predators of the chemical defence producing organism. In addition the test predator must be able to survive in a laboratory aquarium and be amenable to either taking regular food pellets or being manipulated during the assay.

Bursatellin isolated from Kariega Estuary specimens of *B. leachii* was tested in bioassays to determine whether it played a role in chemical defence of the organism. The compound was tested in a fish feeding assay, amphipod feeding assay as well as microbial assays.

4.1.1 Feeding pellet formulation

Feeding pellets were formulated with the ingredients listed in Table 6 and three concentrations of each of bursatellin and ilimaquinone respectively (0.0125%, 0.025%, 0.25% and 0.085%, 0.17% and 1.7%) were added to the wet mixture. Based on the chromatographic isolations which yielded each compound, we predicted that **2.2** and **3.1** were present in concentrations of ~ 0.25% and ~ 0.17% of the dried mass of the sea hare and sponge respectively. We used this assumption to prepare pellets containing half estimated natural concentration, full estimated natural concentration and ten times the estimated natural concentration. Varying the concentration of either **2.2** or **3.1** did not appear to change the colour or consistency of the pellets (Figure 26).

Figure 26: Various concentrations of bursatellin and ilimaquinone containing pellets.

Table 6: Composition of feeding pellets used in fish and amphipod assays.

Ingredients	**%**	**Mass (g)**
Fishmeal	44.574	222.87
Soya	10.722	53.61
Starch (maize)	39.334	196.67
Fish oil	5.258	26.29
Vitamix	0.11	0.55
Water		
TOTAL	100	500

4.1.2 Fish feeding assay

Two species of fish were chosen as representative predators of the sea hare *B. leachii*, namely *Oreochromis mossambicus* (order Perciformes, family Cichlidae) more commonly known as Mozambique tilapia, and *Caffragobius gilchristi* (order Perciformes, family Gobiidae) a hardy intertidal rock pool marine fish. Both predatory fish are found along the length of the Kariega Estuary and were considered suitable predators for this study. *C. gilchristi* was deemed the more ecologically relevant species as it is generally found in close proximity to the sea hare.

Both fish species were held separately in large aerated tanks with a filtering system at 25 °C. The feeding behaviour of both fish species was monitored and compared. The *O. mossambicus* were obtained from a local hatchery while the gobies were caught in rock pools at the mouth of the Kariega Estuary. Initially, both fish species were fed on commercially available fish food during an acclimation period of 2-3 weeks. The *O. mossambicus* learnt to feed on the pellets faster than the gobies as they were raised on a pellet diet, while the wild gobies took a period of five to seven days to associate the pellets with food. After this acclimation period, the fish immediately associated the presence of a hand at the surface of the water with food (Figure 27) and altered their behavior to feed readily when this occurred. The fish were then randomly assigned to individual tanks and left to acclimate again for another three days. Control and treatment diets were then randomly assigned to tanks and the observation of behavioral patterns and feeding commensed. Each fish was offered two pellets and observations over a three minute period.

Figure 27: Oreochromis *mossambicus* during the acclimation period (top left) and feeding of commercially available fish pellets during the acclimation period (top right) and *Caffragobius gilchristi* (bottom).

Ilimaquinone was used as a standard test compound against bursatellin, as it is a known feeding deterrent in the marine environment,[78] although the concentrations at which it deters predation are unknown.

All commercially manufactured pellets were eaten within the first minute during the acclimation period and therefore, a set time of three minutes was used for the feeding observations. Control pellets, half and full concentration pellets of bursatellin and ilimaquinone were all consumed by *Oreochromis mossambicus* immediately, while the tenfold concentrations of both bursatellin and ilimaquinone deterred feeding. There was a difference observed between the high bursatellin concentrations of pellets compared with that of the control pellets (Table 7). In three of the five tanks of bursatellin treatment, the fish took a pellet, but within less than ten seconds the pellet had been regurgitated and no pellets were eaten from that point onward. Ilimaquinone treatments showed similar patterns to that observed in bursatellin, whereby fish did not attempt to feed on the pellets of ten times the isolated concentration, nor did their behaviour change as a result of the detection of a food stimulus (Table 7).

Table 7: Number of pellets eaten by each fish (*Oreochromis mossambicus*) at the varying concentrations of bursatellin and ilimaquinone respectively.

	Number of pellets fed to each fish	Bursatellin % composition in the pellet				Ilimaquinone % composition in the pellet			
Fish		Control	0.0125	0.025	0.25	Control	0.085	0.17	1.7
1	2	2	2	2	0	2	2	2	0
2	2	2	2	2	0	2	2	2	0
3	2	2	2	2	0	2	2	2	0
4	2	2	2	2	0	2	2	2	0
5	2	2	2	2	0	2	2	2	0

4.1.4 Testing pellet concentrations

As a quality assurance measure it was deemed necessary to assay the concentration of both bursatellin and ilimaquinone in the prepared pellets. Ultra violet absorbance readings were recorded at the three concentrations of both ilimaquinone and bursatellin were used to determine the actual concentration of compounds present within the prepared pellets. The arrow indicates the decrease in absorption with subsequent serial dilution (Figures 28 and 29). The direct correlation between absorbance and concentration, allowed for a calibration curve to be estimated and therefore used to determine the concentration of bursatellin within the pellets, through absorption measurements.

Interestingly, it was found that the actual concentrations present within the prepared pellets were approximately tenfold lower than what was expected. This finding may explain why only the “ten times the natural concentration” was sufficient to deter the fish, as this is comparatively similar to that of the isolated concentrations found within the organisms, as observed from testing the pellet concentrations.

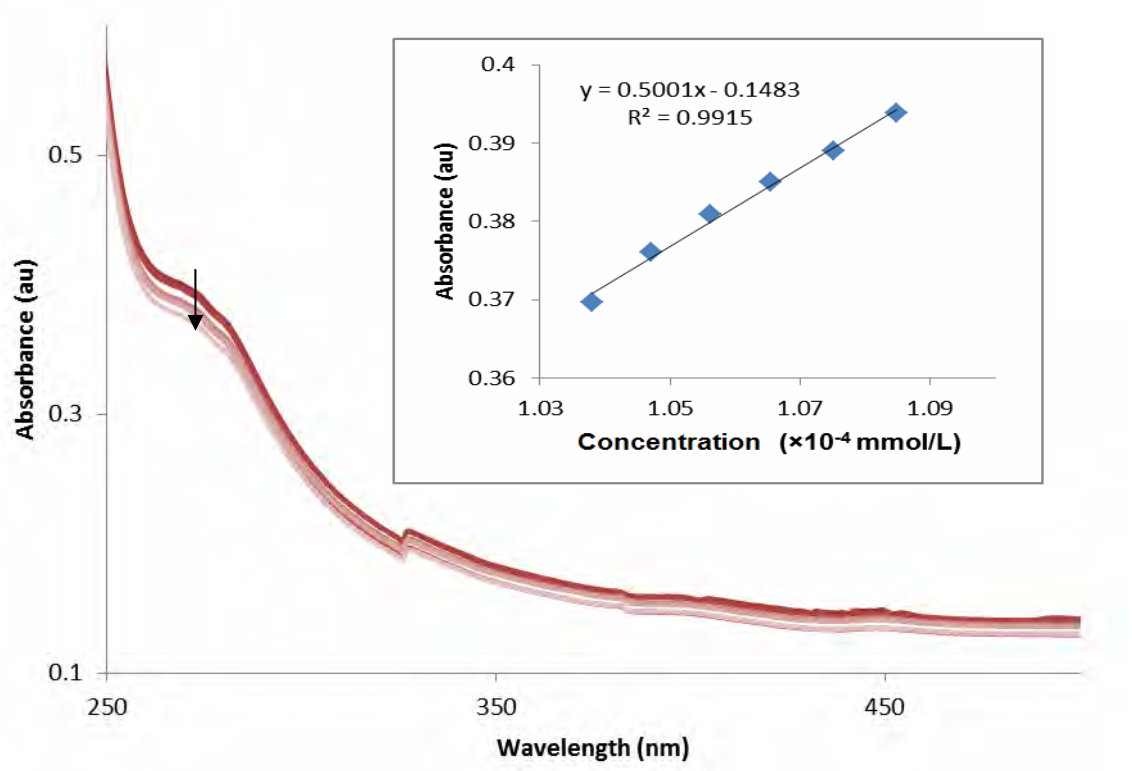


Figure 28: UV absorbance spectra of bursatellin at various concentrations.

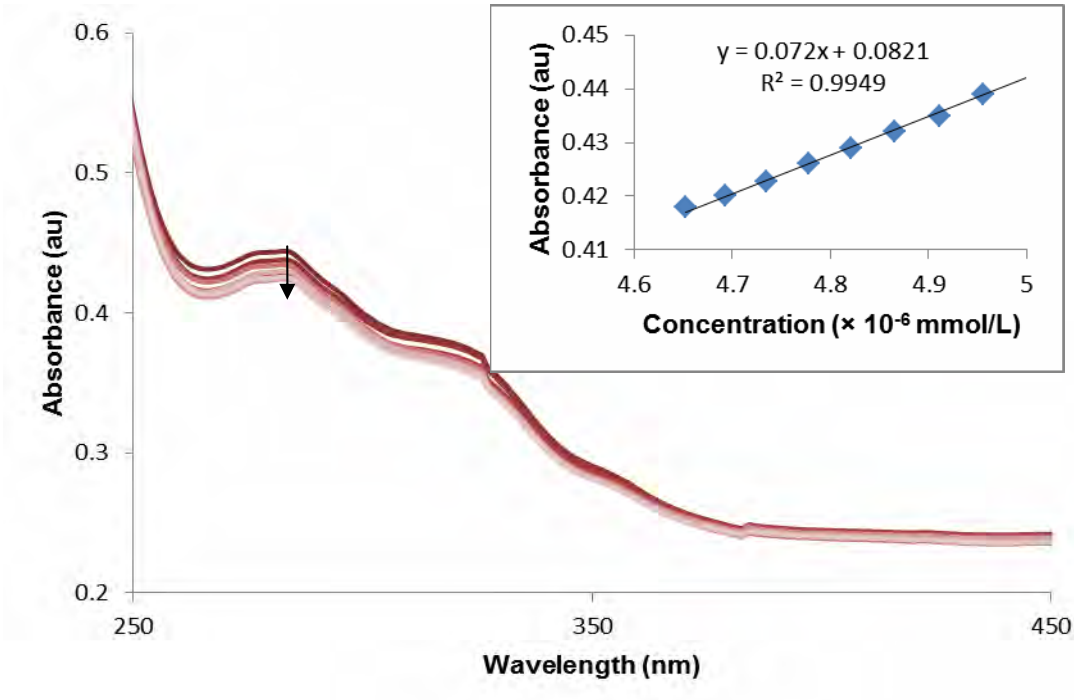


Figure 29: UV absorbance spectra of ilimaquinone at various concentrations.

Interestingly, bursatellin was shown to fluoresce (Figure 31). The fluorescence may be attributed to the aromaticity of this compound and the presence of an electron donating group on the aromatic ring. This may be used as an alternative spectroscopic technique to assay the prepared pellets. Ilimaquinone does not fluoresce.

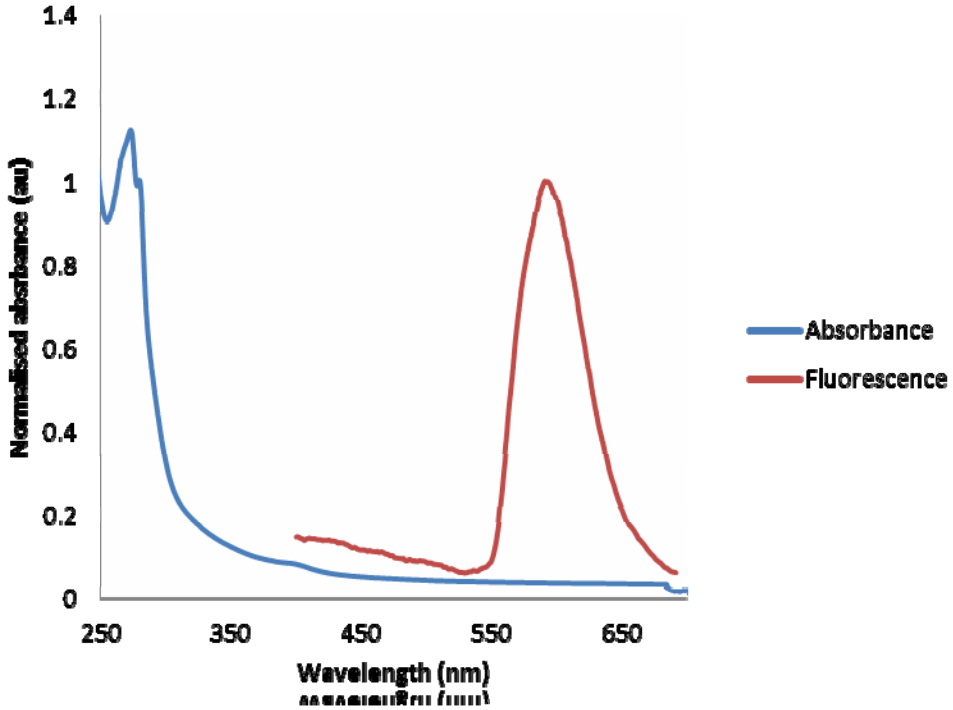


Figure 30: The normalized absorbance spectrum of fluorescence of bursatellin **2.2**.

4.2 Microbial Assay

One of the oldest commercially available anti-bacterial agents is chloroamphenicol which was first isolated from *Streptomyces venezuelae* in 1947.[153] This antibiotic is obtained commercially by chemical synthesis and is biologically active only as its 2*R*, 3*R* enantiomer. It is used clinically as a broad spectrum antibiotic and is particularly useful for the treatment of *Salmonella typhi*, *Rickettsia* and *meningeal* infections, and therefore, is a pharmacologically important natural product.[153] As a result of its close association to bone marrow depression, its use is reserved for serious infections with drug resistant microbes that have been shown to be resistant to all other appropriate anti-microbial agents. Chloramphenicol's mode of action specifically inhibits the synthesis of bacterial protein without affecting other metabolic processes. This property is dependent on the specific configuration and conformation of the molecule.[154]

The chemical structure of bursatellin resembles that of chloramphenicol and therefore, the antibacterial activity of **2.2** was tested and compared to that of chloramphenicol **2.3**. Various concentrations of bursatellin and chloramphenicol were tested against six bacterial strain and two fungal strains (Table 8).

Table 8: Microorganisms and conditions under which they were grown and maintained**.**

Microorganism Test strain	Gram Stain	Morphology	Incubation Temperature	Agar
Bacteria				
Klebsiella pneumoniae	-	Rod	30	Nutrient agar
Pseudomonas sp.	-	Rod	30	Nutrient agar
Serratia marcescens	-	Rod	30	Nutrient agar
Escherichia coli	-	Rod	37	Nutrient agar
Bacillus subtilis	+	Rod, spores	37	Nutrient agar
Staphylococus aureus	+	Coccus	37	Nutrient agar
Fungus				
Fusarium oxysporum			30	Potato dextrose agar
Phytophthora nicotinae			30	Potato dextrose agar

Bacteria can be classified into two large groups based on the empirical method of differentiation based on their morphological and phylogenic characteristics, namely gram positive and gram negative.[155] This method of differentiation developed by Hans Christian Gram allows bacteria to be separated on the cell wall character, with the gram-positive bacteria having a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stain purple while gram-negative bacteria have a thinner cell wall (10% of cell wall), which stain pink.[156] The most common shapes and cell arrangements associated with bacteria are spheres (cocci), rods and spiral shapes.[155]

Escherichia coli are gram-negative, facultative anaerobic, rod-shaped bacteria which live in the intestines of warm-blooded organisms. Although most *E. coli* bacteria are regarded as not harmful, a few strains can cause gastroenteritis. Pathogenic *E. coli bacteria* are usually transmitted through contaminated food and water.[157]

Klebsiella pneumonia, commonly known as Friedlander's bacillus is a small, facultative anaerobic, gram-negative coccobacilli. This bacterium does not form spores, instead it forms capsules. This common bacterium causes urinary tract infections, nosocomial pneumonia, and intraabdominal infections which causes necrotizing lobar pneumonia.[158]

The *Pseudomonas sp.* is a gram-negative rod-shaped saprotrophic soil, bacteria that utilizes aerobic metabolism. The multiple polar flagella are used for motility to propel towards the seeds of the plants which provides nutrients to the bacterial cells.[159] The bacteria demonstrate a very diverse metabolism, including the ability to degrade organic solvents such as toluene. This ability has been put to use in

bioremediation, or the use of microorganisms to biodegrade oil.[160] Use of *P. putida* is preferable to some other *Pseudomonas* species, capable of such degradation as it is a safe bacterial species, unlike *P. aeruginosa*which is an opportunistic human pathogen.[161]

Serratia marcescens is a motile, short rod-shaped, gram-negative, facultative anaerobe bacterium, classified as an opportunistic pathogen. A human pathogen, *S. marcescens* is involved in nosocomial infections, particularly catheter-associated bacteremia, urinary tract infections and wound infections.[162]

Bacillus subtilis is a gram-positive, rod-shaped and endospore-forming aerobic bacterium. It is found in soil and rotting plant material and is non-pathogenic. The ability of *B. subtilis* to differentiate and form endospores, has attracted much interest, and this is why it is one of the most studied gram-positive bacteria.[163]

Staphylococcus aureus is a gram-positive, coccus-shaped facultative anaerobe. S. aureus colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales, including the skin, oral cavity and gastrointestinal tract. *S. aureus* is not always pathogenic although it is a common cause of skin infections, respiratory disease, and food poisoning.[164]

Fungi are most commonly classified into four divisions: the Chytridiomycota (chytrids), Zygomycota (bread molds), Ascomycota (yeasts and sac fungi), and the Basidiomycota (club fungi). The shape and internal structure of the sporangia, which produce the spores, are the most useful character for identifying these various major groups, which is based on the sexual reproduction of the fungus.[165]

Fusarium oxysporum is a member of Ascomycota. *F. oxysporum* strains are ubiquitous soil inhabitants that have the ability to exist as saprophytes, and degrade lignin and complex carbohydrates associated with soil debris. They are also pervasive plant endophytes that can colonize plant roots and may even protect plants or be the basis of disease suppression.[86]

Phytophthora nicotinae is a member of Oomycota. *Phytophthora nicotianae* has a very wide host range and occurs on many plant species. Two spore types are produces namely sporangia, which are asexual spores[166] and chlamydospores, with no release of zoospores. Sporangia also produce germ tubes that penetrate the host epidermis and begin the disease process. Chlamydospores are produced on the inside of diseased leaves and stems, which fall to the ground.[151]

The six bacterial strains were grown aerobically at either 30 °C or 37 °C (Table 8) in nutrient broth prepared according to the manufacturer's specifications. The bacterial broth was shaken up and the optical density observed and adjusted with nutrient broth to 0.8-1.0 in order to yield inoculums of ~104 to 105 CFU (colony forming unit)/mL). 250 µL of bacteria broth was inoculated onto nutrient agar plates and evenly spread across the plate. Discs concentrated (15 µL) with varying concentrations (0.25, 0.5, 1, and

2 mg/mL) of bursatellin were placed on the bacterial mat and left incubated at 30°C for 18 h and then observed. Bacterial growth was monitored and recorded (Figure 32).

The two fungal strains were grown up on potato dextrose agar (PDA), and inoculated with a 5 mm diameter core of fungal subculture in the centre of the agar plate and incubated for two days to allow for initial mycelial growth on the plate. Discs of varying concentrations of bursatellin and chloramphenicol were placed on the plates and incubated for 18 h. Refer to Section 5.4.2.2 for further details. The zone of inhibition was visually qualitatively analyzed for both bacterial and fungal strains (Figure 33).

Zone of Inhibition testing is a fast, qualitative means to measure the ability of an antimicrobial agent to inhibit the growth of microorganisms and qualitatively test the ability of antimicrobial agents to inhibit the growth of microorganisms over an 18-24 hour period of contact.[167] This test is a very useful assay for getting quick measurements of how much general antimicrobial activity there is in a sample. The bacterial growth area is opaque, with only areas around the discs with a high enough concentration of the antimicrobial substances having a clearance zone, free from bacterial growth. The area of the zone of inhibition correlates to the level of antimicrobial activity present in the test compound, sample, with a larger area of inhibition implying that the antimicrobial agent is more potent.[167] Although it is quick and simple, occasionally the zone of inhibition is not always clear and obvious.

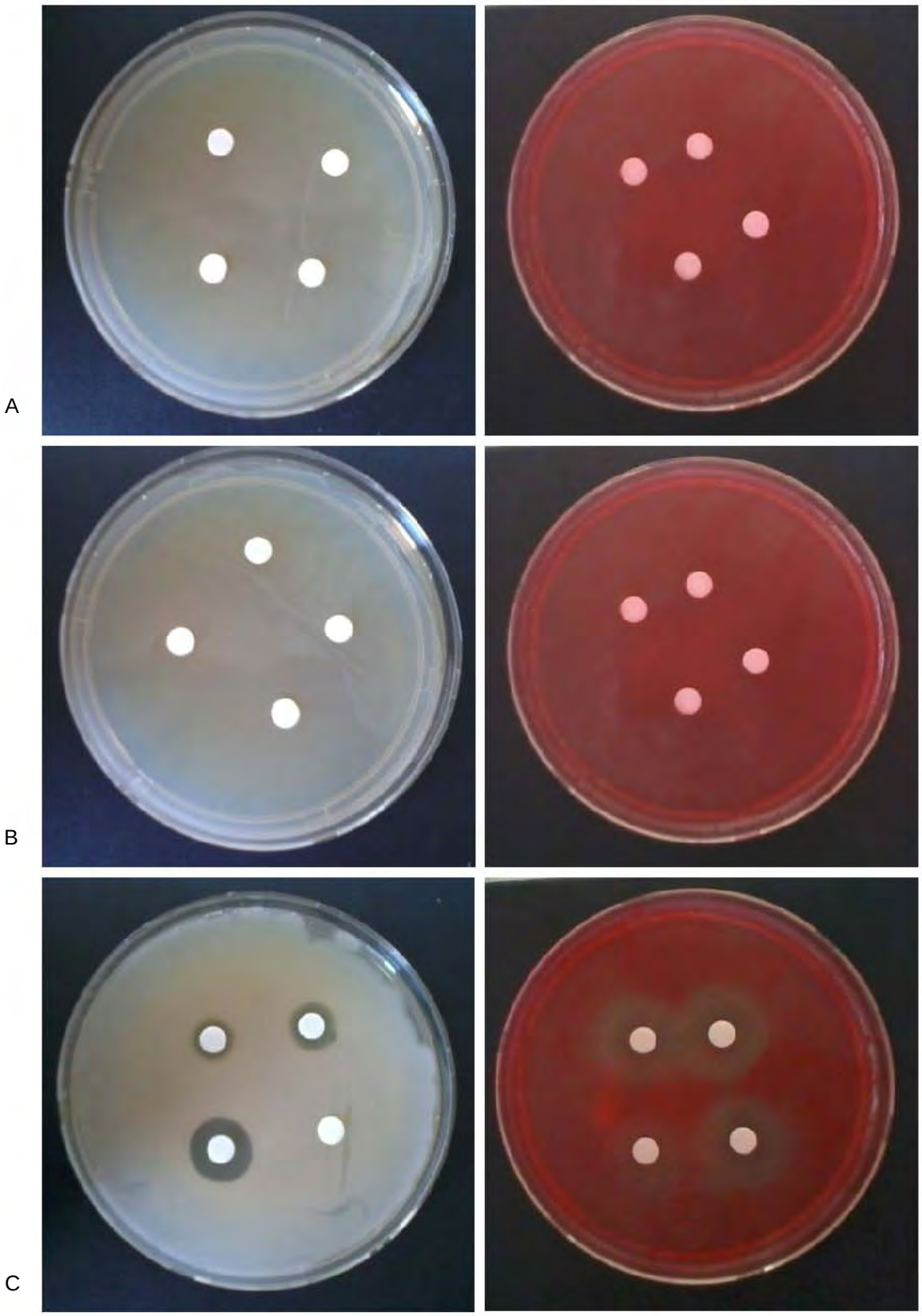


Figure 31: Nutrient agar plates with bacterial growth (A) control, (B) bursatellin and (C) chloramphenicol treatments.

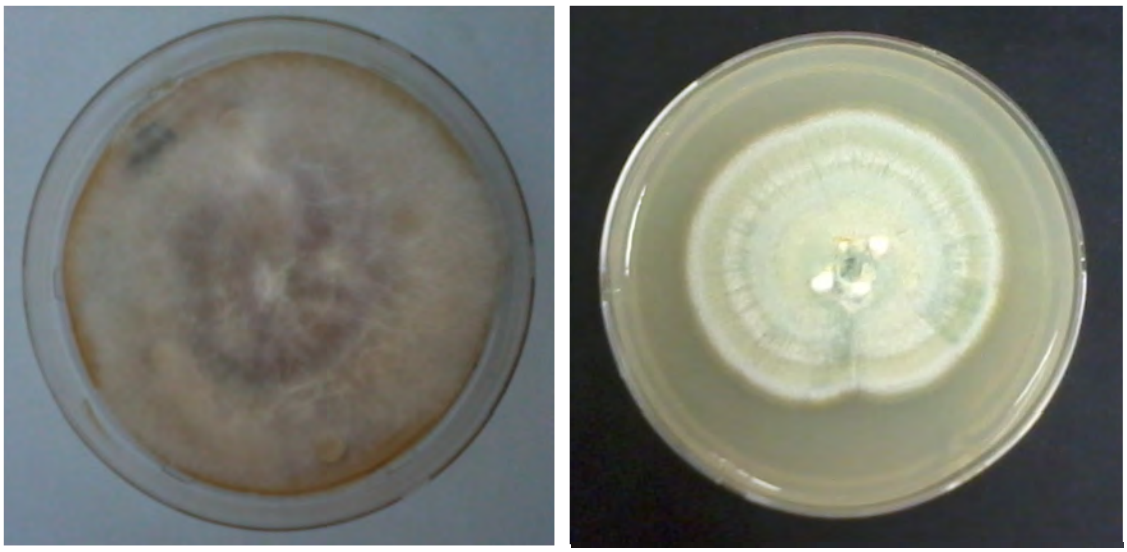


Figure 32: Potato dextrose agar with *F. oxysporum* (left) and *P. nicotinae* fungal growth across the plate.

Table 9: Comparative results of the antibacterial activity of bursatellin and chloramphenicol at varying concentrations with the strength of activity indicated by the symbols (- no activity, + activity).

Test Strain	Results						
Bacteria	Bursatellin Concentrations (mg/mL)				Chloramphenicol Concentrations (mg/mL)		
	0.25	0.5	1	2	0.25	0.5	1
Klebsiella pneumoniae	-	-	-	-	++	+++	++++
Pseudomonas putida	-	-	-	-	++	+++	++++
Serratia marcescens	-	-	-	-	++	+++	++++
Escherichia coli	-	-	-	-	++	+++	++++
Bacillus subtilis	-	-	-	-	++	+++	++++
Staphylococus aureus	-	-	-	+	++	+++	++++
Fungus							
Fusarium oxysporum	-	-	-	-	-	-	-
Phytophthora nicotinae	-	-	-	-	-	-	-

Results of the microbial assays indicated that at low (< 1mg/mL) concentrations of bursatellin, there was no evidence of antimicrobial activity. Indeed, bursatellin antibacterial activity for the *S. aureus* bacterial strain was only recorded at the highest concentration tested (2mg/mL). In comparison, chloramphenicol, a well known antimicrobial agent, showed antimicrobial activity at all the concentrations tested (Table 9). Both bursatellin and chloramphenicol, demonstrated no anti-fungal activity at all concentrations tested.

4.3 Discussion and concluding remarks

The results of the various assays, suggest that at low concentrations (<0.25% dry body mass) bursatellin offers no apparent chemical defence although at much higher concentrations it appears to deter the feeding of fish and inhibits the growth of *Staphylococus aureus*. It is worth noting that the bursatellin concentration in the pellets may have degraded/oxidized, thus results obtained at the lower concentrations may be subject to experimental error. Unfortunately there is no data available to test this hypothesis. The isolated absolute concentrations of bursatellin obtained from the sea hare tissue during this study are like to be underestimates as there were several possible potential sources of error. Upon collection of the sea hares from the estuary, they produced large quantities of a mixture of ink and other mucus substances. The excreted mixture dispersed into the surrounding water. The concentrations of bursatellin found to be present within the organism in the laboratory is thus most likely to be much lower than the actual concentration present within the organism under normal, "non-stressed" conditions. This may account for the fact that only at the highest concentrations did bursatellin show some form of defence against potential fish predators. In addition secondary metabolites often adhere to the chromatographic media and not all the secondary metabolite present in an extract is isolated during chromatography. Isolated yields from chromatography are therefore generally lower than naturally occurring yields.[168] It should be pointed out, however that to fully investigate the potential of bursatellin as a chemical defence strategy for the sea hare, further feeding assays with different predator invertebrates and vertebrates need to be conducted.

Sea hares and other macrobenthic organisms play an important role in linking benthic and pelagic systems. The distribution of benthos within the estuaries is dependent on a variety of factors including habitat availability, physical factors such as temperature and salinity as well as biological factors such as competition, food availability and predation.[169] *Bursatella leachii* are usually found within shallow waters, closely associated with the seagrass, *Nanozostera capensis.*[169] A study of the macrobenthic community of the Kariega Estuary carried out by Hodgson (1987), demonstrated that the high levels of macrobenthic diversity within the littoral zone of the system were a result of the presence of extensive beds of the eelgrass, *Zostera capensis.* The sea grass beds are thought to provide a refuge against predation and are areas of increased food availability. The eelgrass beds may be of particular importance as a refuge area for juvenile *B. leachii* as they do not appear to have bursatellin in their tissues at concentrations which may deter predators. The absence of bursatellin from the juvenile implies that the juveniles feed on a different diet to that of the adults and therefore the bursatellin is not derived from the diet. Bursatellin was only isolated from the body tissues of adult specimens, which suggests that the bursatellin is acquired from the diet of cyanobacteria, and that possibly the cyanobacteria is producing this natural product, after an as yet undetermined development stage of the sea hare, and that possibly, mortality rates of juveniles are much higher than those of the adults.

CHAPTER 5
EXPERIMENTAL PROCEDURES

5.1 General Experimental Procedures

5.1.1 Analytical

NMR spectra were acquired using standard pulse sequences on Bruker Avance 600 MHz Avance II spectrometers. Chemical shifts are reported in ppm and referenced to residual solvent resonances ($CDCl_3$ δ_H 7.26, δ_C 77.0, CD_3OD δ_H 3.31 δ_C 49.9). Coupling constants are reported directly from the NMR spectra and corresponding coupling constants have not been matched. Optical rotations were measured on a Perkin-Elmer 141 polarimeter at the sodium-D line (589 nm). Following standard protocol, the concentration of solutions used to determine optical rotations is expressed in g/100 mL. Infra-red spectra were recorded on a Perkin-Elmer Spectrum 2000 FT-IR spectrometer and Digilab FTS 3100 Excalibur HE Series with compounds as films (neat) on KBr discs. Mass spectrometry was performed on a Waters API Q-TOF Ultima instrument using electron-spray ionization in the positive ion mode (ESI+) at the University of Stellenbosch Central Analytical Facility.

5.1.2 Chromatography

General laboratory solvents were distilled from glass before use. Analytical normal phase thin layer chromatography was performed on DC-Plastikfolien Kieselgel 60 F_{254} plates. Plates were viewed under UV light (254 nm) and developed by spraying with 10% H_2SO_4 in MeOH followed by heating. HP-20 beads used were manufactured by Diaion and supplied by Supelco. Open column chromatography was performed using Discovery® DSC-DIOL supplied by Supelco, and flash chromatography was performed using Kieselgel 60 (230-400 mesh) silica gel. Normal phase DIOL semi preparative HPLC separations were performed on a Machery-Nagel VP 250/10 Nucleosil 100-7 OH column using an Agilent 1100 Series quad pump and a Agilent 1100 diode array detector. Reverse phase semi-preparative HPLC separations were performed on a Phenomenex Onyx Monolithic Semi-PREP C-18 column using an Agilent 1100 Series quant pump and a Agilent 1100 diode array detector.

5.1.3 Synthesis

All reactions requiring anhydrous conditions were conducted in either flame-dried or oven-dried apparatus under an atmosphere of dry argon/nitrogen or using an anhydrous calcium chloride drying tube. Dry solvents were prepared by standard procedures as described by Perin and Armarego[170] and stored over appropriate drying agent under an atmosphere of dry nitrogen. Pyridine and dichloromethane were distilled at reduced pressure from potassium hydroxide and calcium hydride respectively and stored over 4Å molecular sieves under nitrogen. Organic extracts were dried over anhydrous $MgSO_4$ or $NaSO_4$. All reactions were magnetically stirred.

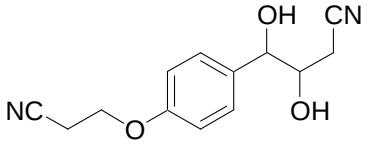
5.1.4 Computer modelling

Theoretical models were constructed and subjected to structural optimization using DFT methods, followed by full frequency analyses to confirm that the final structures were true stationary points. All DFT calculations (energy optimizations and frequency analyses) were performed using the Gaussian 03[171] suite of algorithms with the 6-31G (d) vector basis set and the B3LYP[172] energy gradient correcting functional. Models were constructed and visualized using DS Visualizer.[173]

5.2 Chapter Two Experimental Procedures

5.2.1 Extraction and Isolation of bursatellin from the sea hare *Bursatella leachii*

The sea hares were immediately frozen after collection and later freeze-dried. All of the freeze-dried material was extracted with MeOH to give a brown gum (8.40 g), which was passed through HP-20 yielding bursatellin from the 40% MeOH $_{(aq)}$ fraction (**2.2**, 55.2 mg) as an orange oil. The specimens were then soaked three times in methanol, sonicated and solvent filtered off, and the three methanol fractions were combined and subjected to cyclic loading onto a column of HP-20 beads until an eluent of concentration 12.5% MeOH/H_2O was achieved. The organs obtained from dissected specimens of *B. leachii* were steeped in methanol three times with sonication. The combined methanol extracts were filtered and separately loaded onto individual HP20-resin columns**.** Aqueous methanol fractions 20%, 40%, 60, 80 and 100% methanol were eluted from the column for each of the different organs.

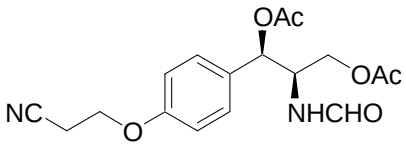


2.1

Bursatellin (2.2) oil, $[\alpha]_D^{22}$ –5.0° (*c* 0.52, $CHCl_3$), lit [133]. –8.8; IR (film) ν_{max} 3414, 3413, 2230, 2930, 1638, 1515, 1283, 1198, 1012 cm^{-1}; ^1H and ^{13}C NMR data see Table 2; HREIMS *m/z* (calcd for $C_{22}H_{30}O_4$ [M^++ Na] (287.0923).

5.2.2 Acetylation of bursatellin

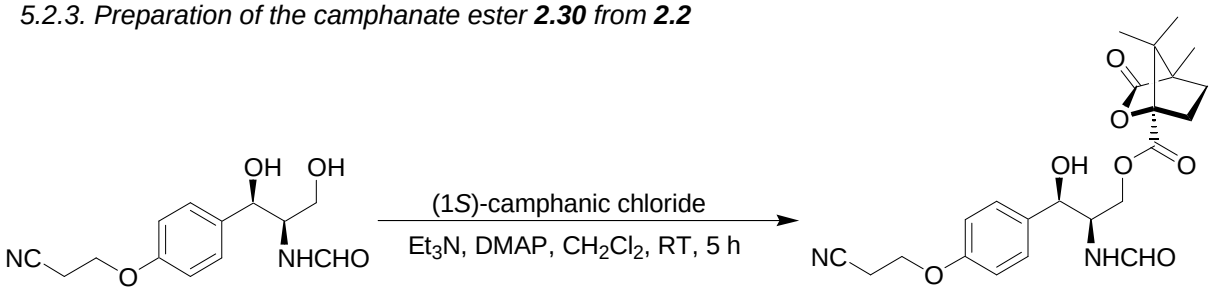
Bursatellin (**2.2**, 4.2 mg, 0.016 mmol) was dissolved in pyridine (0.5 mL) and Ac_2O (0.5 mL) and stirred at room temperature for 24 h. The reaction was quenched with MeOH (2.0 mL) and concentrated *in vacuo* to yield bursatellin acetate (**2.28**, 4.6 mg, 83% yield) as a yellow oil.



2.28

Bursatellin acetate (2.28) orange oil; $[\alpha]_D^{22}$ –17° (*c* 0.52, $CHCl_3$), lit.[131] –23.2°; IR ν_{max} 3414 (br), 2927, 2263, 2075, 1638, 1436, 1377, 1292, 1224, 1026 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ_H 8.21 (1H,s, CHO), 7.71 (2H, d, H-5), 6.90 (2H, d, H-6), 4.73 (1H, d, H-3), 4.22 (1H, m, H-2), 4.20 (2H, t, H-8), 4.08 (1H, dd, 6.0, H-1b), 3.88 (1H, dd, 6.4, H-1a), 2.84 (2H, t, 6.0, H-9), 2.12 (3H, m, 2-OAc), 2.10 (3H, m, 3-OAc) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C 167.8 (2-CHO), 160.8 (q, C-7), 132,5 (q, C-4),128.8 (CH_2, C-5) 115.0 (CH_2, C-6), 73.5 (CH, C-3), 68.2 (CH, C-2), 62.5 (CH_2, C-8), 63.1 (CH_2, C-1a), 63.0 (CH_2, C-1b), 21.3 (CH_3, 3-Me), 21.0 (CH_3, 1-Me), 18.5 (CH_2, C-9) ppm; HREIMS *m/z* 349.1403 (calcd for $C_{17}H_{21}N_2O_6$ [M^++ H], 349.1400).

*5.2.3. Preparation of the camphanate ester **2.30** from **2.2***



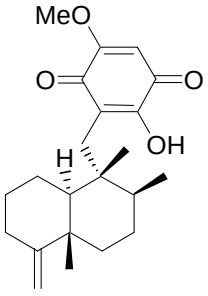
2.2 **2.31**

Bursatellin (**2.2**, 10 mg, 0.039 mmol) was reacted with (1*S*)-camphanic chloride following the reaction procedure described in Section *5.2.3.1* to yield a green solid (12.3 mg), the synthesis of the camphanate ester did not occur.

5.3 Chapter Three Experimental Procedures

5.3.1 Extraction and Isolation of the sponge *Hippospongia metachroma* metabolites

The sponge *Hippospongia metachroma* was immediately frozen after collection and later freeze-dried. Freeze-dried material was extracted with 50:50 MeOH and DCM solution to give a brown gum (17 g), which was then subjected to DIOL bench top column yielding an orange oil, ilimaquinone **3.1** as well as a yellow oil, pelorol **3.19** from the DCM : EtOAc 80 %: 20% fraction.

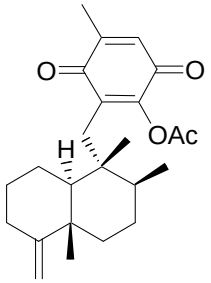


3.1

Ilimaquinone (3.1): orange oil; $[\alpha]_D^{22}$ –22.4° (*c* 0.11, $CHCl_3$), lit.[174] –23.2°; IR ν_{max} 3429 (br), 2975, 2922, 2860, 1634, 1609, 1452, 1383, 1349, 1233, 11951, 1038 cm^{-1}; ^1H & ^{13}C NMR data see Table 4; HREIMS 359.2225 (calcd for $C_{22}H_{31}O_4$ [$(M+H)^+$], 359.2222).

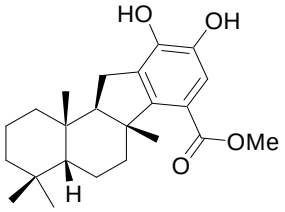
5.3.2 Acetylation of ilimaquinone

Ilimaquinone (**3.1**, 3.6 mg) was dissolved in pyridine (0.5 mL) and Ac_2O (0.5 mL) and stirred at room temperature for 24 h. The reaction was quenched with MeOH (2.0 mL) and concentrated *in vacuo* to yield ilimaquinone monoacetate (**3.21**, 6.5 mg, 82% yield) as a yellow oil.



3.20

Ilimaquinone monoacetate (3.20): yellow oil; $[\alpha]_D^{20}$ –6.2° (*c* 0.31, $CHCl_3$), lit.[175] –8.3°; IR ν_{max} 2928, 2850, 1780, 1672, 1638, 1607, 1450, 1370, 1231, 1150, 1039 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ_H 5.88 (1H, s, H-19), 4.45 (1H, br s, H-11a), 4.43 (1H, br s, H-11b), 3.82 (3H, s, H_3-22), 2.54 (1H, d, *J* = 13.3 Hz, H-15a), 2.41 (1H, d, *J* = 13.3 Hz, H-15b), 2.32 (3H, s, 17-OAc), 2.29 (1H, m, H-3a), 2.07 (1H, m, H-1a), 1.92 (1H, br d, *J* = 14.2 Hz, H-3b), 1.87 (1H, m, H-2a), 1.54 (1H, m, H-6a), 1.47 (1H, br d, *J* = 12.6 Hz, H-1b), 1.46 (2H, m, H_2-7), 1.35 (1H, m, H-6b), 1.21 (1H, m, H-2b), 1.18 (1H, m, H-8), 1.03 (3H, s, H_3-12), 0.91 (3H, d, *J* = 6.4 Hz, H_3-13), 0.83 (3H, s, H_3-14), 0.71 (1H, dd, *J* = 11.7, 1.2 Hz, H-10) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ_c 181.8 (q, C-21), 179.3 (q, C-18), 167.9 (q, 17-OAc), 159.7 (q, C-20), 159.6 (q, C-4), 151.3 (q, C-17), 133.9 (q, C-16), 105.4 (CH, C-11), 103.0 (CH, C-19), 56.7 (CH_3, C-22), 50.8 (CH, C-10), 43.9 (q, C-9), 40.5 (q, C-5), 38.1 (CH, C-8), 36.5 (CH_1, C-6), 33.4 (CH_2, C-3), 32.8 (CH_2, C-15), 28.5 (CH_2, C-2), 27.8 (CH_2, C-7), 23.4 (CH_2, C-1), 20.5 (q, 17-OAc) 20.4 (CH_3, C-12), 18.1 (CH_3, C-13), 17.1 (CH_3, C-14) ppm; HRFABMS *m/z* 401.2312 (calcd for $C_{24}H_{33}O_5$ [M^++H], 401.2328).



3.19

Pelorol (3.19) oil, $[\alpha]_D^{20}$ –67.8 ° (*c* 0.11, $CHCl_3$), lit[174] –69.4°; IR ν_{max} 3415 (br), 2972, 2927, 2075, 1639, 1638, 1436, 1377, 11292, 1224, 1026 cm^{-1}; ^1H & ^{13}C NMR data see Table 5; HRFABMS *m/z* 373.2371 (calcd for $C_{23}H_{33}O_4$ $[M^+ + H]$, 373.2379).

5.4 Bioassays

5.4.1 Feeding Assays

5.4.1.1 Pellet formulation

Commercially available fish pellets were used to make a standard stock solution, in which the compounds bursatellin **2.2** and ilimaquinone **3.1** were then mixed into the dry ingredients (Table 6) together with water which ensured binding of the ingredients to shape and maintain the pellets. The pellets were prepared at varying concentrations (0.0125%, 0.025%, 0.25% and 0.085%, 0.17% and 1.7%) of bursatellin and ilimaquinone using methanol and chloroform respectively based on the isolated yield obtained from the dried mass of the organisms. Control pellets were also made using only the solvents specific for each compound. Pellets were left in the oven for 12h at 37 °C and lyophilized to ensure that all solvents had been removed.

5.4.1.2 Fish feeding assay

Juvenile *Oreochromis mossambicus* (males, 70-100mm in length) were obtained from a local hatchery and *Caffragobius gilcristi* were caught in rock pools around the Kariega Estuary and both species were placed in fresh water and sea water fish tanks respectively (60 cm × 40 cm),with air stones, a filtering system and flowing water at 25 °C. They were left to acclimate for 2-3 weeks and fed on commercially available fish pellets during this period. After the acclimation period each fish was randomly assigned to a separate tank (20 cm × 20 cm) and left to acclimate again for another three days. Fish were randomly assigned to the four different treatments (bursatellin (1), bursatellin control (2), ilimaquinone (3) and ilimaquinone control (4)). Each treatment was given to five fish. Two pellets were fed to each fish which

was then observed for a 3 min period. The behaviour of the fish and time taken to eat the pellets was noted.

5.4.2 Microbial assays

5.4.2.1 Bacterial assays

S. aureus, K. pneumonia, P. sp, S. marcescens, E. coli and *B. subtilis* were grown aerobically at 30 °C and 37°C accordingly (Table 8), in nutrient broth prepared according to manufacturer's specifications. The bacterial broth solutions were shaken up and the optical densities observed. The optical density at 600 nm (OD 600) was adjusted to 0.8-1.0 with nutrient broth to give inoculums of ~104 to 105 CFU (colony forming unit)/mL. Nutrient agar plates were inoculated with the six bacterial isolates and spread evenly across the agar plate. Triplicate plates of each bacterial strain were tested. Discs concentrated (15 µL) with varying concentrations (0.25, 0.5, 1, and 2 mg/mL) of bursatellin and chloramphenicol and saline solution (control) were placed on the inoculated nutrient agar plates, which were incubated at 30°C for 18 h and the bacterial growth observed.

5.4.2.2 Fungal assays

Both strains of fungus (*Fusarium oxysporum* and *Phytophthora nicotinae*) were cultured and the cultures were maintained on potato dextrose agar (PDA). PDA plates were inoculated with the fungal isolates by placing a 5 mm diameter core of fungal subculture into the centre of the PDA plate and incubated at 30°C for two days to allow for initial growth, after which discs with varying concentrations of bursatellin, chloramphenicol and saline solution were placed on the plates and incubated for another 18 h. Triplicate cultures of each fungus were tested. The radial growth of the mycelia was observed over a 3-5 day period, and qualitatively analyzed.

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