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Investigations of Natural Products from Bryozoa Inhabiting Aotearoa New Zealand

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

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at

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by

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Abstract

Two species of bryozoa, *Pterocella vesiculosa* and *Plumatella repens*, were investigated in an endeavour to isolate and characterise novel secondary metabolites.

Analysis of fractions derived from chromatography of a dichloromethane extract of *Pterocella vesiculosa*, indicated the probable presence of β -carboline and pterocellin alkaloids. Continued separation led to further purification of some pterocellin alkaloids, however the characteristic ultraviolet chromatogram that is produced by pterocellins could not be found in any of the fractions analysed. Despite this, further investigations by thin layer chromatography, tandem mass spectrometry and high resolution mass spectrometry suggested structural similarities between metabolites from polar fractions and known pterocellin alkaloids. In the process of isolating these polar, pterocellin-like alkaloids, a new thin layer chromatography solvent system was developed, which better differentiates the more polar metabolites in extracts compared to previously utilised solvent schemes.

LCMS analysis of chromatographic fractions resulted in the detection of eight β -carboline alkaloids, as well as the identification of the previously published 5-bromo-8-methoxy-1-methyl- β -carboline. Further purification of the alkaloid containing fractions by size exclusion chromatography was achieved but individual alkaloids were unable to be isolated due to time constraints associated with the project. Molecular formulae for the eight alkaloids were obtained by the use of high resolution mass spectrometry. Tandem mass spectrometry helped to confirm these formulae and also suggested the types of functional groups attached to the β -carboline skeleton.

A biologically active extract of the freshwater species, *Plumatella repens* was investigated chemically as a continuation of a pilot study. A series of fractions from the pilot study were separated by size exclusion chromatography, with monitoring of activity against the murine P388 lymphocytic leukaemia cell line. Results of the assay indicated the presence of a highly active metabolite/s,

however isolation of the metabolite/s responsible for the activity was not achieved. This was a result of delays in finding facilities to undertake the biological assays and varying degrees of P388 activity, which may suggest metabolite instability.

As part of this project, tissue culture was undertaken at AgResearch, at the Ruakura Research Centre, Hamilton. A method for assay of *Plumatella repens* samples was developed for bioassay guided fractionation.

The sterol composition of *Plumatella repens* was also investigated. Three sterols were identified and an additional two sterols were present, the structures of which could not be confirmed. The relative concentration of each of the sterols was also determined.

Acknowledgments

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The research undertaken in this thesis would have not been possible without Dr Abigail Smith's (the University of Otago) collection of the *Plumatella repens* specimen and Marilyn Manley-Harris' (the University of Waikato) provision of the *Pterocella vesiculosa* specimen.

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List of Abbreviations

DAD	Diode Array Detector
ED ₅₀	Effective Dose for 50 % of Tested Subjects
FABMS	Fast Atom Bombardment Mass Spectrometry
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
HRESIMS	High Resolution Electrospray Ionisation Mass Spectrometry
HRFABMS	High Resolution Fast Atom Bombardment Mass Spectrometry
IC ₅₀	Concentration at 50 % of Growth Inhibition
IR	Infrared
LC	Liquid Chromatography
LREIMS	Low Resolution Electron Impact Mass Spectrometry
MIC	Minimum Inhibitory Concentration
MID	Minimum Inhibitory Dose
MS	Mass Spectrometry
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance
PKC	Protein Kinase C
PTLC	Preparative Thin Layer Chromatography
R _f	Retention Factor
SCUBA	Self Contained Underwater Breathing Apparatus
TAM	Turbidity Adjusted Measurement
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
UV	Ultraviolet
XRD	X-Ray Diffraction

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Chapter One: Introduction

1.1 Bryozoa

Bryozoa, also known as Ectoprocta, are a phylum of aquatic, filter feeding invertebrates. There are over 5 000 known living species of bryozoa,¹ making up three classes; Gymnolaemata, Phylactolaemata and Stenolaemata. Gymnolaemata is the most populous, containing over 3 000 species which are separated into the two orders Ctenostomata and Cheilostomata, as represented in Figure 1.^{2, 3} Some species of bryozoa inhabit freshwater ponds, rivers and lakes, with some preferring brackish water over fresh.⁴ The vast majority however, live in marine environments, particularly near the coast and on reefs, above a depth of 100 metres, although many deep sea species have also been described.⁵

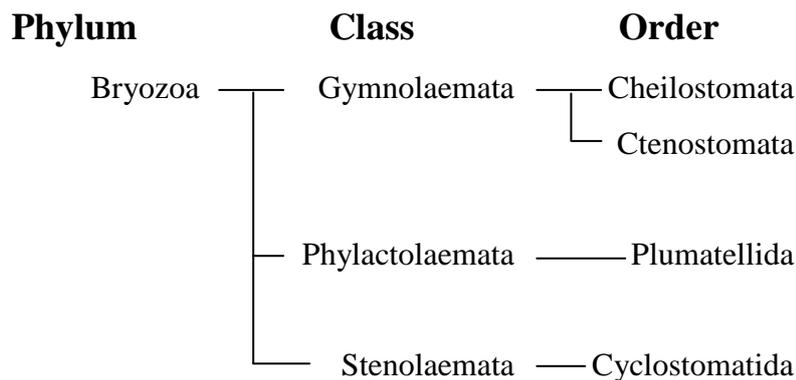
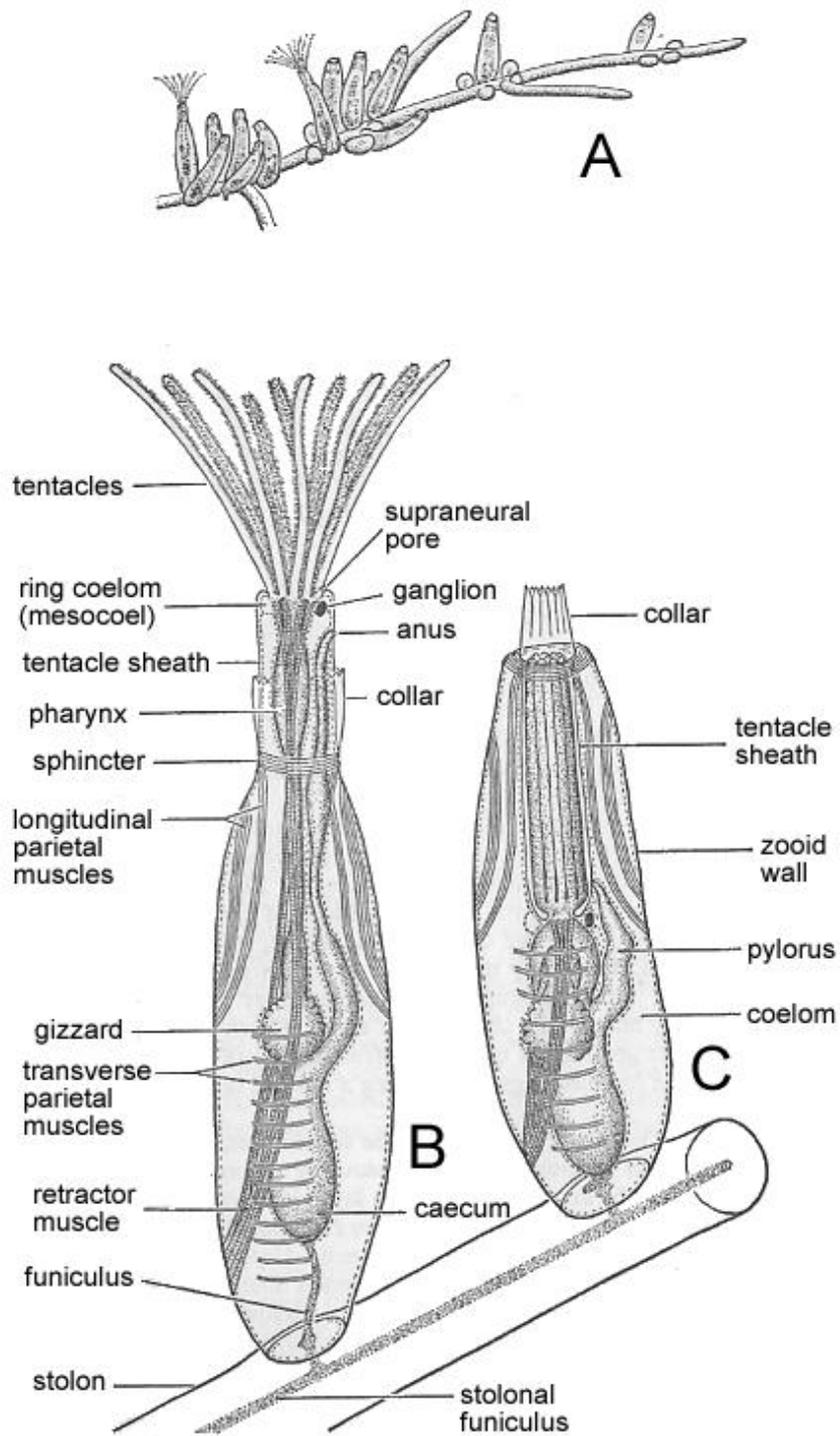


Figure 1: Bryozoa taxa

As filter feeding animals, bryozoa use a lophophore, which is an array of hollow tentacles, to abstract food from their aquatic environments. In almost all cases, bryozoa form colonies, some of which can be several metres across⁶ and have been estimated to contain over two million microscopic individuals, known as zooids.⁷ Colony formation is typical for all but one genus, monobryozoon, which does not form colonies but instead maintains a solitary growth habit. The zooids of bryozoa are primarily feeding zooids (known as autozooids), which are responsible for dietary intake and excretion.^{8, 9}



A - Part of colony showing zooids clustered along stolon
B - Zooid with tentacles expanded
C - Zooid with tentacles partially retracted

Figure 2: Zooid structure (Adapted from Reference 9).

Most species of bryozoa have the ability to undergo polymorphism. This allows individual zooids to undergo physiological changes that help support the colony in ways other than that of autozooids. Such changes allow zooids to take on roles such as protection, cleaning and anchoring.^{2, 10} Although this is the case for most bryozoa, the class Phylactolaemata, made up of species that live exclusively in freshwater, have not evolved polymorphism, which results in all their zooids being autozooids.¹¹

The reproduction method of bryozoa can be either sexual or asexual, depending on the species and maturity of a colony.¹² Asexual reproduction of bryozoa occurs either through budding of an individual zooid into a clone of itself, or by the formation of a statoblast.^{12, 13} Statoblasts are produced exclusively by the class Phylactolaemata, most abundantly towards autumn. Statoblasts are hardy, disc shaped objects that are dormant but in the right circumstances, develop into zooids.¹³ On account of the ability of statoblasts to remain dormant for extended periods of time, as well as being able to withstand a varying array of environmental conditions, it is possible for a colony's lineage to survive through conditions that might destroy the colony itself.⁹ It has been demonstrated in laboratory conditions that *Plumatella repens* statoblasts can still be viable after having been frozen at -10°C for 50 hours.¹⁴ In fact, when the germination rates of statoblasts that had been frozen were compared to those of statoblasts stored at 24°C, it was noted that the effect of freezing was negligible.¹⁴ It was calculated that one square metre of bryozoa inhabited plant zone could release up to 800 000 statoblasts.¹⁴

Sexual reproduction occurs by fertilisation of an egg of one zooid by sperm released by another zooid. This can occur either through the release of both eggs and sperm (external fertilisation), or by the capture of sperm by the lophophore of zooids that are producing eggs (internal reproduction).⁹ With internal reproduction, the egg is fertilised either within the zooid's coelom (Illustrated in Figure 2) or in the ovary.¹⁵ In non-brooding species, the egg is then released via the intertentacular organ, a small organ protruding between a pair of tentacles. The intertentacular organ is also where eggs are released in other species for external reproduction. Although in most cases zooids contain either testes or ovaries, they are actually protandric sequential hermaphrodites, meaning that during their

lifetime they will first possess male sex organs before in later life developing female sex organs and losing the male reproductive organs.¹² In this process, a colony will usually simultaneously possess zooids with either male or female reproductive organs. This is the case for many marine species, however for the exclusively freshwater dwelling class Phylactolaemata, the zooids are simultaneous hermaphrodites, containing both male and female reproductive organs at one time.⁹

The geographical distribution of bryozoa is vast; inhabiting both temperate and tropical marine water.¹⁶ There are also species that have been described in Antarctic waters, freshwater and, because of their independence from photosynthesis as a source of energy, bryozoa are also found in deep sea environments, below 2 000 metres,⁹ far from the reach of direct solar energy.^{9, 17} Their means of reproduction and tolerance to a range of different environments has allowed bryozoa to populate the globe.⁹

1.2 Natural Products

Metabolites are compounds which are used by all organisms and can be classified as either primary or secondary. Primary metabolites are compounds which are involved in the growth, development and reproduction of organisms. These compounds are typically not species specific and often have evident roles in the biology of the organism which produces them, whereas secondary metabolites are characterised as molecules of great structural diversity, typically of low molecular weight (generally < 3 000 Da).¹⁸ More importantly, unlike primary metabolites, secondary metabolites have no obvious biological role in the organism which produces them.¹⁸

Natural products, a term which is used interchangeably and is synonymous with secondary metabolites, frequently have biologically active (bioactive) properties, meaning that the metabolite exerts some physiological effect on another organism in either a beneficial or detrimental manner. This has led secondary metabolites and their derivatives to become widely used pharmaceuticals for the treatment of a variety of conditions, such as pain, mental illness, bacterial infections and cancer.

The vast majority of natural products isolated to date have come from terrestrial flora and fauna rather than from aquatic environments. This is a result of both the ease of access to the terrestrial environment and the historical uses of terrestrial species in many traditional medical remedies and spiritual ceremonies.¹⁹ The knowledge that medicinal preparations of some terrestrial species can have positive effects in the treatment of certain conditions made them sensible choices for early work in natural product isolation.

One of the most well known groups of natural products from a terrestrial species are the penicillins, isolated from *Penicillium chrysogenum* of the Trichocomaceae family of fungi in 1928.^{20, 21} The penicillins are still in common use today and have been utilised to treat an array of different types of bacterial infections. The core structure of the penicillins is illustrated in Figure 3.

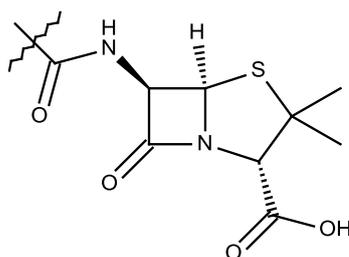
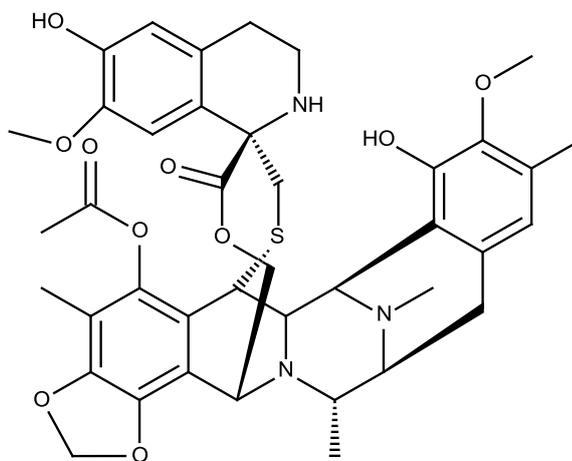


Figure 3: Core structure of the penicillins

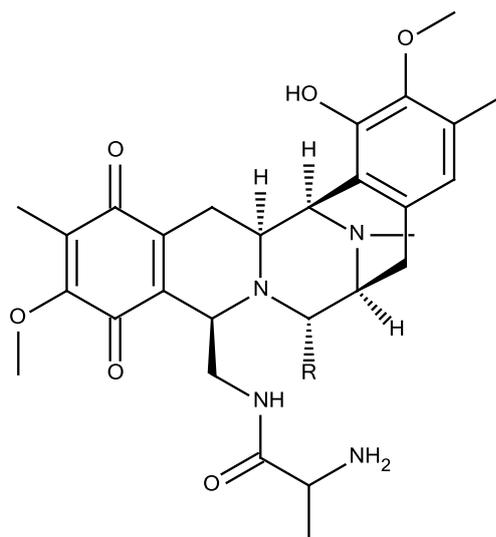
Although most secondary metabolites isolated to date have been isolated from species which inhabit terrestrial environments, the relatively small number discovered in aquatic environments is not due to lack of biodiversity. With greater than 70 % of the Earth's surface covered in water, aquatic environments are some of the most biological diverse areas to be found. This is reflected by the fact that the number of new natural products being discovered from marine species is on the increase, with over 1 000 being reported yearly from 2008 to 2010, up 11 % on the number published in 2007.^{22, 23}

An example of a widely used pharmaceutical from an aquatic species is ecteinascidin 743 (**1**). The antitumour activity of an extract from the marine

tunicate *Ecteinascidia turbinata*, was first observed in 1969. The structures of the active constituents were not determined until more than ten years later. This was due to the low abundance of the active compounds, ecteinascidin 743 (**1**) being the most abundant with a 0.0001 % yield.²⁴ Many bioactive natural products constitute only a small quantity of the total mass of the organism. This can be a problem when needing large quantities for pharmaceutical applications, as was the case for ecteinascidin 743 (**1**). The unique structure of ecteinascidin 743 (**1**), containing three tetrahydroisoquinolines and seven chiral centres, was a challenge for synthesis and although the first enantioselective total synthesis was reported in 1996, the multistep synthesis was not practical for large scale production.²⁴⁻²⁶ This problem was overcome in 2000 when the semi-synthesis of ecteinascidin 743 (**1**) from cyanosafrafin B (**2**) was reported.²⁷ Safrafin B (**3**) could be obtained in kilogram amounts from the cultured bacterium *Pseudomonas fluorescens*, resulting in large amounts of a cheap starting material from a reliable renewable resource.²⁷



(1)



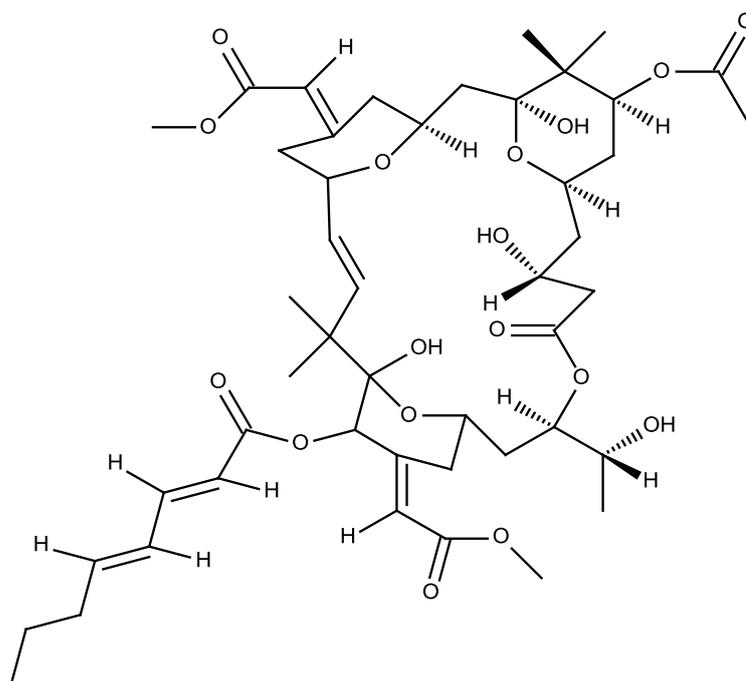
(2) R = -CN

(3) R = -OH

1.3 Natural Products Isolated from Bryozoa

On account of the limited amount of research that has been undertaken on bryozoa in comparison to other phyla of aquatic organisms such as Porifera and Cnidaria, the number of natural products that have been characterised from the phylum Bryozoa is comparatively low. However, although the actual quantity of natural products discovered from this phylum is quite low, a significant number of them have proven to be of great interest, either for their bioactivity or the uniqueness of their structures, or in some cases both.

One of the most well studied classes of compounds isolated from bryozoans is the bryostatins. In 1968, Pettit *et al.*²⁸ discovered that an extract from the species *Bugula neritina* collected in the eastern Pacific Ocean, had significantly potent antineoplastic activity. Fourteen years later, in 1982, Pettit *et al.* published the chemical structure of bryostatin 1 (**4**), the main active constituent of the *B. neritina* extract.²⁸ The structure contained a complex, 20-membered macrolide ring, which is characteristic of all of the bryostatins and has been given the name bryopyran (illustrated in Figure 4).²⁸ Bryostatin 1 (**4**) displayed an ED₅₀ value of 0.86 $\mu\text{g mL}^{-1}$ against the National Cancer Institute (NCI) murine P388 lymphocytic leukaemia cell line.²⁸



(4)

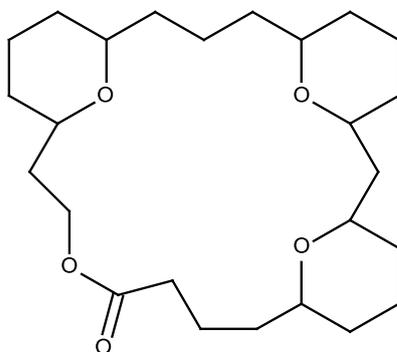
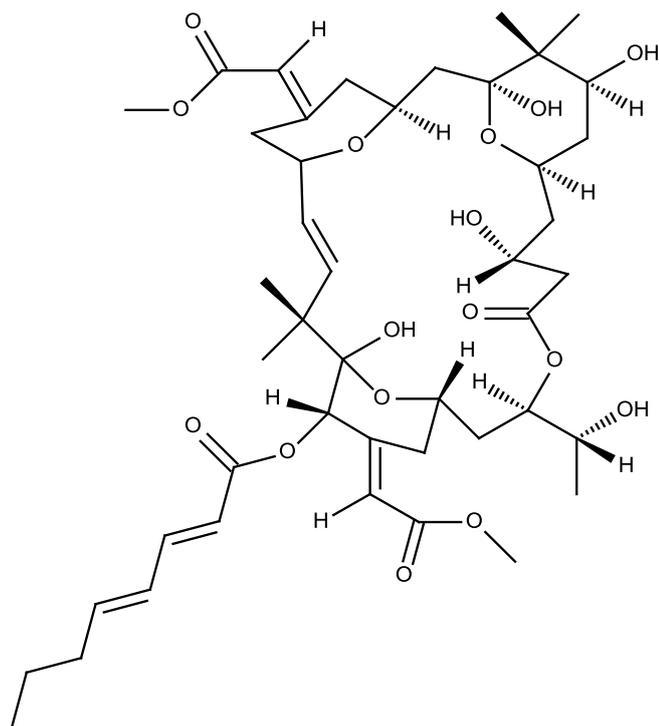
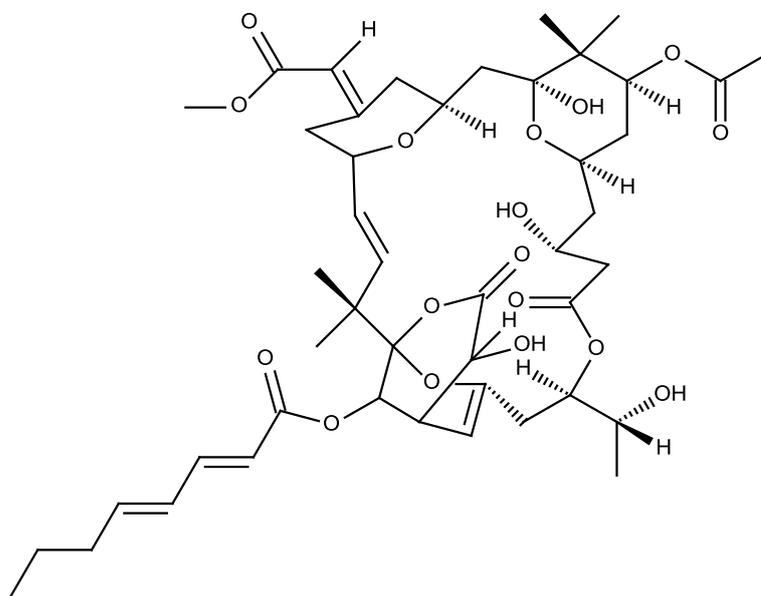


Figure 4: Bryopyran ring system

The year following the publication of bryostatin 1 (4), structures of bryostatin 2 (5) and bryostatin 3 (6) were published, both of which were isolated from *B. neritina* obtained from the eastern Pacific Ocean.^{29, 30} Structures of (5) and (6) were determined with the use of various techniques including fast atom bombardment mass spectrometry (FABMS), infrared (IR) spectroscopy, and ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy.^{29, 30}



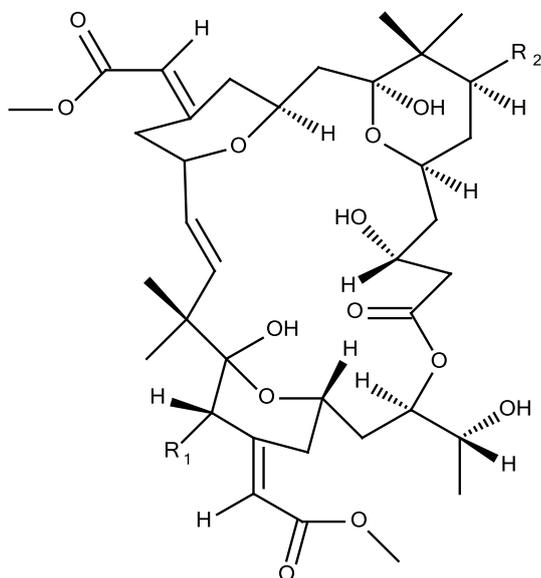
(5)



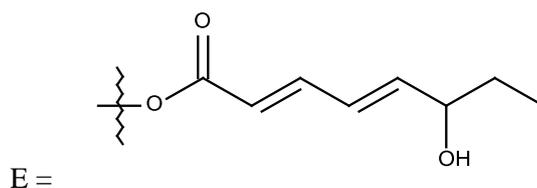
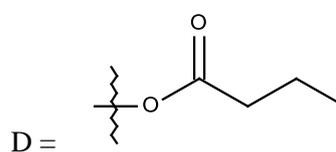
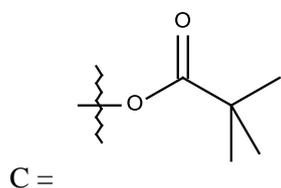
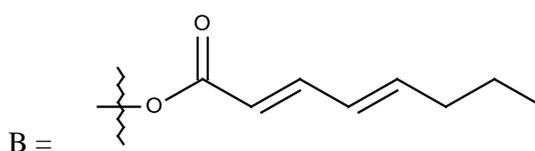
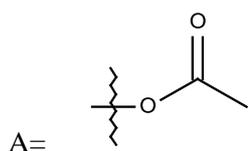
(6)

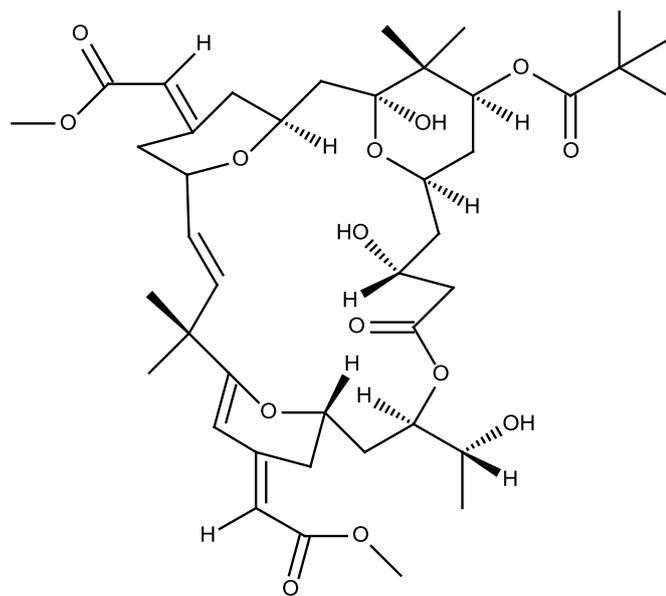
Over the next thirteen years, the structures of a further 15 bryostatins (**7-21**) were published, all of which were extracted from samples obtained from the Gulfs of California, Mexico and Sagami.³¹⁻³⁶ Unlike all other known bryostatins, bryostatin

8 (**11**) was not first isolated from *B. neritina*, but from the species *Amathia convoluta*.^{37, 38} Bryostatins 2-18 (**5-21**) displayed levels of antineoplastic activity comparable to bryostatin 1 (**4**).

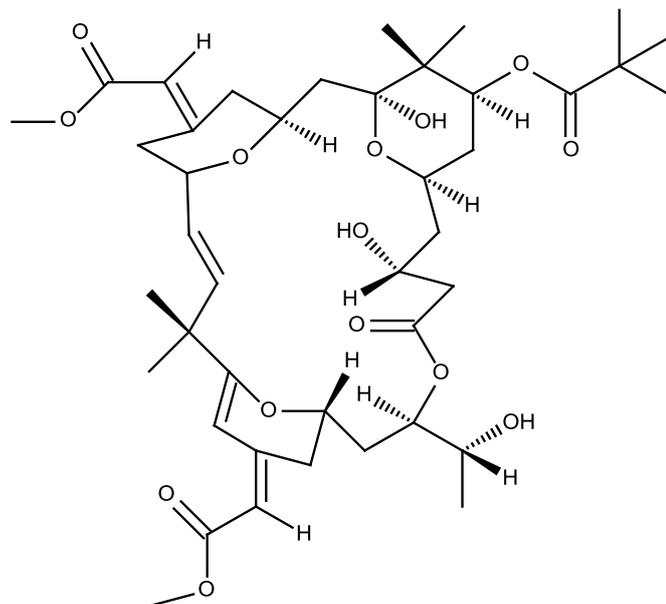


- | | | | |
|------|--|------|--|
| (7) | R ₁ = D; R ₂ = C | (13) | R ₁ = -H; R ₂ = C |
| (8) | R ₁ = A; R ₂ = C | (14) | R ₁ = -H; R ₂ = A |
| (9) | R ₁ = A; R ₂ = D | (15) | R ₁ = B; R ₂ = D |
| (10) | R ₁ , R ₂ = A | (16) | R ₁ = -H; R ₂ = D |
| (11) | R ₁ , R ₂ = D | (17) | R ₁ = -OH; R ₂ = C |
| (12) | R ₁ = D; R ₂ = A | (18) | R ₁ = E; R ₂ = A |

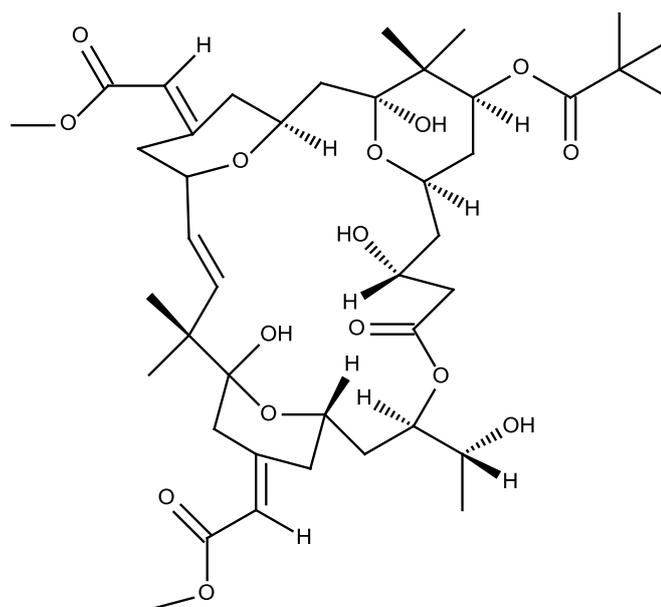




(19)

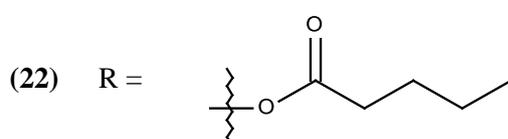
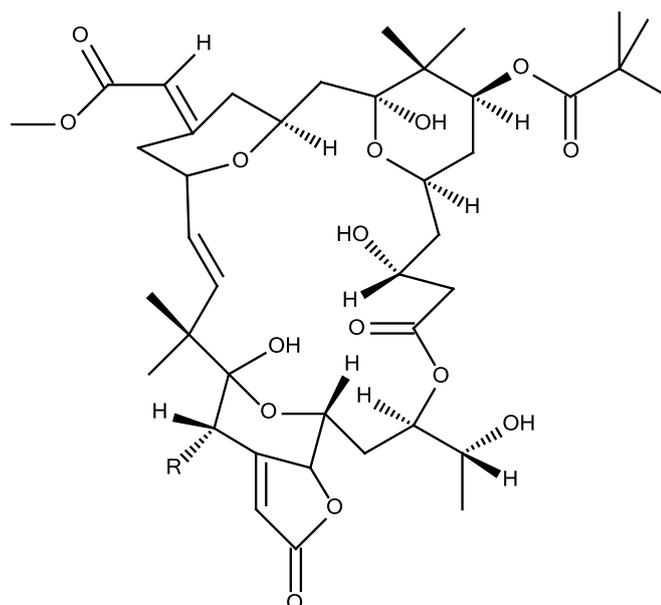


(20)



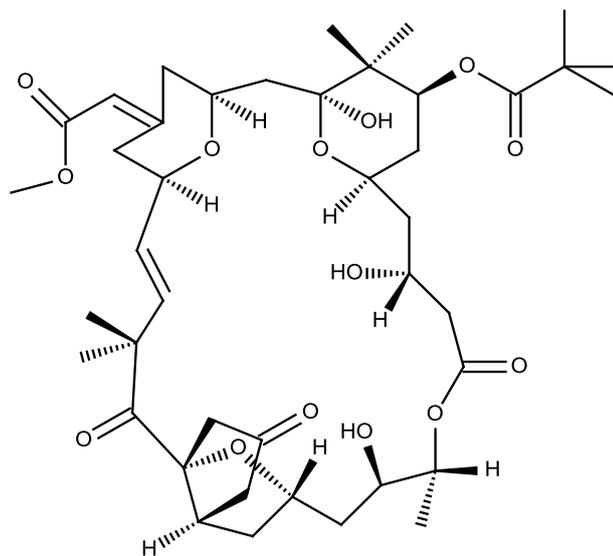
(21)

Bryostatin 19 (**22**) was also isolated from *B. neritina*, but from a collection obtained from the South China Sea,³⁹ whilst bryostatin 20 (**23**) was isolated from a collection of *B. neritina*, obtained from the east coast of the United States of America. The presence of another, as yet unidentified bryostatin was also noted.⁴⁰



It has been shown that the bryostatins are able to bind to the enzyme protein kinase C (PKC). Activation of this enzyme by phorbol esters promotes the growth of tumour cells and although the bryostatins have the same binding site as phorbol esters, they produce antineoplastic activity.⁴¹ Most studies to date have involved the interaction between bryostatin 1 (**4**) and PKC. These studies have shown bryostatin 1 (**4**) to initially promote the activation of PKC for a short period followed by its significant deregulation.^{41, 42} The deregulation results in growth inhibition, cell differentiation and programmed cell death. Another study has shown that bryostatins 5 (**8**) and 8 (**11**) have similar antitumour properties to bryostatin 1 (**4**) but may possess different or fewer side effects.⁴¹ Bryostatin 1 (**4**) has been in over 50 phase I and II NCI clinical trials investigating its potential for treatment of various cancers and is currently in a phase I trial to determine the maximum dosage for patients with unresectable (unable to be removed by surgery) or metastatic solid tumours.⁴³

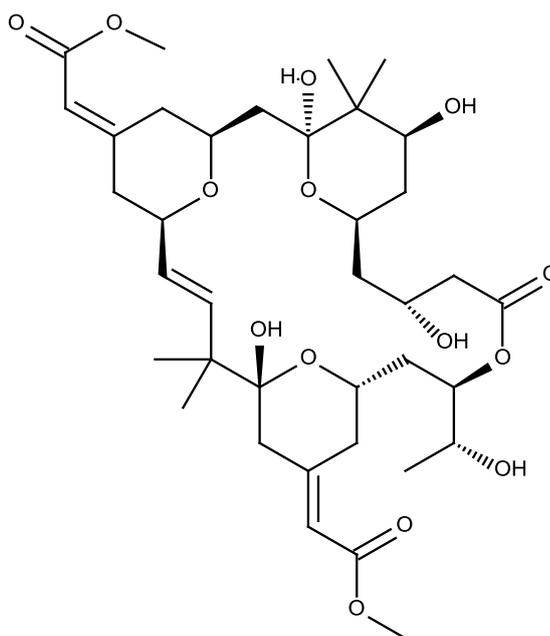
A possible biological precursor to the bryostatins, neristatin 1 (**24**) was isolated from *B. neritina* using bioassay guided separation and as for the bryostatins, neristatin 1 (**24**) also possesses significant antineoplastic properties against P388 cell lines.⁴⁴



(24)

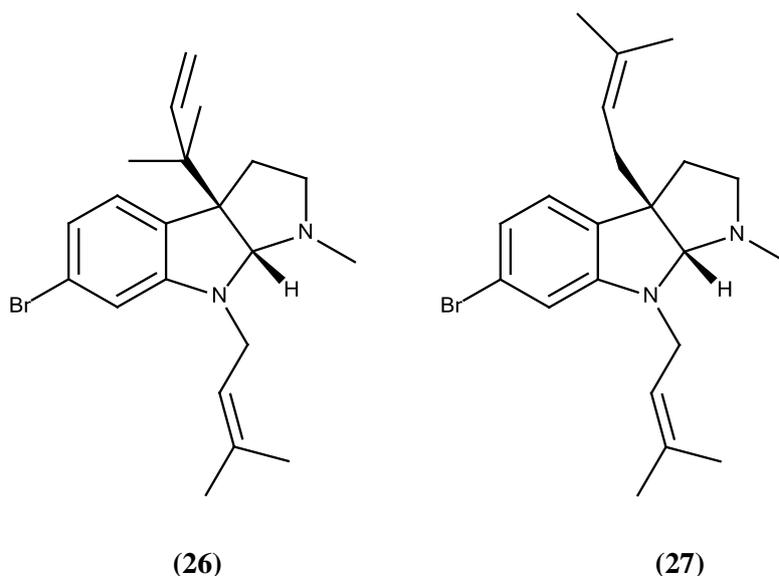
Although all bryostatins have been isolated from bryozoan species, a number of studies have indicated that the bryostatins are of bacterial origin, from the

symbiotic bacterium *Candidatus Endobugula sertula*.^{45, 46} Bryostatins are similar in structure to complex polyketides that are known to be produced by bacteria. Results have been published that showed genetic variation in *E. sertula* found in *B. neritina* correlates with the variation in the types of bryostatins found in a given colony.⁴⁷ It has also been found that North Atlantic *B. neritina*, which does not have the *E. sertula* symbiont, does not possess bryostatins.⁴⁶ *B. neritina* with the symbiotic bacterium present have been grown in the laboratory and it was found that substantially less bryostatins are produced when the bryozoan is treated with antibiotics, with 22-60 % less bryostatin activity for treatment of 7-14 days.⁴⁵ Another study, which investigated the genes of *E. sertula*, concluded that the bacterium had the potential to synthesise the hypothetical compound bryostatin 0 (**25**). It was postulated that all other known bryostatins could be synthesised from this metabolite.⁴⁶

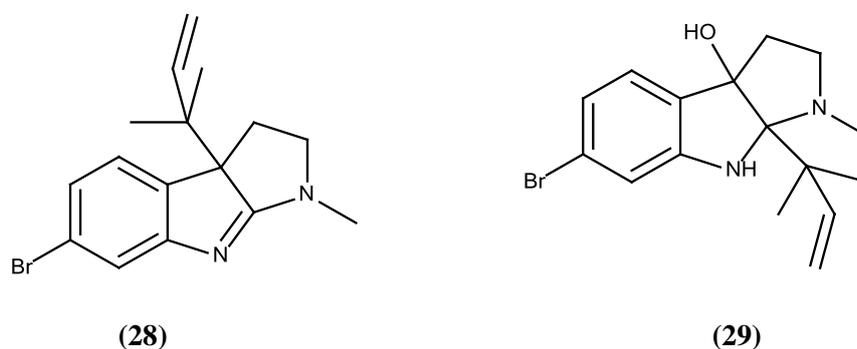


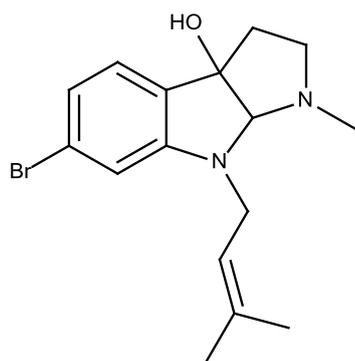
(25)

Two bromo-substituted indole alkaloids, flustramines A (**26**) and B (**27**), were isolated from a petroleum ether extract of the bryozoan *Flustra foliacea*. Purification of the compounds was achieved through silica gel chromatography and the structures were elucidated through the use of high resolution mass spectrometry (MS) and NMR spectroscopy.^{48, 49}

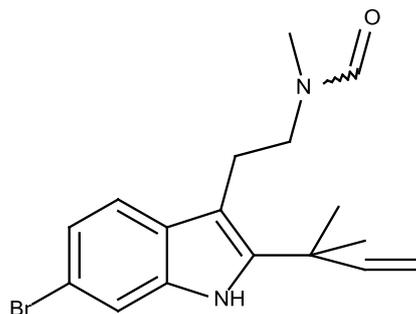


Flustramine C (**28**), flustraminols A (**29**) and B (**30**), flustrabromine (**31**), flustramide A (**32**) and a bromo-substituted tryptamine, 6-bromo-*N*_b-methyl-*N*_b-formyltryptamine (**33**) were also isolated from *F. foliacea* obtained in Scandinavian waters.⁵⁰⁻⁵³ A reduction reaction was performed on (**28**) using lithium aluminium hydride to give debromo-8,8a-dihydroflustramine C (**34**).⁵⁰ The first naturally occurring bromo-substituted quinoline, 7-bromo-4-(2-ethoxyethyl)-quinoline (**35**) was also isolated from *F. foliacea*.⁵⁴ However, the extract from which (**35**) had been isolated had come into contact with ethanol and therefore the 2-ethoxyethyl group on the quinoline could possibly be an artefact of the isolation method and may have replaced a more reactive functional group.⁵⁴

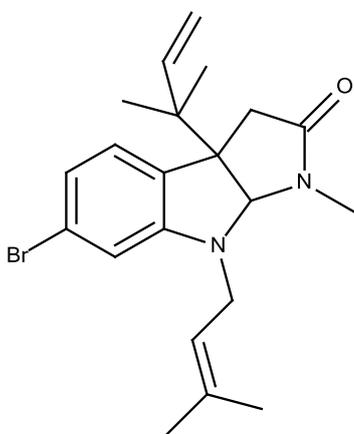




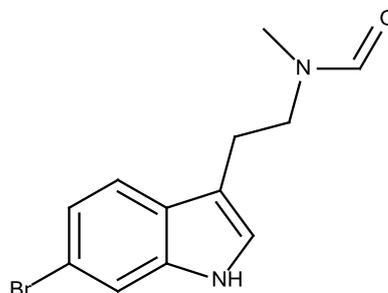
(30)



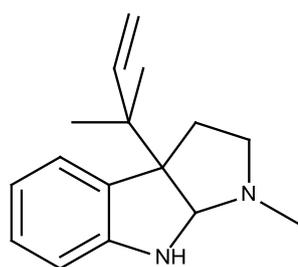
(31)



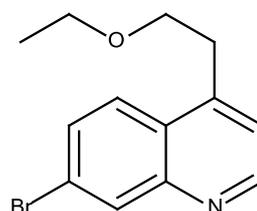
(32)



(33)



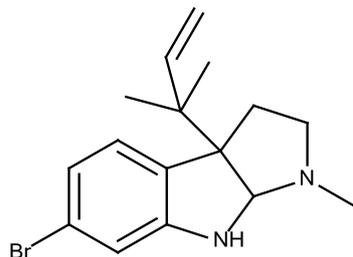
(34)



(35)

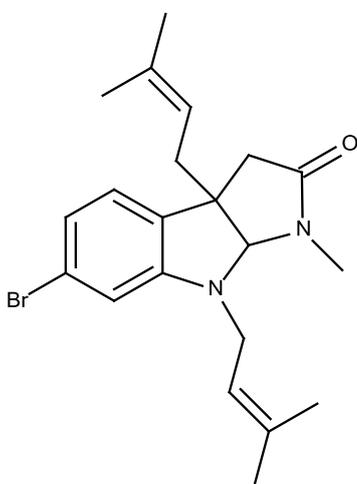
Dihydroflustramine C (**36**) was isolated from a dichloromethane extract of *F. foliacea*, collected from the Minas Basin, Nova Scotia.⁵⁵ The extract displayed strong antibacterial activity against *Bacillus subtilis*. Isolation of the compound was achieved by bioassay guided separation, using thick layer chromatography

and high-performance liquid chromatography (HPLC) and the structure was determined with the use of ultraviolet (UV) spectroscopy, high resolution MS and various NMR spectroscopy techniques.⁵⁵

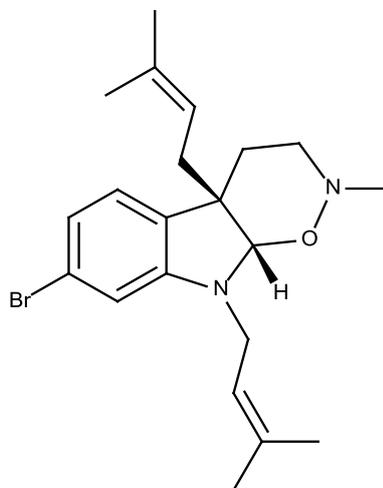


(36)

Flustramide B (37) and flustrarine B (38) were isolated from a petroleum ether and an ethyl acetate extract of *F. foliacea*, respectively.⁵⁶ Both were separated using normal phase liquid chromatography (LC) and finally HPLC, to result in samples pure enough for NMR spectroscopic analysis.⁵⁶



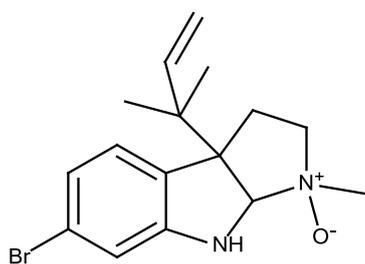
(37)



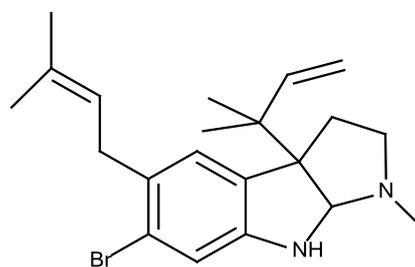
(38)

An aqueous methanol extract of *F. foliacea* collected from Scandinavian waters, was shown to have significant antibacterial activity. Further purification using bioassay guided fractionation, yielded four new natural products, dihydroflustramine C *N*-oxide (39), flustramine D (40), flustramine D *N*-oxide

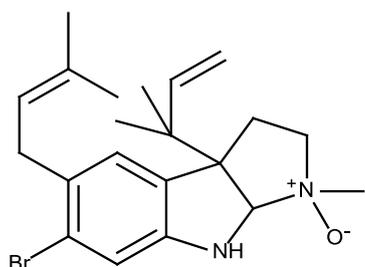
(41), and isoflustramine D (42).⁵⁷ They were isolated based on activity against *B. subtilis* and purification was achieved using reversed phase LC and HPLC.



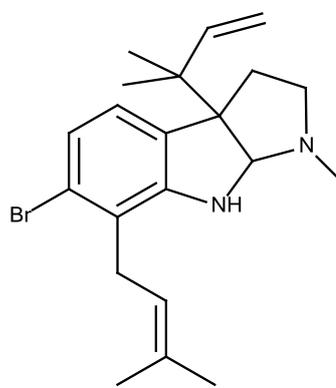
(39)



(40)



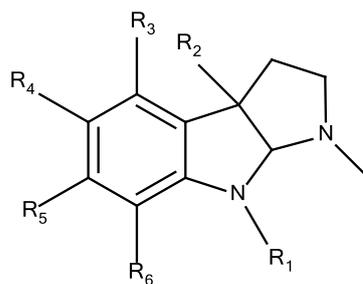
(41)



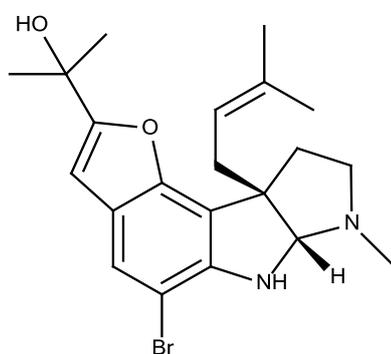
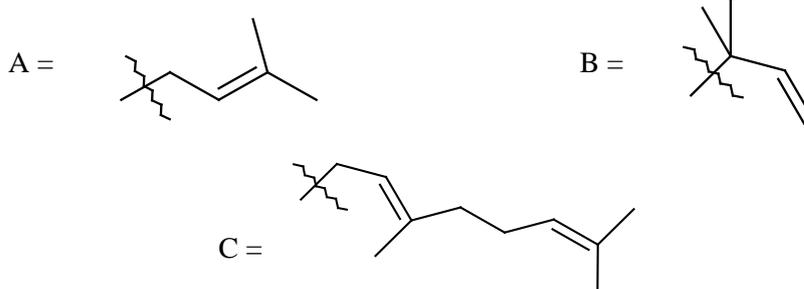
(42)

Debromoflustramine B (43) and flustramine E (44) were isolated from a North Sea collection of *F. foliacea*.⁵⁸ Eleven further flustramines, flustramines, F-P (45-55) were isolated from a dichloromethane extract of *F. foliacea* collected from the Minas Basin off the New Brunswick and Nova Scotian shores. Two of these compounds, flustramine O (54) and P (55) are dimers and it was noted that they could possibly be isolation artifacts.⁵⁹

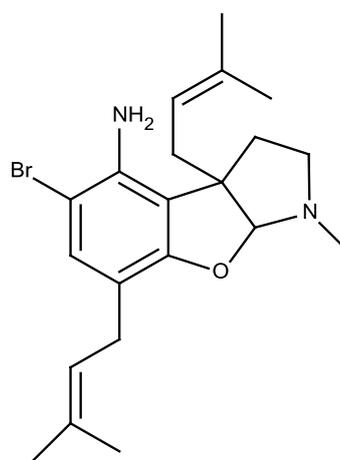
Isolation of deformylflustrabromine (56) was achieved by liquid-liquid extraction, size exclusion chromatography and HPLC of an organic extract of a *F. foliacea* specimen collected in the southern North Sea.⁶⁰



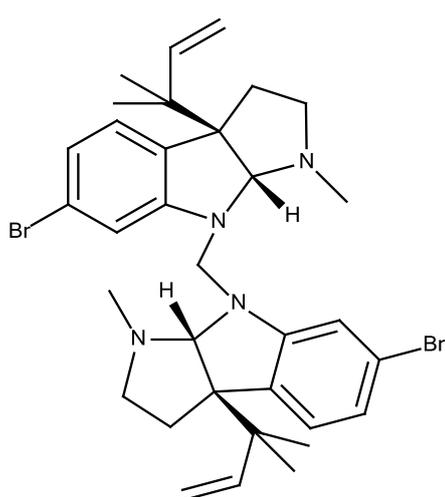
- (43) $R_1, R_2 = A$; $R_3, R_4, R_5, R_6 = -H$
 (44) $R_1, R_3, R_4, R_6 = -H$; $R_2 = A$; $R_5 = -Br$
 (45) $R_1 = -Ac$; $R_2 = B$; $R_3, R_4, R_6 = -H$; $R_5 = -Br$
 (46) $R_1, R_3, R_6 = -H$; $R_2 = B$; $R_4, R_5 = -Br$
 (47) $R_1, R_4, R_5 = -H$; $R_2 = C$; $R_3 = -OH$; $R_6 = -Br$
 (48) $R_1, R_4, R_5 = -H$; $R_2 = A$; $R_3 = -OH$; $R_6 = -Br$
 (49) $R_1, R_5 = -H$; $R_2 = C$; $R_3 = -OH$; $R_4, R_6 = -Br$
 (50) $R_1, R_5 = -H$; $R_2 = A$; $R_3 = -OH$; $R_4, R_6 = -Br$
 (51) $R_1, R_5 = -H$; $R_2, R_4 = A$; $R_3 = -OH$; $R_6 = -Br$



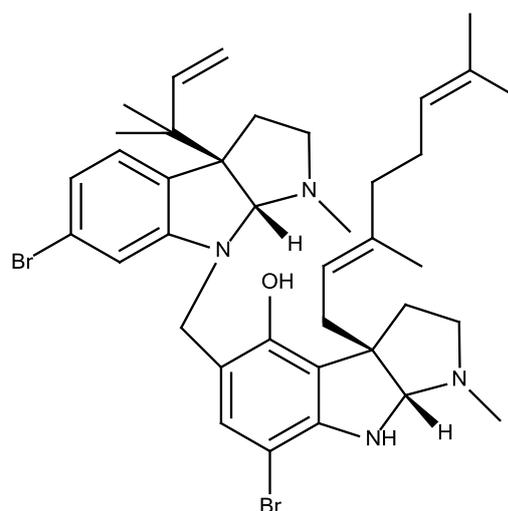
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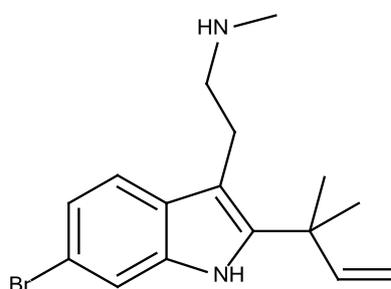
(53)



(54)

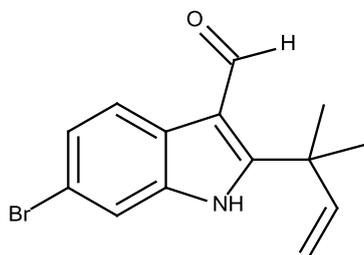


(55)

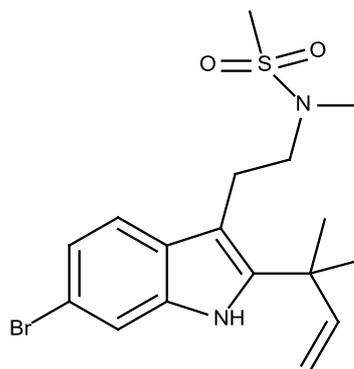


(56)

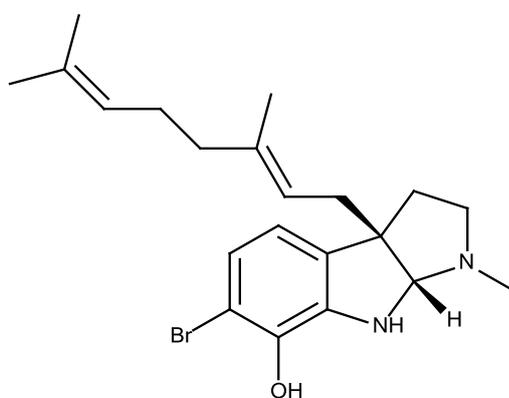
Three novel natural products based around an indole structure, 6-bromo-2-(1,1-dimethyl-2-propenyl)-1*H*-indole-1-carbaldehyde (**57**), *N*-(2-[6-bromo-2-(1,1-dimethyl-2-propenyl)-1*H*-indol-3-yl]ethyl)-*N*-methylmethanesulfonamide (**58**), and (3*aR**,8*aS**)-6-bromo-3*a*-[(*2E*)-3,7-dimethyl-2,6-octadienyl]-1,2,3,3*a*,8,8*a*-hexahydropyrrolo[2,3-*b*]-indol-7-ol (**59**) were isolated from an organic extract of a *F. foliacea* sample collected from Steingrund, North Sea, Germany and purified by silica gel vacuum LC, solid phase extraction and normal and reversed phase HPLC.⁶¹



(57)

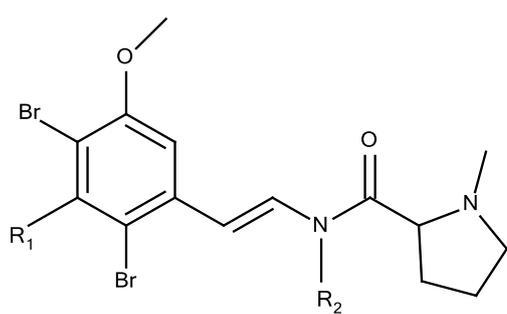


(58)



(59)

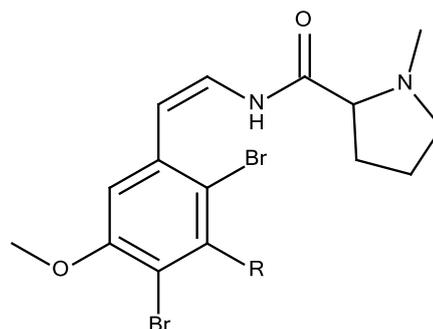
A new class of natural products, known as amathamides, were isolated from a Tasmanian collection of *Amathia wilsoni*. Amathamides A-G (**60-66**) are phenylethylamine-like compounds containing an *N*-methyl-pyrrolidine functional group along with varying halogenations from dibromination in amathamides A (**60**) and B (**61**), to tribromination in amathamides C-G (**62-66**). It has been hypothesised that the amino acids proline and phenylalanine are precursors to the amathamides, given the structural similarities.⁶²⁻⁶⁵



(60) $R_1, R_2 = -H$

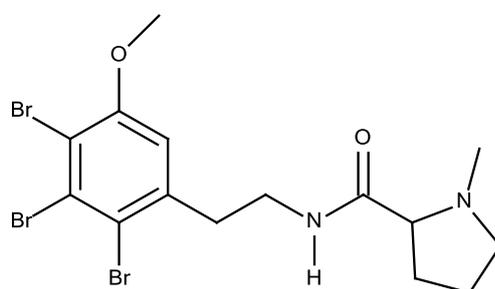
(62) $R_1 = -Br; R_2 = -CH_3$

(64) $R_1 = -Br; R_2 = -H$

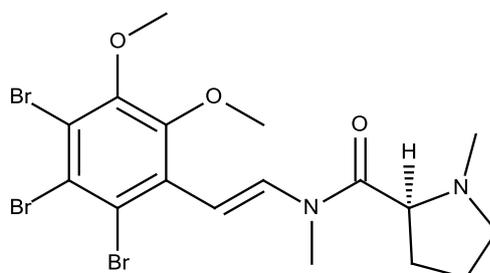


(61) $R = -H$

(65) $R = -Br$



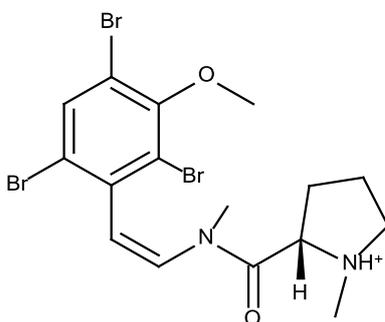
(63)



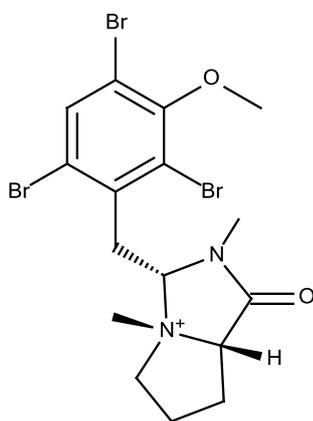
(66)

An organic extract of *A. wilsoni*, collected from Point Peur, southeast Tasmania, Australia yielded amathamide H (**67**), in addition to wilsoniamines A (**68**) and B (**69**).⁶⁶ The previously reported amathamide C (**62**) was also obtained and upon structural determination, Carroll *et al.*⁶⁶ concluded that amathamide C (**62**) contains a 2,4,6-tribromo-3-methoxyphenyl substitution pattern rather than the 2,3,4-tribromo-5-methoxyphenyl substitution that was previously reported. The

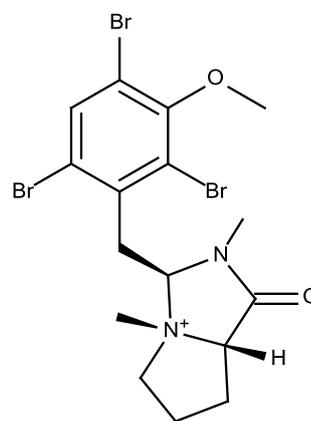
revision of the structures of amathamides D-F (**63-65**) was also suggested, as they too likely contain the 2,4,6-tribromo-3-methoxyphenyl moiety.⁶⁶



(67)

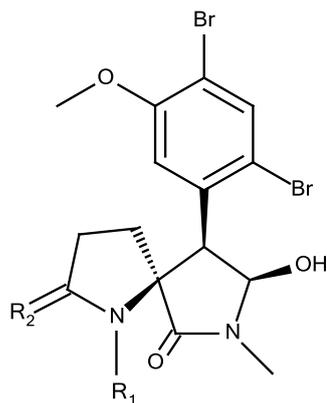


(68)



(69)

A series of six γ -lactam containing metabolites, known as amathaspiramides, were isolated from a New Zealand collection of *A. wilsoni*, collected from the waters off Barrett Reef in Wellington, New Zealand. Amathaspiramides A-F (**70-75**) each contain a γ -lactam group as well as a dibrominated, methoxy-substituted phenyl group.⁶⁷ They were isolated from an organic extract which was separated with the use of multiple LC techniques with final purification by HPLC.⁶⁷

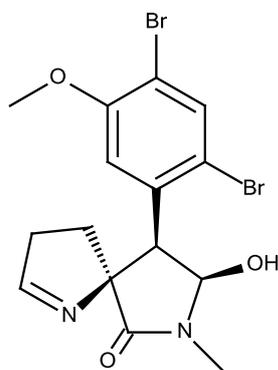


(70) $R_1 = -CH_3$; $R_2 = -H_2$

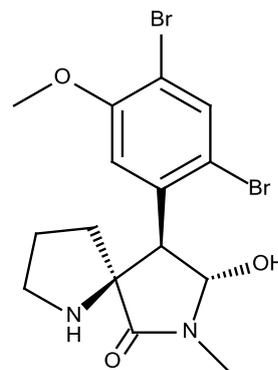
(71) $R_1 = -CH_3$; $R_2 = =O$

(72) $R_1 = -H$; $R_2 = -H_2$

(73) $R_1 = -H$; $R_2 = =O$



(74)

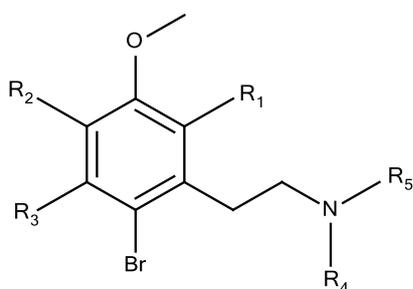


(75)

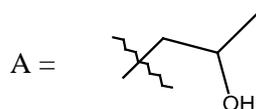
Convolutamines A-J (**76-85**) are a series of novel β -phenylethylamines, containing methoxy substitutions on the phenyl group.⁶⁸⁻⁷¹ The ten natural products have varying degrees of halogenation, from mono- and dibromination in convolutamines A-C (**76-78**) and F-H (**81-83**) to tribromination in convolutamines D-E (**79-80**) and I-J (**84-85**). Convolutamines A-H (**76-83**) were isolated from organic extracts of *Amathia convoluta* (A-G (**76-82**) were isolated from a collection obtained off the coast of Florida, United States of America, while H (**83**) was isolated from a collection from the east coast of Tasmania, Australia).⁶⁸⁻⁷⁰ Convolutamine I (**84**) and J (**85**) were isolated from an organic extract of *Amathia tortusa* collected in Bass Strait, Tasmania, Australia.⁷¹

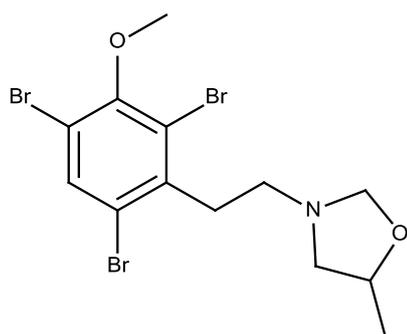
Isolated from the same extract as convolutamine H (**83**), was the tribrominated, *N*-methoxy substituted indole alkaloid convolutindole A (**86**).⁶⁸ Convolutamine H (**83**) and convolutindole A (**86**) displayed significant nematocidal activity, each possessing greater potency than that of the commercial nematocide, levamisole. The bioactivity of (**83**) and (**86**) also appeared to be quite selective, with neither displaying any antibacterial or antifungal activity when assayed against *B. subtilis* and *Saccharomyces cerevisiae*.⁶⁸

Convolutamines I (**84**) and J (**85**) also displayed considerable bioactivity. In an assay against *T. b. brucei*, a parasite known to cause the disease Human African trypanosomiasis, both (**84**) and (**85**) were active, with IC₅₀ values of 1.1 μM and 13.7 μM respectively.⁷¹

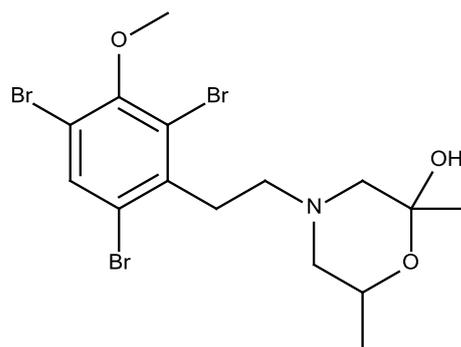


- (76) R₁, R₂ = -Br; R₃ = -H; R₄ = -CH₃; R₅ = A
 (77) R₁ = -Br; R₂, R₃ = -H; R₄ = -CH₃; R₅ = A
 (78) R₁, R₂ = -Br; R₃, R₄ = -H; R₅ = A
 (81) R₁ = R₂ = -Br, R₃ = R₄ = -H, R₅ = -CH₃
 (82) R₁ = -Br; R₂, R₃, R₄ = -H; R₅ = -CH₃
 (83) R₁ = -OCH₃; R₂, R₃ = -Br; R₄ = -H; R₅ = -CH₃

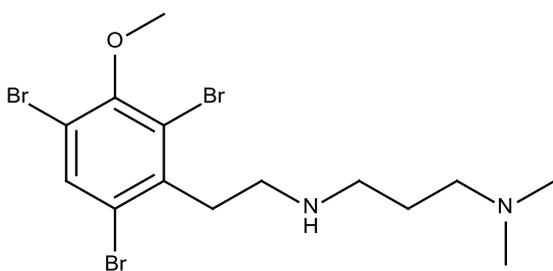




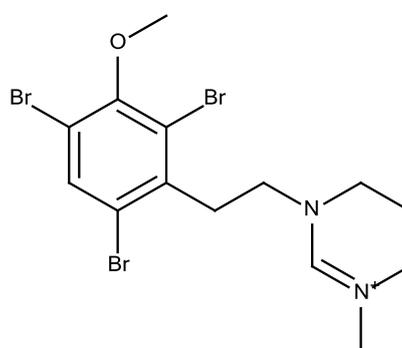
(79)



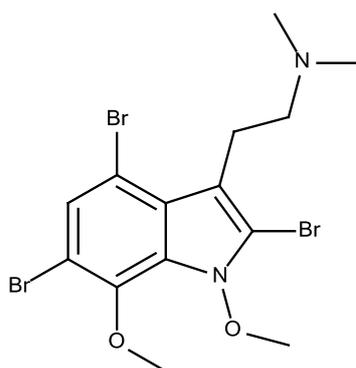
(80)



(84)



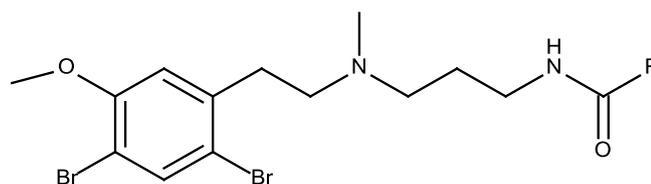
(85)



(86)

Volutamides A-E (**87-91**) were isolated from a collection of *A. convoluta* obtained off the Atlantic coast of North Carolina. All of the structures are derived from amino acids, with phenyl substitutions reminiscent of the convolutamines. Each of the volutamides have been shown to be effective antifeedants towards fish and sea

urchins.⁷² An organic extract of *A. convoluta* was placed into squid-based food at natural concentrations. This extract-infused diet reduced feeding of the pinfish *Lagodon rhomboides* by 93 %. The isolation of the five metabolites was achieved by typical chromatographic techniques and their structures were determined using a range of methods, including high resolution fast atom bombardment mass spectrometry (HRFABMS), low resolution electron impact mass spectrometry (LREIMS) and in the case of volutamide D (**90**), single crystal X-ray diffraction (XRD) analysis.⁷²

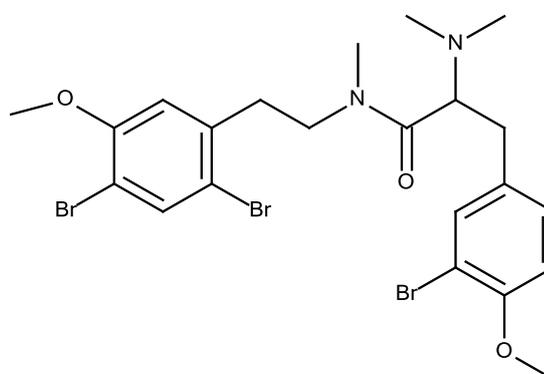
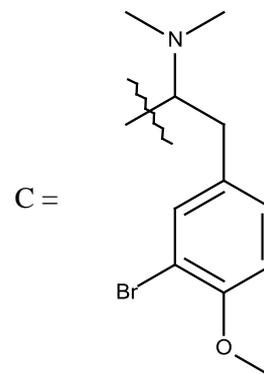
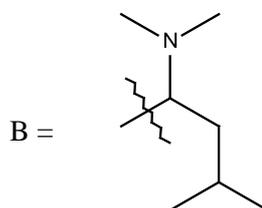
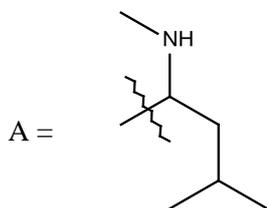


(87) R = -CH₃

(88) R = A

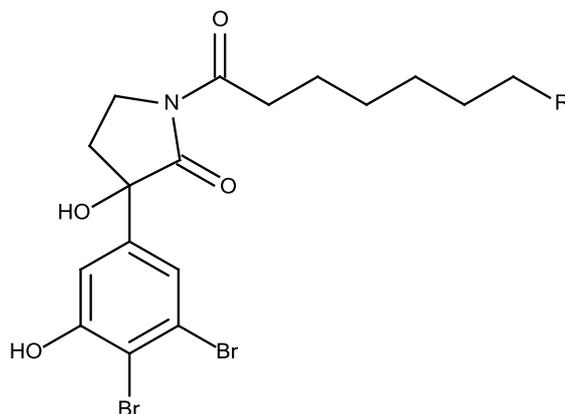
(89) R = B

(91) R = C



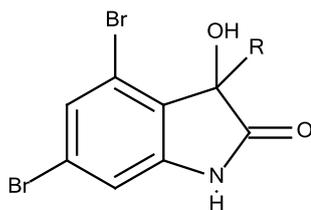
(90)

Six halogenated compounds, convolutamides A-F (**92-97**) were isolated from a collection of *A. convoluta* obtained off the coast of Florida, United States of America. The same species yielded the convolutamines and the volutamides. The convolutamides possess a dibrominated phenol as well as a γ -lactam group. The metabolites were isolated from an organic extract which was subjected to typical separation techniques, with final purification achieved by preparative thin layer chromatography (PTLC) and reversed phase HPLC.⁷³



- (**92**) R = $-(\text{CH}_2)_6\text{CH}_3$
 (**93**) R = $-\text{CH}_2\text{CH}=\text{CH}(\text{CH}_2)_5\text{CH}_3$
 (**94**) R = $-(\text{CH}_2)_8\text{CH}_3$
 (**95**) R = $-\text{CH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{CH}_3$
 (**96**) R = $-(\text{CH}_2)_{10}\text{CH}_3$
 (**97**) R = $-\text{CH}=\text{CH}(\text{CH}_2)_{10}\text{CH}_3$

Also isolated from a Floridian collection of *A. convoluta*, were convolutamydines A-E (**98-102**). Their structures resemble that of convolutindole A (**86**), being based on an indole, however they do not possess the 1-methoxy substitution but rather contain the more typical secondary amine in this position. The structures of each convolutamydine are very similar, only varying in the substitution at one of the indole carbons.^{70, 74, 75}



(98) R = -CH₂COCH₃

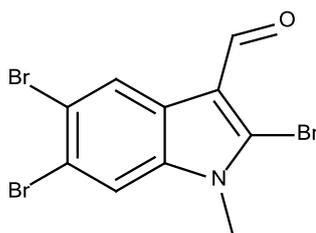
(99) R = -CH₂CH₂Cl

(100) R = -CH₃

(101) R = -CHCH₂

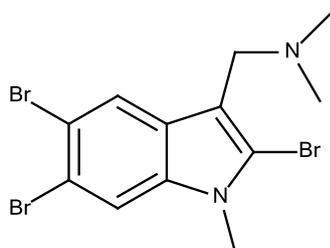
(102) R = -CH₂CH₂OH

2,5,6-Tribromo-*N*-methylindole-3-carbaldehyde (**103**), was isolated from an organic extract of *Zoobotryon verticillatum* collected from Cádiz and Huelva off the Spanish coast⁷⁶ and inhibited egg development of the sea urchin *Paracentrotus lividus*, with an IC₅₀ value of 52 μM.⁷⁷

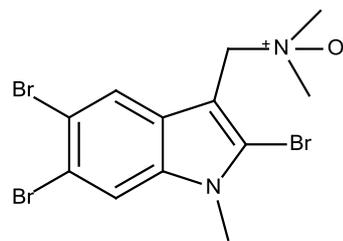


(103)

Another indole containing metabolite, 2,5,6-tribromo-1-methylgramine (**104**), along with its *N*-oxide derivative (**105**), were isolated from *Z. verticillatum* collected from the San Diego region, California, United States of America. Although these compounds were not new, this was the first example of them being isolated as natural products.⁷⁸

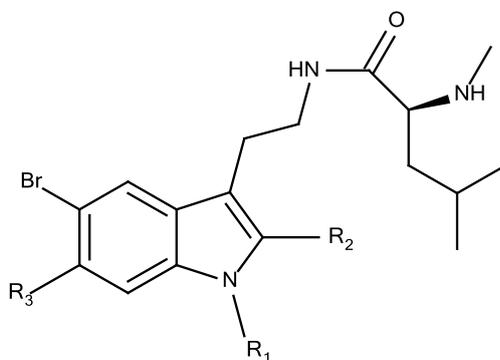


(104)



(105)

Four new tryptamine derived compounds, alternatamides A-D (**106-109**), were obtained from an organic extract of *Amathia alternata*. The samples were collected by self contained underwater breathing apparatus (SCUBA) off the coast of North Carolina, United States of America. Each of the alternatamides are dibrominated, with a bromo-substitution on C5 of the indole and an additional bromo-substitution on either C2 (alternatamides A-C (**106-108**)) or C6 (alternatamide D (**109**)) of the indole. Alternatamides A-C were tested against the Gram-positive bacteria *Staphylococcus aureus*, *S. epidermidis*, *S. haemolyticus*, *Bacillus subtilis*, *Enterococcus faecalis*, *E. faecium* and *Streptococcus pyogenes*, with each showing moderate activity (minimum inhibitory concentration (MIC) values ranging from 4 $\mu\text{g mL}^{-1}$ to 32 $\mu\text{g mL}^{-1}$).⁷⁹



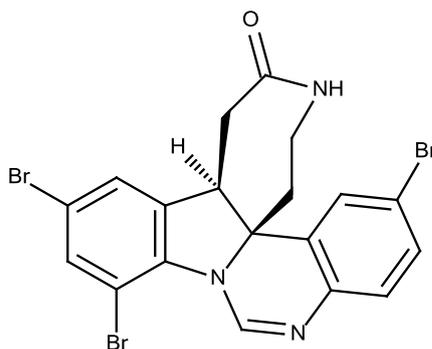
(**106**) $R_1 = -\text{CH}_3$; $R_2, R_3 = -\text{Br}$

(**107**) $R_1 = -\text{H}$; $R_2, R_3 = -\text{Br}$

(**108**) $R_1, R_3 = -\text{H}$; $R_2 = -\text{Br}$

(**109**) $R_1, R_2 = -\text{H}$; $R_3 = -\text{Br}$

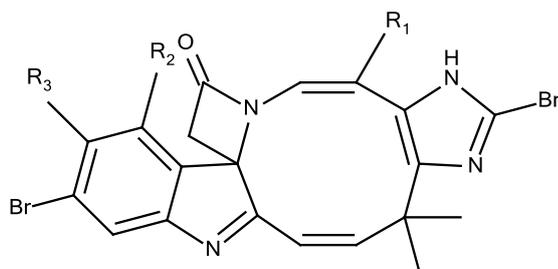
Hinckdentine A (**110**), a tribrominated, indole-containing alkaloid, was isolated from *Hincksinoflustra denticulata* collected from the east coast of Tasmania, Australia. The metabolite was obtained from an organic extract and isolated with the use of HPLC.⁸⁰ Over twenty years after the publication of (**110**), the first total synthesis of the compound was reported.⁸¹



(110)

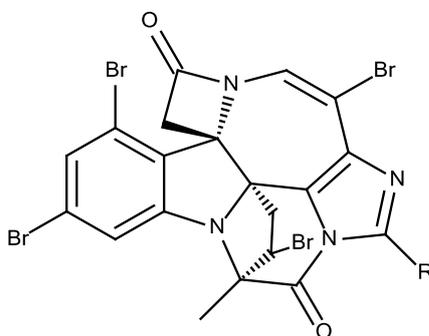
Three novel macrocycles, chartellines A-C (**111-113**), were isolated from *Chartella papyracea* collect by SCUBA, off Roscoff, France. Chartelline A (**111**), a pentahalogenated alkaloid, is monochlorinated and tetrabrominated and, typical of all chartellines, has an unusual structure which consists of an indole, β -lactam, and imidazole group. The compound was isolated from a dichloromethane extract that was subjected to fractionation by liquid chromatographic techniques before final purification by PTLC. The structure of chartelline A (**111**) was determined by high resolution MS, various NMR spectroscopic techniques, and single crystal XRD.⁸²

Chartellines B (**112**) and C (**113**) were isolated from an ethyl acetate extract which had been defatted using petroleum ether. This extract was then fractionated by cellulose chromatography followed by recrystallisation from ethyl acetate and purification by HPLC.⁸³ The presence of an isolation artefact, dechloro-3-methoxychartelline A (**114**), was also noted.⁸³



- (111) $R_1 = -Cl$; $R_2, R_3 = -Br$
 (112) $R_1 = -Cl$; $R_2 = -Br$; $R_3 = -H$
 (113) $R_1 = -Cl$; $R_2, R_3 = -H$
 (114) $R_1 = -OCH_3$; $R_2, R_3 = -Br$

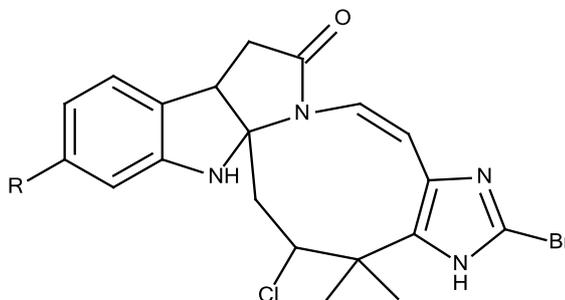
Further β -lactam containing alkaloids, chartellamides A (**115**) and B (**116**), were isolated from a collection of *C. papyracea* obtained off the coast of Roscoff, France. The chartellamides are closely related to the chartelline alkaloid family, possessing similar indole, imidazole, and β -lactam groups. The metabolites were isolated from an ethyl acetate extract which was fractionated using cellulose chromatography, silica gel chromatography and HPLC. The structures of (**115**) and (**116**) were determined by typical spectroscopic techniques.⁸⁴



- (115) $R = -H$
 (116) $R = -Br$

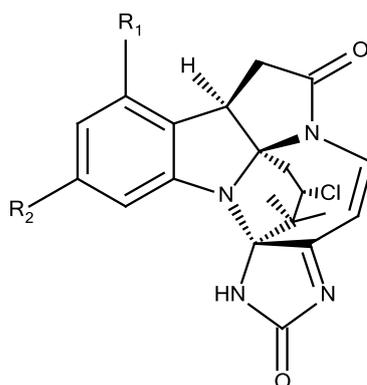
Nine novel natural products, securamines A-G (**117-123**) and securines A (**124**) and B (**125**) were isolated from organic extracts of *Securiflustra securifrons*, collected from the Danish west coast in the North Sea.^{85, 86} The structures of the securamines have similarities to the chartellines, in that they each possess an indole, imidazole and lactam group, however the securamines possess a γ -lactam group rather than the β -lactam group possessed by the chartellines.^{85, 86} The securines contain a macrocyclic lactam ring rather than the γ -lactam substituent

seen in the securamines. It has been hypothesised that the securines could act as precursors to not only the securamines but also to the chartellines. It was also hypothesised by Rahbaek *et al.* that securamine A is a precursor to the other three securamines.⁸⁵



(117) R = -H

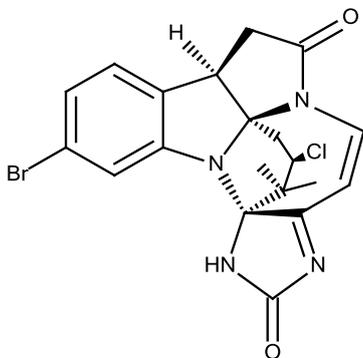
(118) R = -Br



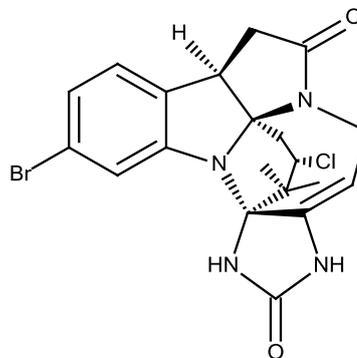
(119) R₁ = -H; R₂ = -Br

(120) R₁, R₂ = -H

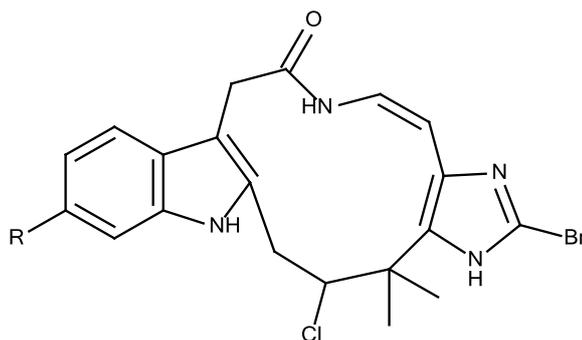
(121) R₁, R₂ = -Br



(122)



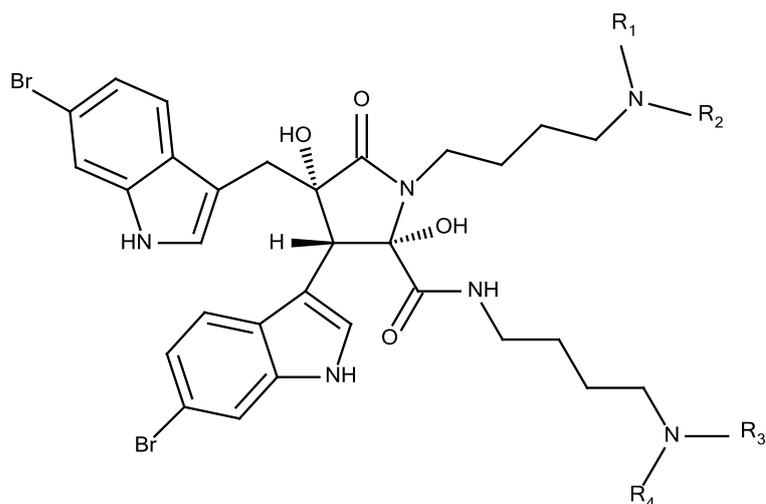
(123)



(124) R = -H

(125) R = -Br

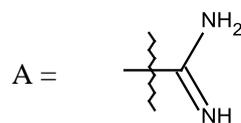
Four novel eusynstyelamides have also been isolated from bryozoa. The metabolites eusynstyelamides D-F (**126-128**), as well as ent-eusynstyelamide B (**129**), an enantiomer of the previously published eusynstyelamide B (**130**) were isolated from the Arctic dwelling species *Tegella cf. spitzbergensis*.⁸⁷ The eusynstyelamides are tryptophan-derived compounds containing two brominated indole groups with a γ -lactam group between the two. The previously published eusynstyelamides were isolated from the ascidian *Eusynstyela latericius*.⁸⁸ There is some ambiguity in the absolute structure of ent-eusynstyelamide B (**129**), as its published structure is identical to that of eusynstyelamide B (**130**), however, here it is assumed that (**129**) is simply the enantiomer of (**130**) published by Tapiolas *et al.*^{87, 88}

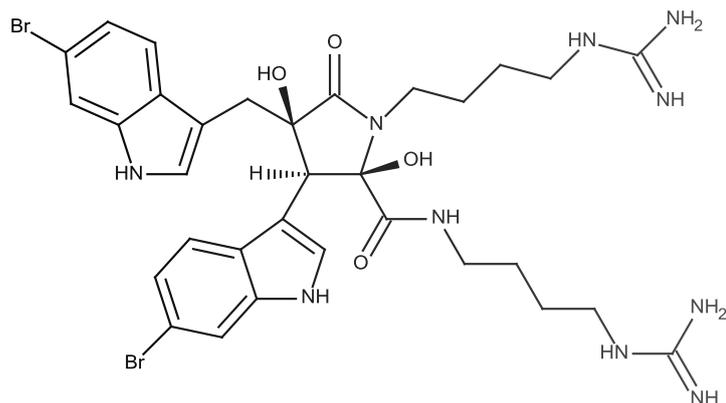


(126) R₁, R₂, R₃, R₄ = -H

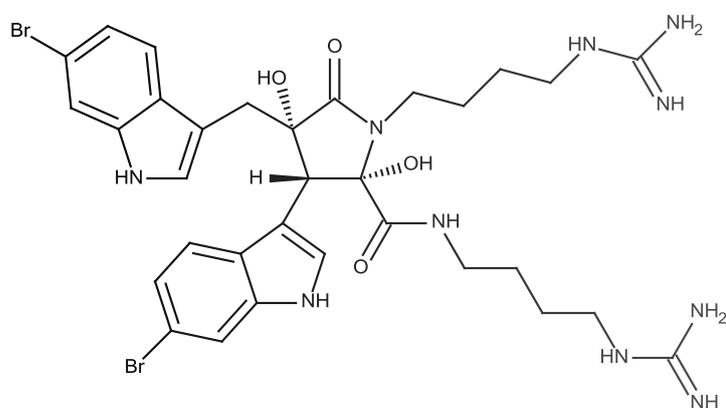
(127) R₁, R₃, R₄ = -H; R₂ = A

(128) R₁, R₂, R₄ = -H; R₃ = A



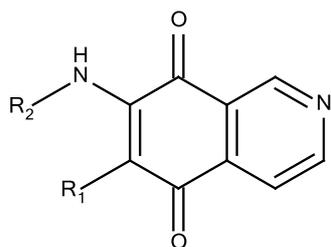


(129)

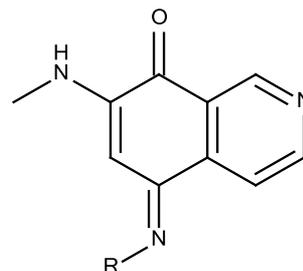


(130)

Bioassay guided fractionation of an aqueous extract of *Caulibugula intermis* collected from the Indo-Pacific region near Palau, led to the isolation of six new secondary metabolites, caulibugulones A-F (**131-136**). The extract was subjected to liquid-liquid extraction, with final isolation of the metabolites achieved by HPLC and structural determination via techniques including HRFABMS and NMR spectrometry. The six metabolites were assayed against the NCI murine IC-2^{WT} cell line and displayed significant activity, with IC₅₀ values ranging from 0.03 $\mu\text{g mL}^{-1}$ for caulibugulone E (**135**) to 1.67 $\mu\text{g mL}^{-1}$ for caulibugulone D (**134**).⁸⁹

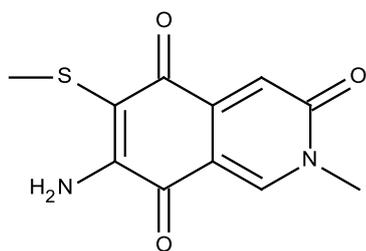


- (131) $R_1 = -H$; $R_2 = -CH_3$
 (132) $R_1 = -Br$; $R_2 = -CH_3$
 (133) $R_1 = -Cl$; $R_2 = -CH_3$
 (134) $R_1 = -H$; $R_2 = -CH_2CH_2OH$

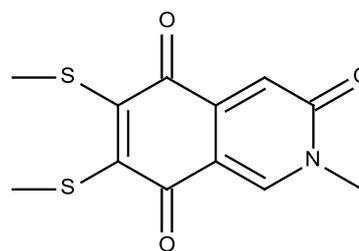


- (135) $R = H$
 (136) $R = -CH_2CH_2OH$

Two new isoquinoline quinones were isolated from an organic extract of *Membranipora perfragilis*, gathered by SCUBA at Rapid Bay in South Australia. The structures of the two compounds, perfragilins A (**137**) and B (**138**), closely resemble those of caulibugulones A-D (**131-134**) and were separated by typical chromatographic techniques, with purification achieved with gel permeation chromatography and PTLC.⁹⁰

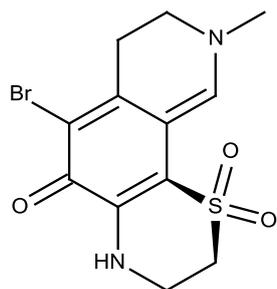


(137)

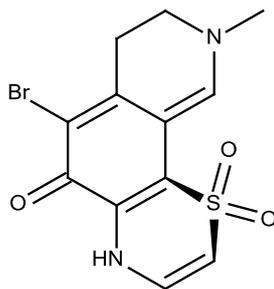


(138)

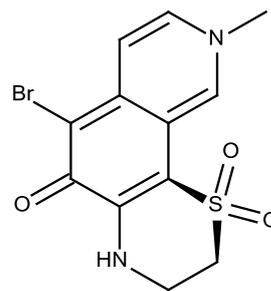
The euthyroideones were isolated from an organic extract of *Euthyroides episcopalis*, which was collected by SCUBA from Fiordland, South Island, New Zealand. The structures of these monobrominated metabolites, euthyroideones A-C (**139-141**), are very similar, only differing in the degree and location of saturation.⁹¹



(139)



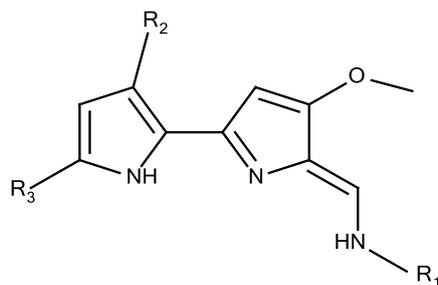
(140)



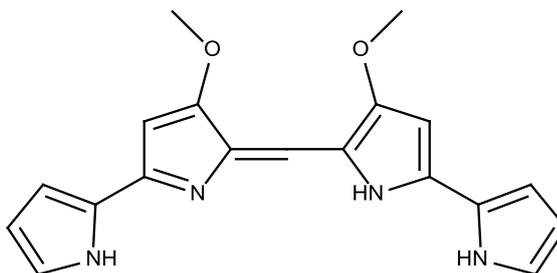
(141)

Tambjamines A-K (**142-152**) have been isolated from various marine sources including bryozoa. Tambjamines A-D (**142-145**) were isolated from the nudibranchs, *Tambje abdere*, *T. eliora*, and *Roboastra tigris* collected in the Gulf of California, United States of America.⁹² It was also found that the tambjamines were present in extracts of the bryozoan *Sessibugula translucens*, a prey of the nudibranchs, suggesting that the tambjamines were being sequestered as part of their dietary intake.⁹² Tambjamines E (**146**) and F (**147**) were first isolated from species of the phylum Chordata and tambjamines G-J (**148-151**) were isolated from the bryozoa *Bugula dentata* along with tambjamine E (**146**).^{93, 94} Tambjamine K (**152**) was isolated from the nudibranch *Tambja ceutae* and was also found in *B. dentata* in much smaller amounts. This suggests that tambjamine K (**152**), like tambjamines A-D (**142-145**), is also of dietary origin.⁹⁵ All of the tambjamines share a methoxy-substituted bipyrrrole structure and the majority of them are brominated.

A structurally related tetrapyrrole, blue pigment (**153**) has also been isolated from *B. dentata*. The pigment had previously been isolated from the bacterium *Serratia marcescens* and was shown to display significant antimicrobial activity.⁹⁶

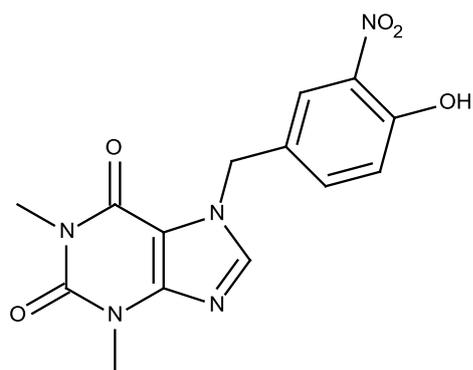


- (142) $R_1, R_2, R_3 = -H$
 (143) $R_1, R_2 = -H; R_3 = -Br$
 (144) $R_1 = -CH_2CH(CH_3)_2; R_2, R_3 = -H$
 (145) $R_1 = -CH_2CH(CH_3)_2; R_2 = -Br; R_3 = -H$
 (146) $R_1 = -CH_2CH_3; R_2, R_3 = -H$
 (147) $R_1 = -CH_2CH_2Ph; R_2, R_3 = -H$
 (148) $R_1 = -CH_2CH_3; R_2 = -H; R_3 = -Br$
 (149) $R_1 = -CH_2CH_2CH_3; R_2 = -H; R_3 = -Br$
 (150) $R_1 = -CH_2CH(CH_3)_2; R_2 = -H; R_3 = -Br$
 (151) $R_1 = -CH_2CH(CH_3)CH_2CH_3; R_2 = -H; R_3 = -Br$
 (152) $R_1 = -CH_2CH_2CH(CH_3)_2; R_2, R_3 = -H$



(153)

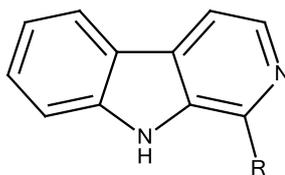
Phidolopin (**154**), a compound based on a xanthine structure derived from purine, was isolated from *Phidolopora pacifica* collected off the coast of Barkley Sound, British Columbia, Canada. Its structure represents a significant addition to the xanthine class of compounds as it is from animal origin, whereas the vast majority of xanthenes that have been discovered, such as caffeine and theobromine, are from the *Plantea* kingdom. A proportion of the collection was extracted using organic solvents and separated further using LC and PTLC to give (**154**). The structure of (**154**) was determined by single crystal XRD of its *p*-bromophenacyl derivative.⁹⁷



(154)

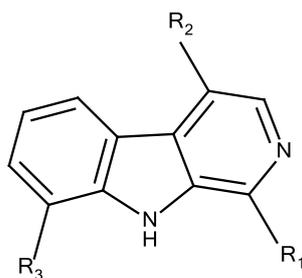
Like phidolopin (**154**), other secondary metabolites that are related to natural products found in terrestrial species have also been found in marine bryozoa. β -Carboline alkaloids, a class of compounds that are found in numerous terrestrial species, including plants, fungi and animals,⁹⁸⁻¹⁰⁰ have been isolated from an array of bryozoan species, in some cases with functional groups unique to the aquatic animals.

The first β -carboline alkaloids to be obtained from a bryozoan extract were isolated from *Costaticella hastata* collected from Tasmania, Australia.¹⁰¹ Of the four compounds isolated, three had been previously isolated from terrestrial species, while the other was a novel metabolite. The known compounds were 1-methyl- (**155**), 1-ethyl- (**156**) and 1-vinyl- β -carboline (pavettine) (**157**), the first having been isolated from numerous terrestrial species, including plants and fungi¹⁰² and the other two having been isolated from the plant species *Hannoa klaineana*¹⁰³ and *Pavetta lanceolata*,^{100, 103} respectively. The novel compound (*S*)-1-(1'-hydroxyethyl)- β -carboline (**158**) was obtained from a sample that was successively extracted with various organic solvents. It was isolated by typical chromatographic techniques and purified by PTLC.¹⁰¹



- (155) R = -CH₃
 (156) R = -CH₂CH₃
 (157) R = -CHCH₂
 (158) R = -CH(OH)CH₃

Two more novel β -carboline alkaloids, 8-hydroxy-1-vinyl- β -carboline (**159**) and 1-ethyl-4-methylsulfone- β -carboline (**160**) were obtained from an organic extract of *Cribricellina cribraria* from Kaikoura, South Island, New Zealand. Separation of the crude extract was achieved by C₁₈ chromatography and in the case of 1-ethyl-4-methylsulfone- β -carboline (**160**), reversed phase HPLC. Fractionation was guided by both thin layer chromatography (TLC) and bioassay against P388 cell lines.¹⁰⁴ The same publication compared the biological activities of these two metabolites with those of a large number of previously published β -carboline alkaloids. The results of these comparisons suggested that the 1-vinyl substitution was of particular significance for activity against the P388 cell line, with 8-hydroxy-1-vinyl- β -carboline (**159**) and 1-vinyl- β -carboline (**157**) having IC₅₀ values of 100 ng mL⁻¹ whereas 1-ethyl- β -carboline (**156**) and 1-methyl- β -carboline (**155**) had IC₅₀ values of 25 000 ng mL⁻¹.¹⁰⁴ Preparation of derivatives, some with and some without 1-vinyl substitutions, confirmed the importance of this group for increased potency against the P388 cell line.¹⁰⁴ Synthetic β -carboline compounds without the vinyl substitution had little or no activity in the assay, providing further confirmation.¹⁰⁴ The same trend was observed when the metabolites were assayed for antiviral activity.¹⁰⁴ The trend did not hold up however when the metabolites were assayed for antimicrobial and antifungal activity and it was suggested that in this case, solubility of the compounds had a greater correlation to MID values than the 1-vinyl substitutions.¹⁰⁴

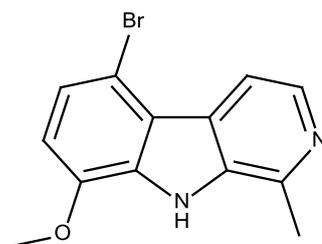


(159) R₁ = -CH=CH₂, R₂ = -H, R₃ = -OH

(160) R₁ = -CH₂CH₃, R₂ = -S(O₂)CH₃, R₃ = -H

8-Hydroxy-1-vinyl- β -carboline (**159**) has also been isolated from an organic extract of a *Catenicella cribraria* specimen collected at Cape Vlamingh, Rottnest Island, Western Australia. Isolation was carried out by bioassay guided fractionation against LOX IMVI melanoma and U251 CNS cell lines.¹⁰⁵

Another novel β -carboline, 5-bromo-8-methoxy-1-methyl- β -carboline (**161**), was isolated from *Pterocella vesiculosa* collected from the Alderman Islands off the North Island of New Zealand. (**161**) was tested for bioactivity against the P388 cell line, the bacterium *Bacillus subtilis* and the fungi *Candida albicans* and *Trichophyton mentagrophytes* and proved to be moderately potent, with an IC₅₀ value of 5 089 ng mL⁻¹ and minimum inhibitory doses (MID) of, 2-4 μ g mL⁻¹, 4-5 μ g mL⁻¹ and 4-5 μ g mL⁻¹, respectively.¹⁰⁶

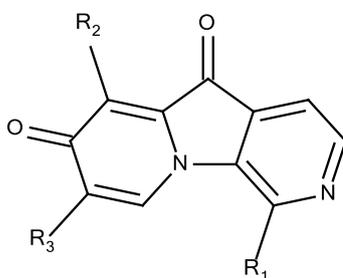


(161)

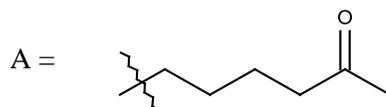
A series of alkaloids, the pterocellins, were isolated from *P. vesiculosa*. Pterocellins A (**162**) and B (**163**) were isolated from an organic extract of *P. vesiculosa* collected from the Hen and Chicken Islands off the North Island of New Zealand.¹⁰⁷ Both compounds were assayed for antitumour, antibacterial, antifungal and antiviral activity and possessed relatively strong activity against the P388 cell line with IC₅₀ values of 477 ng mL⁻¹ and 323 ng mL⁻¹ for pterocellins A

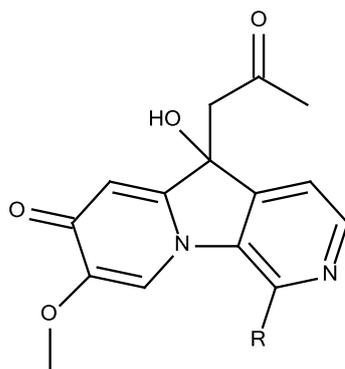
(**162**) and B (**163**) respectively. Both compounds also showed strong activity against the Gram-positive bacterium *Bacillus subtilis* and the fungus *Tricophyton mentagrophytes*, in addition to cytotoxicity to the BSC-1 cell line. The structures of the pterocellins are reminiscent of β -carboline alkaloids but rather than being based around an indole structure, the pterocellins are based on a 4-pyridone group and a pyridine group bound together through a five membered ring. The similarities between β -carboline alkaloids and pterocellin alkaloids have led to the hypothesis that the pterocellin compounds may be biosynthetically derived from β -carboline alkaloids.¹⁰⁷

Further pterocellins, pterocellins C-I (**164-170**), were isolated from the same collection from which pterocellins A (**162**) and B (**163**) were obtained.¹⁰⁸⁻¹¹⁰ Pterocellins C (**164**), E (**166**), F (**167**), H (**169**) and I (**170**) possessed little or no bioactivity against the P388 cell line, unlike pterocellins A (**162**) and B (**163**) which are potent inhibitors of this cell line.¹⁰⁷⁻¹⁰⁹ Pterocellin D (**165**) displayed moderate inhibition of the P388 cells with an IC_{50} value of $4\ 773\ \text{ng mL}^{-1}$.¹⁰⁸ Unlike all other pterocellin alkaloids, pterocellins E (**166**) and F (**167**), are dimers.^{108, 109}



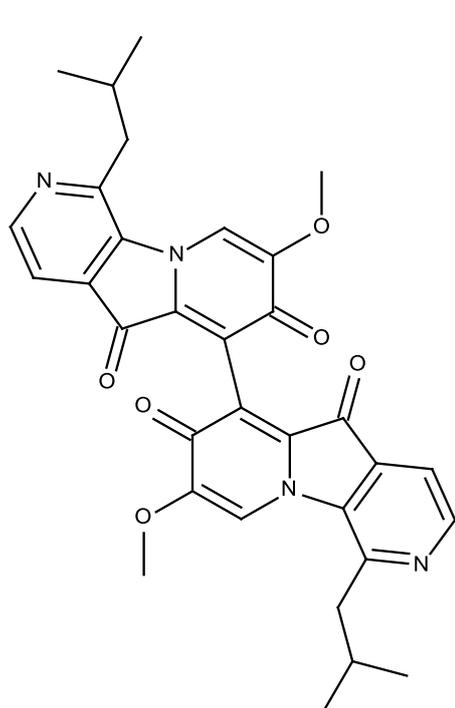
- (**162**) $R_1 = -\text{CH}_2\text{CH}(\text{CH}_3)_2$, $R_2 = -\text{H}$, $R_3 = -\text{OCH}_3$
 (**163**) $R_1 = -\text{CH}_2\text{Ph}$, $R_2 = -\text{H}$, $R_3 = -\text{OCH}_3$
 (**164**) $R_1 = -\text{CH}_2\text{CH}(\text{CH}_3)_2$, $R_2 = \text{A}$, $R_3 = -\text{OCH}_3$
 (**165**) $R_1 = -\text{CH}_2\text{CH}(\text{CH}_3)_2$, $R_2 = -\text{CH}(\text{CH}_3)_2$, $R_3 = -\text{OCH}_3$
 (**168**) $R_1 = -\text{CH}_2\text{CH}(\text{CH}_3)_2$, $R_2 = -\text{H}$, $R_3 = -\text{OH}$



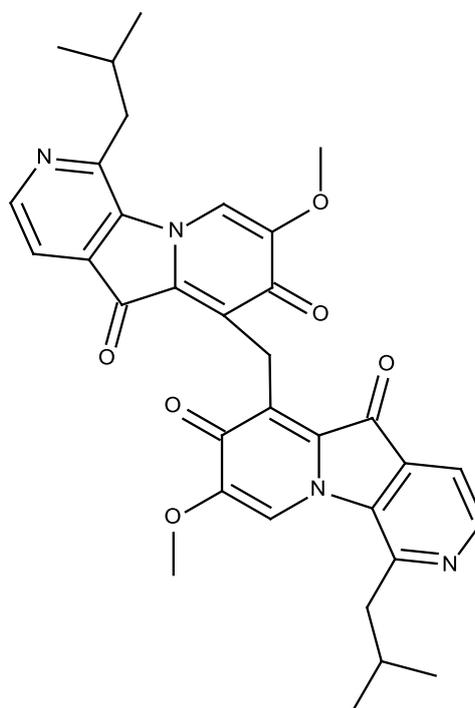


(169) R = $-\text{CH}_2\text{CH}(\text{CH}_3)_2$

(170) R = $-\text{CH}_2\text{Ph}$

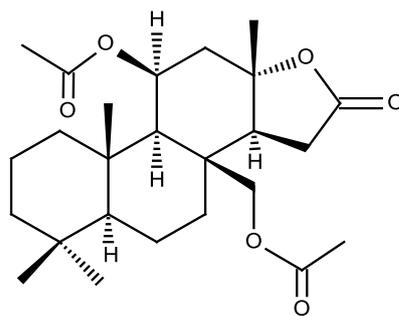


(166)



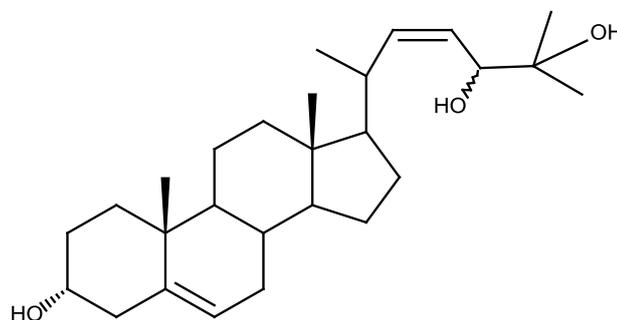
(167)

Secondary metabolites other than alkaloids have also been isolated from bryozoa. Murrayanolide (**171**) is a tetracyclic C_{21} - C_{23} -ene terpenoid containing a γ -lactone group, which was isolated from *Dendrobeatia murrayana* collected off the east coast of Canada. The determination of its structure was achieved with the use of 2D NMR spectroscopic techniques. Murrayanolide (**171**) is a significant compound in that very few terpenes (with similar structures) have been isolated from marine organisms, including bryozoa and it displayed moderate inhibition of metalloprotease collagenase IV, with 54 % inhibition at $25 \mu\text{g mL}^{-1}$.¹¹¹

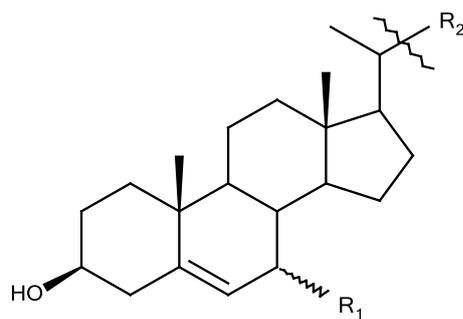


(171)

An oxygenated sterol, (22Z)-3 α ,24 ξ ,25-trihydroxycholesta-5,22-diene (**172**), was isolated from *Biflustra grandicella*, collected off Huang Island in the Peoples Republic of China. Three previously reported sterols, cholesta-5-ene-3 β ,7 α -diol (**173**), cholesta-5-ene-3 β ,7 β -diol (**174**) and 24-methylcholesta-5,22(*E*)-dien-3 β ,7 α -diol (**175**) were also obtained. Each of the compounds was isolated from an organic extract which was subjected to typical chromatographic separation.¹¹²



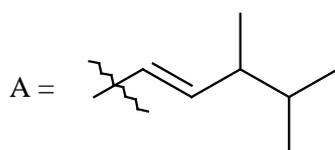
(172)



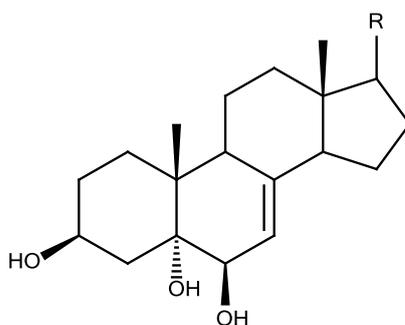
(173) R₁ = α -OH; R₂ = $-(\text{CH}_2)_3\text{CH}(\text{CH}_3)_2$

(174) R₁ = β -OH; R₂ = $-(\text{CH}_2)_3\text{CH}(\text{CH}_3)_2$

(175) R₁ = α -OH; R₂ = A



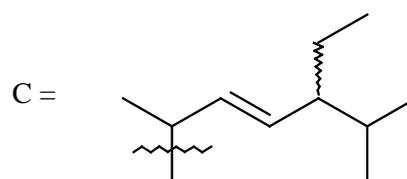
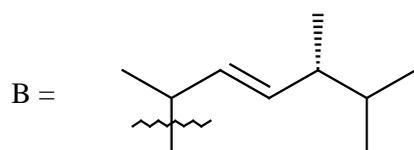
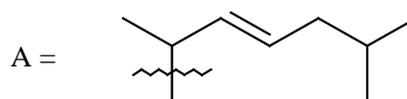
Three new $3\beta,5\alpha,6\beta$ -trihydroxysterols, (22*E*)-cholesta-7,22-dien- $3\beta,5\alpha,6\beta$ -triol (176), (22*E,24R*)-24-methylcholesta-7,22-dien- $3\beta,5\alpha,6\beta$ -triol (178) and (22*E,24E*)-24-ethylcholesta-7,22-dien- $3\beta,5\alpha,6\beta$ -triol (179) were isolated from a chloroform extract of the Mediterranean bryozoan *Myriapora truncata*. The specimen was collected off the coast of the Bay of Naples, Italy.¹¹³



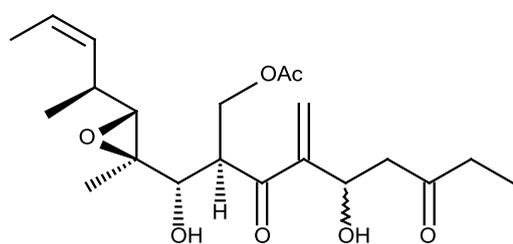
(176) R = A

(177) R = B

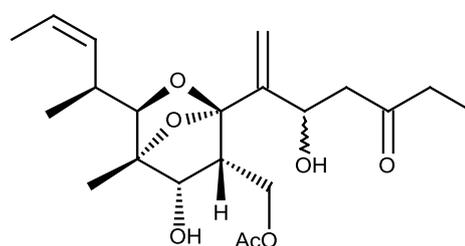
(178) R = C



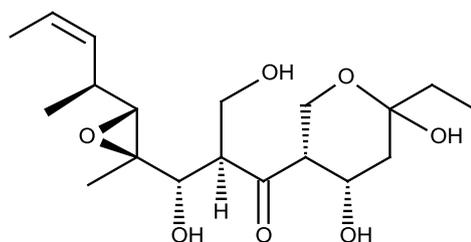
Four polyketide-derived compounds, myriaporones 1-4 (**179-182**) were also isolated from *M. truncata* collected from the Western Mediterranean Sea. The separation and structural determination of myriaporones 1 (**179**) and 2 (**180**) proved to be much more straightforward than that of myriaporones 3 (**181**) and 4 (**182**). This was because (**181**) and (**182**) are isomers that interconvert, reaching an equilibrium of 3:1 of (**181**):(**182**). As these two compounds could not be separated, their structures had to be determined by analysis of a mixture. Of the four metabolites, the equilibrium mixture of (**181**) and (**182**) proved to be the most bioactive against L1210 murine leukaemia cells, displaying growth inhibition of 88 % at 0.2 $\mu\text{g mL}^{-1}$.¹¹⁴



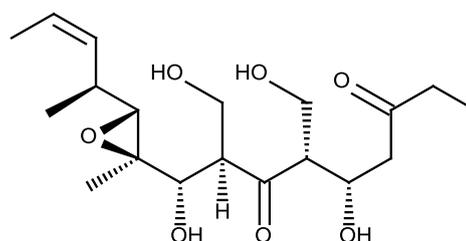
(**179**)



(**180**)



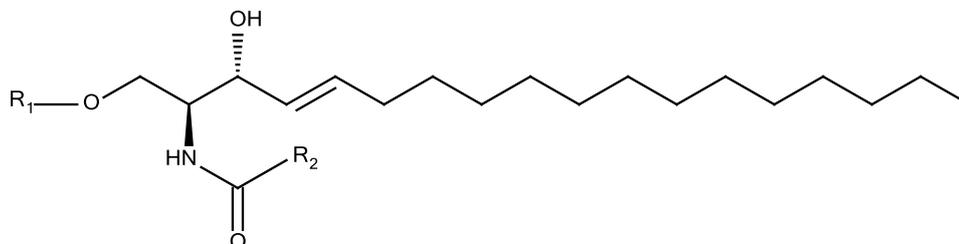
(**181**)



(**182**)

Three disulfides, pentaporins A-C (**183-185**), were isolated from an organic extract of *Pentapora fascialis* prepared from a specimen collected in the Mediterranean Sea. Isolation of the pentaporins was achieved by bioassay-guided fractionation utilising the parasitic worm *Trichinella spiralis*, however specific activities of individual metabolites were not mentioned in the publication.¹¹⁵ Isolation and structural determination of the metabolites was achieved by various techniques, including size exclusion chromatography and NMR spectroscopy.¹¹⁵

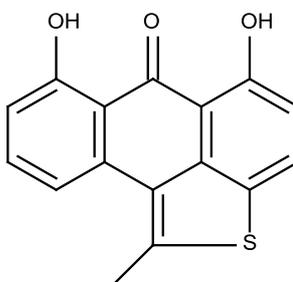
Another ceramide, (2*S*,3*R*,4*E*)-2-(14'-methyl-pentadecanoylamino)-4-octadecene-1,3-diol (**188**) and a cerebroside, 1-*O*-(β -D-glucopyranosyl)-(2*S*,3*R*,4*E*)-2-(heptadecanoylamino)-4-octadecene-1,3-diol (**189**), were isolated from a collection of *Bugula neritina*, obtained from Daya Bay, Shenzhen, Guangdong Province, Peoples Republic of China in the South China Sea.¹¹⁷



(**188**) $R_1 = -H$; $R_2 = -(CH_2)_{12}CH(CH_3)_2$

(**189**) $R_1 = \text{glc}$; $R_2 = -(CH_2)_{15}CH_3$

A novel antiangiogenic metabolite, bryoanthrathiophene (**190**), was isolated from an acetone extract of *Watersipora subtorquata*, collected from Tsutsumi Island, Fukuoka Prefecture, Japan and its structure was determined by typical techniques.¹¹⁸ (**190**) displayed significant bioactive properties, with an IC_{50} value of 0.005 μmol against bovine aortic endothelial cells.¹¹⁸



(**190**)

The number of natural products that have been isolated and characterised from any freshwater species of animal or plant, let alone freshwater bryozoa, is relatively small in comparison to marine natural products. The main reason for the large distinction is that of all the water on Earth, less than 3 % of it is fresh.¹¹⁹ Most of the water that makes up that 3 % is either in groundwater or locked up in ice caps and glaciers. Less than 0.01 % of all water on Earth is found in

freshwater rivers and lakes and this has a significant effect on the amount of biodiversity that is found in fresh water compared to marine environments.¹¹⁹

This lack of biodiversity is reflected in the number of freshwater bryozoa species compared to their marine counterparts. To date, only one order of freshwater bryozoa has been described, containing only five extant families, compared to three orders with over 100 families containing mostly marine species.⁹

1.4 Bioassays

Fractionation of biological extracts is often guided by bioassays in an attempt to isolate the most potent active metabolite. A wide range of assays (*in vitro*, *in vivo* or *ex vivo*) can be used, depending on the intention of the research being undertaken. *In vitro* experiments are biological assays conducted without the use of a whole animal, typically utilising cultures of specific cell types, whereas *in vivo* experiments are performed on whole, living organisms and *ex vivo* experiments utilise tissue outside of the organism, typically an organ or a part of one.

One of the main advantages of using *in vitro* assay techniques in natural product isolation is the rapid turnover and reproducibility of results. This helps to speed up the isolation of active metabolites and give a good basis to compare compounds. Although *in vitro* experiments are the most common type of assay utilised in bioactive natural product isolation, they do have disadvantages when compared to *in vivo* experiments. As *in vivo* assays utilise the whole, living organism, the compounds of interest have the potential to interact with all of the metabolic processes which can lead to unpredictable side-effects which would not be evident in *in vitro* experiments. Also with *in vivo* experiments, there are multiple means of administration of the compounds of interest, such as intravenous, intramuscular, oral and transdermal, each of which can produce different results depending on the compounds being administered.¹²⁰

Bioassays utilised in the work outlined in this thesis were *in vitro*, detecting antitumour activity using murine P388 lymphocytic leukaemia cells and were carried out at both the University of Canterbury by Gill Ellis and at AgResearch,

at the Ruakura Research Centre in Hamilton by the author of this thesis, under the supervision of Megan Callaghan. The P388 assay is commonly used when trying to isolate natural products with antitumour properties, due to its ease of use and relatively rapid turnover rate (72 hours).

The metabolised indicator used for this assay was MTS tetrazolium (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) which is metabolised by healthy cells to MTS formazan (Figure 5). This process of metabolism of MTS tetrazolium produces a colour change from yellow to a dark red/brown. By comparing the colour change in blank and positive control wells to that of wells containing active compounds, it is possible to determine the concentration at which 50 % of the P388 cell lines are inhibited. Four hours after MTS tetrazolium is added to each well of the microtitre plate, the plate is scanned by a plate reader at 490 nm and at 360 nm. MTS formazan absorbs at 490-500 nm, however any turbidity in the wells will give inaccurate readings. To compensate for the turbidity in the wells, a background reading is taken at 360 nm and this turbidity reading is then subtracted from the reading at 490 nm to give a turbidity adjusted measurement of metabolised MTS tetrazolium.

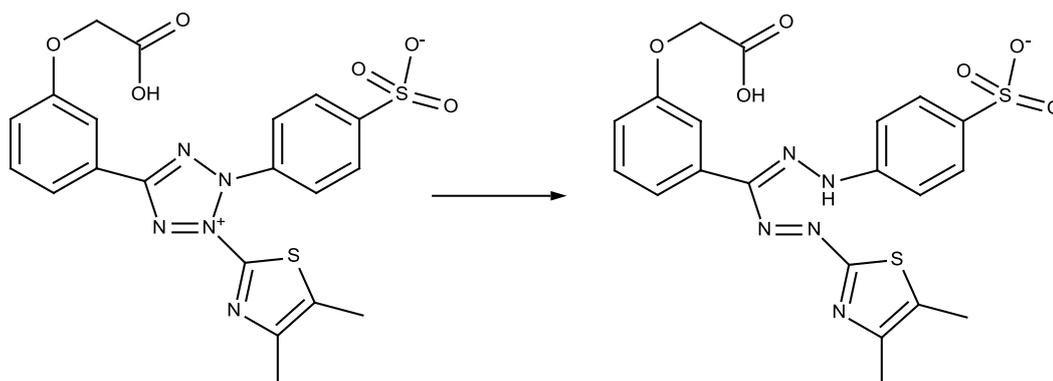


Figure 5: The conversion of MTS tetrazolium (yellow) to its formazan product (red)

Samples are tested in eight two-fold serial dilutions and run in duplicate, starting at a concentration of $12\ 500\ \text{ng mL}^{-1}$ to the least concentrated dilution of $98\ \text{ng mL}^{-1}$.

A typical preparation for bioassay involved diluting the fraction for bioassay to a known concentration in methanol and delivering a proportion of this into a clean weighed vial. The methanol was then evaporated from this subsample, leaving a known mass for bioassay, typically 1 or 0.1 mg depending on the stage of chromatographic separation.

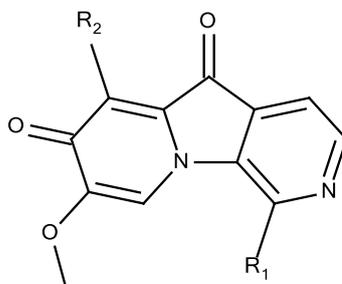
Chapter Two: *Pterocella vesiculosa*

2.1 Introduction

Natural products form the basis of many pharmaceutical drugs in use today, most of which have been isolated or derived from organisms inhabiting terrestrial environments, rather than from species of marine or freshwater origins.

Organisms which inhabit aqueous environments are greatly underutilised in the area of secondary metabolite research. The research in this thesis attempts to tap into this large and largely unexplored, resource.

Pterocella vesiculosa is a species of bryozoan from the class Gymnolaemata. Like most bryozoa, it forms colonies and has a wide habitual range. *P. vesiculosa* was the source of pterocellins, A to I (**162-170**), as well as the brominated β -carboline alkaloid, 5-bromo-8-methoxy-1-methyl- β -carboline (**161**). The presence of additional pterocellin and β -carboline alkaloids has also been noted in extracts of this species, including pterocellins J-L (**191-193**).^{109, 110} The *P. vesiculosa* specimen used in this research was collected from the Alderman Islands, off the North Island of New Zealand.



- (191) $R_1 = -CH_2CH(CH_3)_2$; $R_2 = -CH_2CH_2CH_3$
(192) $R_1 = -CH_2Ph$; $R_2 = -CH_2CH_2CH_3$
(193) $R_1 = -CH_2Ph$; $R_2 = -CH_2CH_2CH_2CH_3$

Research performed by Marisa Till as part of an MSc thesis, resulted in the isolation of the new pterocellin alkaloids H (**169**) and I (**170**) from a proportion of the Alderman Islands specimen.¹⁰⁹ Following on from this research, further investigations on metabolites from the same Alderman Islands specimen were carried out for an undergraduate research project by Katie Moore. A sample of the

P. vesiculosa specimen was extracted with dichloromethane and then halved (Extracts A and B) for ease of handling. Extract A was separated with C₁₈ LC to give a series of fractions which were further investigated as described in this thesis.

Isolation of greater amounts of pterocellins H (**169**) and I (**170**) as well as identification of further pterocellin and β -carboline alkaloids were principle aims of this research. The pterocellin alkaloids that have been previously isolated were easily distinguishable as distinct, rose pink to deep red spots on TLC plates.

2.2 Pterocellin Alkaloids

A proportion of the crude dichloromethane extract (Extract B) that was previously prepared by Katie Moore, was attained for further investigation. This particular extract was salvaged from a failed C₁₈ column and was first cleaned of C₁₈ particulates by filtering the extract through a Büchner funnel and washing with methanol and dichloromethane. The extract was then fractionated by reversed phase C₁₈ chromatography.

Fractions from the C₁₈ column were analysed by TLC and compared to the TLC results of Extract A fractions and a pterocellin A (**162**) reference sample.

Fractions which appeared to contain pterocellin-like compounds and those that had similar TLC results were combined and separated further by size exclusion chromatography. TLC analysis of fractions from one of these columns resulted in the identification of three late eluting fractions which contained pink metabolites with relatively high R_f values (approximately 0.7 with 5:1 ethyl acetate:methanol on silica). The late elution of these metabolites during size exclusion chromatography (LH-20) would suggest they are compounds of small mass and/or contain aromatic substituents. These fractions (AA1-42.8 to 10, Appendix: A1.2) were combined and separated further by size exclusion chromatography. TLC of the resulting fractions suggested the presence of two pterocellin-like metabolites, indicated by pink spots on the TLC plate with R_f values of ~ 0.75 and ~ 0.2. Although the purpose of the size exclusion column was to isolate the metabolite with the R_f value of ~ 0.75, TLC analysis of the column fractions indicated that separation of individual compounds had not been achieved.

To gather more information on the metabolite composition of these fractions, they were subjected to analysis by LCMS. Over the fourteen fractions assessed, there were eleven prominent ions observed (Appendices: A1.6 to A1.8), along with multiple minor ions, two of which, by interpretation of their isotope patterns, were brominated (Table 1). Included in the eleven prominent ions was the m/z 377.1 ion, an ion which is indicative of the $[M+H]^+$ ion of pterocellin I (**170**). Of the eleven metabolites, the m/z 243 and 411 ions most closely correlated with the ~ 0.2 and ~ 0.75 R_f spots seen in TLC analysis. Unfortunately all fractions assessed were still complex mixtures of metabolites, which was also suggested by TLC analysis.

Table 1: Significant ions observed in AA1-58 fractions, including minor brominated m/z 277/279 and 362/364 ions

Observed Ions (m/z)	Time of elution on LCMS (minutes)
183	11.8
229	10.8
243	13.1
257	10.4
261	10.1
333	12.7
377	11.2
379	11.5
411	13.4
501	15.4
535	16.3
277/279	11.0
^a 362/364 (384/386)	11.8

^a $[M+H]^+$ ($[M+Na]^+$)

Although pterocellins generally display a distinctive UV spectrum with several prominent adsorptions (Figure 6) that can help identify them at the early stages of separation, the UV chromatograms of the above fractions showed no significant features.

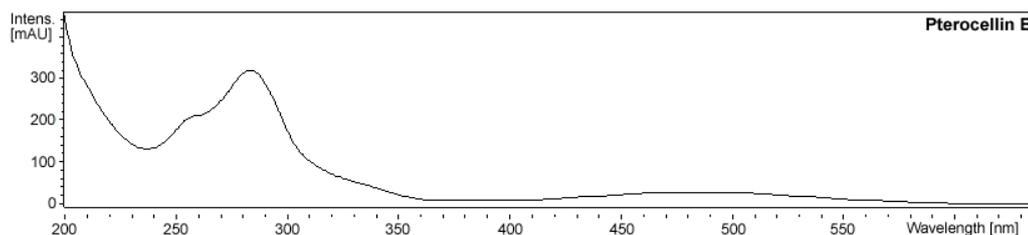


Figure 6: UV spectrum of pterocellin B

An attempt was made to obtain molecular formulae of the significant ions with the use of high resolution electrospray ionisation mass spectrometry (HRESIMS), however the complex mixtures of the fractions gave poor ionisation of the metabolites of interest and no reliable molecular formulae were obtained.

During the separation process, it was noted that a number of fractions contained metabolite/s that would not move from the baseline of the TLC plate using the standard solvent scheme (5:1, ethyl acetate:methanol on silica), indicating the presence of relatively polar compound/s. The colour of the baseline spot was not too dissimilar to the characteristic red of pterocellin alkaloids. To obtain better differentiation between compounds making up the polar spot, the development of a new solvent scheme was needed. After trying various solvent combinations, the best results were achieved using a mixture of ethanol and methanol (1:1). This resulted in the elevation and resolution of the polar metabolites and was still sufficiently non-polar to not dissolve the silica on the TLC plate. The new solvent method is a significant development, since previously, the most polar metabolites were not observable with TLC and were not considered targets for further separation because of the lack of resolution of metabolites.

After using the new solvent method, it was possible to conclude that the baseline spot on the original TLC plate was comprised of at least three compounds, two of which displayed pink spots (R_f values of ~ 0.13 and ~ 0.1) on the TLC plate similar to known pterocellins, and the third being a shade of orange (R_f value of < 0.1).

A total of six fractions (AA1-38.3 to 4, AA1-40.3 to 4 and AA1-42.3 to 4; Appendix: A1.3) were identified containing compounds with similar low R_f

values. Subsamples of each were prepared for LCMS analysis with diode array detection (DAD) to determine any similarities in their metabolite compositions, as well as to detect potential pterocellins by analysis of UV spectra. LCMS analysis of the fractions revealed that each displayed similar chromatograms. However, the chromatograms also indicated that each of the fractions were still quite complex mixtures and analysis of the UV chromatograms did not reveal the presence of any significant pterocellin-like metabolites. Based on this information, the six fractions were combined into two samples which were then separated further by size exclusion chromatography. TLC analysis of the fractions from the size exclusion columns (AA1-75.1 to 29 and AA1-78.1 to 28; Appendix: A1.4) indicated further purification of the polar compounds, however it also suggested that the isolation of individual compounds was unlikely due to the presence of multiple spots in each fraction and low fraction masses.

LCMS analysis with DAD of the same fractions confirmed what was seen on TLC plates, that, although the fractions were further purified, isolation of specific compounds was not achieved. Examination of the total ion chromatogram (TIC) from each of the fractions indicated the presence of ten prominent ions (Appendices: A1.9 and A1.10). Five of the ions had retention times similar to pterocellins A (**162**) and B (**163**) and on interpretation of chromatograms, two of the ions were presumed to be (**162**) and (**163**), with mass to charge ratios of 285 and 319, respectively. Other ions observed in the TIC eluted earlier, as would be expected of more polar compounds (illustrated in Table 2) and although it was expected to find polar compounds with similarities to pterocellin alkaloids, none of these metabolites displayed a UV chromatogram characteristic of the pterocellins. Small traces of the m/z 377 ion were noted however, the same mass to charge ratio typical of the pterocellin I (**170**) molecular ion. Also found within these fractions was the m/z 379 ion, with the same retention time as the m/z 379 ion found in AA1-58 fractions.

Table 2: Metabolites observed from polar fractions (AA1-75 and AA1-78)

Compound	Major Ion (m/z)	Time of elution (minutes)
Unknown	317	9.2
Unknown	345	9.5
Unknown	275	10.0
Unknown	476	10.0
Unknown	379	11.5
Unknown	557	13.9
Unknown	675	13.7
Unknown	501	15.4
Pterocellin A	285	14.4
Pterocellin B	319	15.5

Of the seven unknown ions, those of most interest were ions with odd mass to charge ratios, indicating an even number of nitrogens, typical of the pterocellins. Two of these ions, m/z 345 and 379, were of particular interest as the mass to charge ratios were comparable to those displayed by monomer pterocellins and were observed in fractions which correlated with polar, pink TLC spots. In addition, the mass difference between them was 34 Da, the same as the mass difference between pterocellins A (**162**) and B (**163**).

All eight unknown metabolites were then subjected to tandem MS analysis to gather further information on their structures. The m/z 345 and 379 ions, each lost 60 Da to yield ions with m/z of 285 and 319 respectively, the same m/z ratio of the $[M+H]^+$ ions of pterocellin A (**162**) and B (**163**) respectively. This indicated that the two compounds are probably related to each other and also likely related to pterocellins A (**162**) and B (**163**). The m/z 345 ion also produced a minor m/z 271 ion, a loss of 74 Da. The overall loss of 74 Da was mirrored in the MS³ spectrum of the m/z 379 ion with the presence of a m/z 305 fragment ion, further confirming a relationship between the two metabolites.

The m/z 557 ion was also of significant interest as it eluted at a similar time to other pterocellins and its mass would closely match a possible dimer of a simple

pterocellin. Tandem MS analysis of this ion however did not produce fragmentation patterns which would be expected of a dimer, such as the cleaving of the dimer into two monomers, but resulted in a m/z 513 fragment, a loss of 44 Da. This ion was further fragmented, with the resulting ions illustrated in Table 3.

Table 3: MS³ fragments from m/z 513.1 ion of AA1-78

Major Ion (m/z)	MS ² ion (m/z)	MS ³ fragments (m/z)	Mass Loss (Da)
557.1	513.1	495.1	18
		471.1	42
		411.1	102
		285.1	228

The initial loss of 44 Da from the m/z 557 ion could possibly be attributed to the loss of CO₂, indicating the presence of a carboxylic acid group. The loss of 18 Da from the 513.1 fragment could be attributed to the loss of H₂O, indicating the presence of an hydroxyl group. The formation of a m/z 285.1 ion which is typical of the [M+H]⁺ ion of pterocellin A (**162**), could indicate a possible structural relationship between the two. Although tandem MS evidence of the m/z 557 ion suggests possible substituents, it is not possible to speculate on an absolute structure for the metabolite.

Further investigation of the m/z 345 and 379 metabolites was undertaken by HRESIMS in an attempt to obtain molecular formulae. Poor ionisation of metabolites of interest led to no molecular formulae with reasonable errors. The m/z 379 ion was not present in the mass spectrum and the m/z 345 ion gave a molecular formula of C₂₀H₂₉N₂O₃ with a relatively large error of 8.9 ppm. Information from tandem MS experiments indicated that the two metabolites are structurally related and the presence of m/z 285 and 319 fragments indicate they are also structurally related to the pterocellins. Based on this information and interpretation of the molecular formula from the m/z 345 ion, the structures of the m/z 345 and 379 ions have been hypothesised (Figure 7).

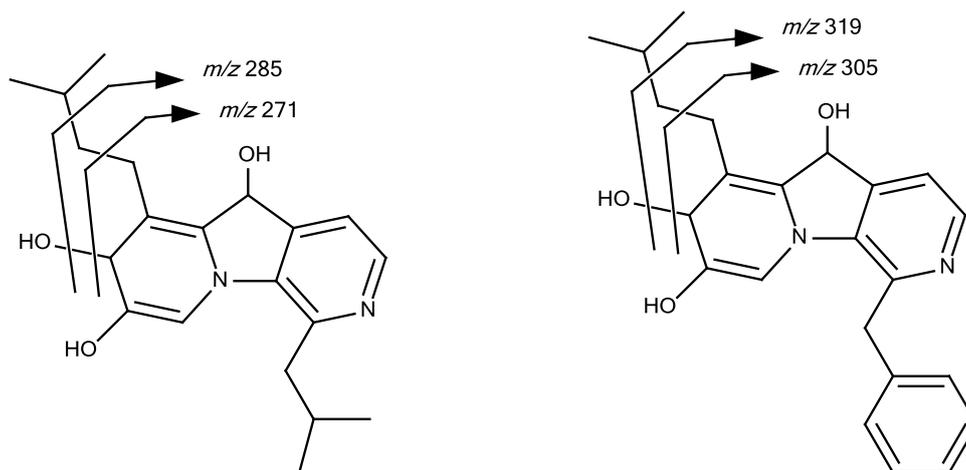


Figure 7: Possible structures of metabolites responsible for m/z 345 and 379 ions with tandem MS fragmentations illustrated

The trihydroxy substitutions, if present, could explain the polarity of the two low R_f pink spots present on TLC. These structures are highly speculative and would require further evidence to confirm their presence.

HRESIMS was also attempted on the m/z 557 ion, however, as the fraction containing the metabolite was still quite a complex mixture, the results of this experiment were inconclusive.

Two fractions (AA1-38.5 and 6; Appendix: A1.3) which appeared to contain pterocellin A (**162**) and/or B (**163**) by TLC were chosen in an attempt to obtain a UV chromatogram of either metabolite for comparative purposes. LCMS analysis of these fractions revealed the presence of the m/z 319 ion in both fractions, although only in trace amounts in one (AA1-38.5). The m/z 319 ion represents the $[M+H]^+$ ion of pterocellin B (**163**) and the UV chromatogram confirmed its presence. Further analysis of the chromatograms revealed significant quantities of a metabolite with a m/z 343 ion and two with m/z 377 ions, eluting with the standard solvent gradient at 10.5 minutes, 11.2 minutes and 12.4 minutes respectively. The m/z 343 molecular ion is indicative of pterocellin H (**169**) and the m/z 377 molecular ion is indicative of pterocellin I (**170**). The results of this analysis indicate the possible presence of pterocellin H (**169**), eluting at 10.5 minutes and pterocellin I (**170**) eluting at either 11.2 or 12.4 minutes. Analysis of minor peaks in the same LCMS chromatograms also revealed the presence of

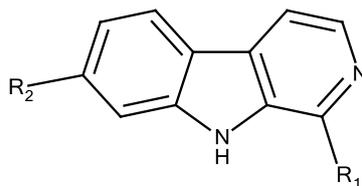
three brominated m/z ions, 535/537, 417/419 and 303/305 (Appendix: A1.12), however considering the low intensities of these ions and the general ease of ionisation of brominated metabolites, it is likely that the abundance of these compounds is very low.

HRESIMS of AA1-38.5 showed a strong peak at m/z 377 which gave a molecular formula of $C_{22}H_{21}N_2O_4$ with an error of 1.5 ppm, the same molecular formula as pterocellin I (**170**). The presence of only one peak in the HRESIMS presumably indicates that the two m/z 377 metabolites are structural isomers. HRESIMS of the m/z 343 peak resulted in a large error of 46.3 ppm for the molecular formula of pterocellin H (**169**).

Isolation of each of these metabolites would have been of high priority, although time constraints resulted in no further purification being achieved.

2.3 β -Carboline Alkaloids

The β -carboline alkaloid, 5-bromo-8-methoxy-1-methyl- β -carboline (**161**) has previously been isolated from *P. vesiculosa* (as a novel compound) and at the time, evidence of the presence of two more β -carboline alkaloids was also noted.¹⁰⁹ The other two alkaloids present were suspected to be the demethoxy- and debromo-analogues of 5-bromo-8-methoxy-1-methyl- β -carboline (**161**) based on UV and LCMS data.¹⁰⁹ Analysis of other extracts of *P. vesiculosa* at the University of Waikato has led to the partial characterisation of another four β -carboline alkaloids, 1-ethyl-7-hydroxy-, 7-hydroxy-1-propyl-, 7-bromo-1-ethyl- and 7-bromo-1-propyl- β -carboline (**194-197**).¹¹⁰ As a result of this work, isolation of greater amounts of these and any other β -carboline alkaloids present was of significant interest.



(194) R₁ = -CH₂CH₃; R₂ = -OH

(195) R₁ = -CH₂CH₂CH₃; R₂ = -OH

(196) R₁ = -CH₂CH₃; R₂ = -Br

(197) R₁ = -CH₂CH₂CH₃; R₂ = -Br

A dichloromethane extract of *P. vesiculosa* was subjected to separation by C₁₈ reversed phase flash LC. Fractions from this column were analysed by TLC and it was noted that a number of fractions contained yellow spots with R_f values of approximately 0.5, as well as spots which appeared fluorescent under 254 nm light. These characteristics are typical of those produced by some β-carboline alkaloids. The fractions containing these compounds were combined to give two samples that were further separated with the use of size exclusion LC. Fractions from each of the samples were again assessed by TLC.

Upon analysis of the fractions, it was noted that six fractions contained similar spots, reminiscent of β-carboline alkaloids. These fractions (AA1-34.8 to 10 and AA1-36.8 to 10; Appendix: A1.5) were analysed by LCMS which confirmed the presence of β-carboline alkaloids, since a number of metabolites yielded a typical UV chromatogram. The presence of up to eight different β-carboline alkaloids across the six fractions, some of which were brominated, was indicated, however there was little separation of the compounds between individual fractions. Seemingly included among these alkaloids were the previously characterised 5-bromo-8-methoxy-1-methyl-β-carboline and the two previously reported, demethoxy- and debromo- derivatives, based on the presence of *m/z* 291/293, 261/263 and 213 ions and observed UV chromatograms.

The six fractions were then combined based on LCMS results and sample size, to give two samples that were separated again using size exclusion chromatography (Appendix: A1.5). LCMS analysis of the fractionated samples revealed that the compounds of interest were further purified but not individually isolated. Across the fractions, a total of eleven possible β-carboline alkaloids (Appendices: A1.13

and A1.14) were indicated by an analysis of UV chromatograms (Figure 8), including 5-bromo-8-methoxy-1-methyl- β -carboline (m/z 291/293 molecular ion) and the previously reported demethoxy- counterpart, with $[M+H]^+$ ion of m/z 261/263. In addition to these alkaloids, the m/z 213 ion was observed, which would correspond to either of the two previously noted metabolites, 8-methoxy-1-methyl- β -carboline or 1-ethyl-7-hydroxy- β -carboline (**194**), as well as the m/z 227 and 275/277 ions which correspond to the previously noted alkaloids 7-hydroxy-1-propyl- β -carboline (**195**) and 7-bromo-1-ethyl- β -carboline (**196**) respectively. Also present were the m/z 277/279 and 303/305 ions, seen in previously analysed fractions (Appendices: A1.8 and A1.12, respectively), however at much high concentrations here. The m/z 277/279 ion could correspond to the demethyl-counterpart of 5-bromo-8-methoxy-1-methyl- β -carboline (**161**).

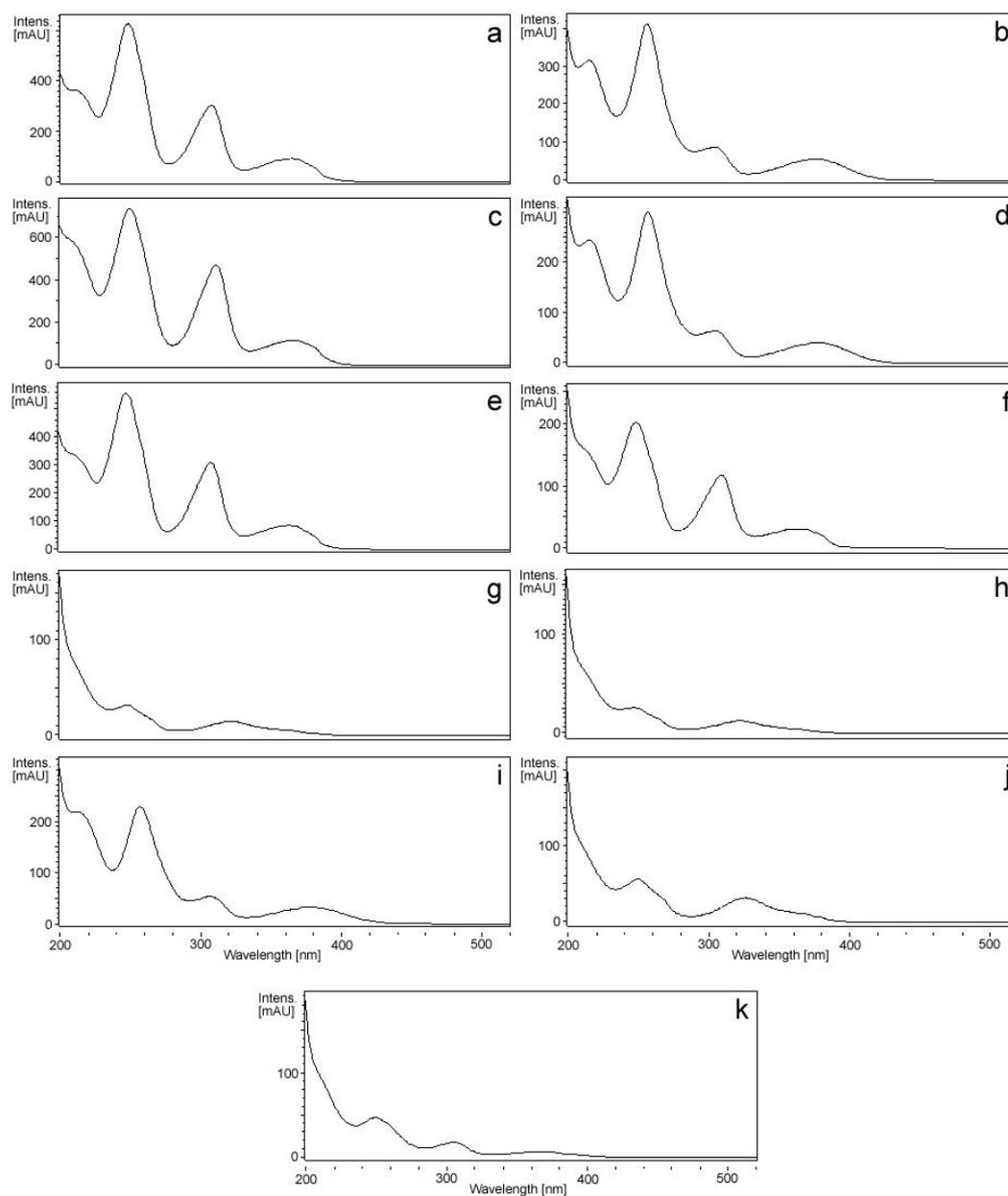


Figure 8: UV chromatograms of possible β -carboline alkaloids from the fractions AA1-94 and AA1-114.

- | | | |
|------------------|------------------|------------------|
| a) m/z 261/263 | e) m/z 275/277 | i) m/z 277/279 |
| b) m/z 291/293 | f) m/z 303/305 | j) m/z 289 |
| c) m/z 337/339 | g) m/z 227 | k) m/z 213 |
| d) m/z 305/307 | h) m/z 255 | |

The presence of four minor metabolites (Table 4) was also noted and analysis of isotope patterns indicated that each was brominated (Appendix: A1.15). Although these minor metabolites would be compounds of interest, the relative abundance of each made further investigation during this research project impractical.

Table 4: Minor brominated metabolites from late eluting fractions of AA1-94.28 to 30

Brominated Ions (<i>m/z</i>)	Time of elution (minutes)
353/355	18.8
455/457	18.9
552/554	15.2
582/584	15.3

Subsamples of two β -carboline alkaloid containing fractions were prepared for HRESIMS analysis, with the intention of obtaining molecular formulae for the eleven potential alkaloids. The fractions chosen, together contained each of the possible β -carboline alkaloids of interest. HRESIMS data from the fractions gave molecular formulae for eight of the eleven compounds, each with reputable errors ranging from 0.61 ppm to 4.43 ppm. The molecular formulae generated by HRESIMS (Table 5), closely match those of hypothesised structures for the eight compounds.

Table 5: Possible β -carboline alkaloid molecular formulae with errors

Mass [M+H]⁺ (<i>m/z</i>, error in ppm)	Molecular Formula [M+H]⁺
227.1169 \pm 4.43	C ₁₄ H ₁₅ N ₂ O
255.1489 \pm 1.32	C ₁₆ H ₁₉ N ₂ O
261.0011 \pm 4.23	C ₁₂ H ₁₀ N ₂ ⁷⁹ Br
275.0177 \pm 0.61	C ₁₃ H ₁₂ N ₂ ⁷⁹ Br
289.1333 \pm 0.98	C ₁₉ H ₁₇ N ₂ O
303.0487 \pm 1.46	C ₁₅ H ₁₆ N ₂ ⁷⁹ Br
305.0275 \pm 2.99	C ₁₄ H ₁₄ N ₂ O ⁷⁹ Br
337.0320 \pm 4.27	C ₁₈ H ₁₄ N ₂ ⁷⁹ Br

To determine possible functional groups present in the β -carboline alkaloids, each metabolite was subjected to tandem MS analysis. The results of the tandem MS

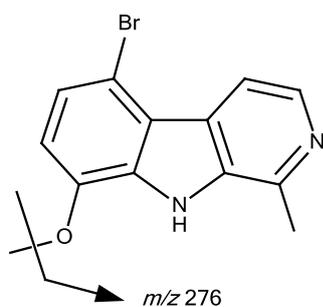
analyses are summarised below in Table 6, including suspected mass fragments. For comparative purposes, tandem MS was also performed on the molecular ion of 5-bromo-8-methoxy-1-methyl- β -carboline (**161**), m/z 291.

Table 6: Summary of tandem MS results from β -carboline alkaloid containing fractions

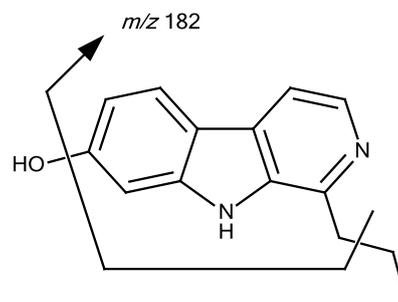
$[M+H]^+$ ion (m/z)	MS/MS fragments (m/z)	Theorised Fragmentations $[M - X + H]^+$
291	276	$[M - CH_3 + H]^+$
227	182	$[M - C_2H_5O + H]^+$
255	240	$[M - CH_3 + H]^+$
	212	$[M - C_3H_7 + H]^+$
261	182	$[M - ^{79}Br + H]^+$
275	260	$[M - CH_3 + H]^+$
	196	$[M - ^{79}Br + H]^+$
289	274	$[M - CH_3 + H]^+$
	211	$[M - C_6H_5 + H]^+$
303	260	$[M - C_3H_7 + H]^+$
	182 (minor ion)	$[M - C_3H_7Br + H]^+$
305	290	$[M - CH_3 + H]^+$
337	259	$[M - C_6H_5 + H]^+$

From the analysis of TLC, UV, LCMS, tandem MS, and HRESIMS data, the presence of eight β -carboline alkaloids has been observed and their molecular formulae postulated. Based on this information and knowledge of typical substitution patterns in β -carboline alkaloids from this species, the structures of the eight metabolites have been hypothesised and are illustrated in Figure 9, along with the explanations of fragmentations found in tandem MS experiments above.

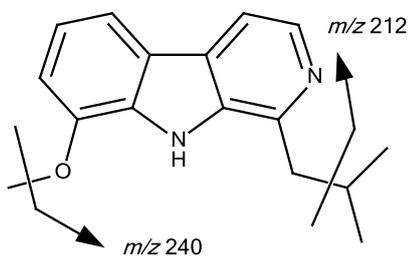
In the case of the m/z 227 molecular ion, it has been hypothesised that, unlike the other oxygen-containing metabolites, it does not contain a methoxy substitution as there was no evidence of the characteristic loss of 15 Da. This further confirms the likelihood that this metabolite is the previously noted compound 7-hydroxy-1-propyl- β -carboline (**195**). With m/z 255 and 303 ions, an isobutyl group has been suggested, although an n -butyl substitution is also possible. It should be noted that in the structures below, fragmentation has been explained with β -cleavage of the side chains from the core β -carboline structure, as seen in ion m/z 227, rather than α -cleavage; however fragmentations observed in tandem MS spectra could also be explained with α -cleavage with an additional methyl substitution elsewhere on the β -carboline core.



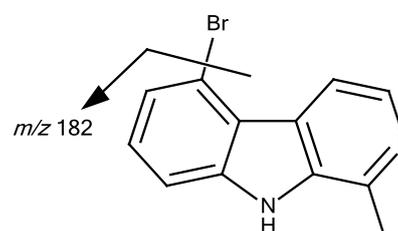
a) m/z 291/293 metabolite



b) m/z 227 metabolite



c) m/z 255 metabolite



d) m/z 261/263 metabolite

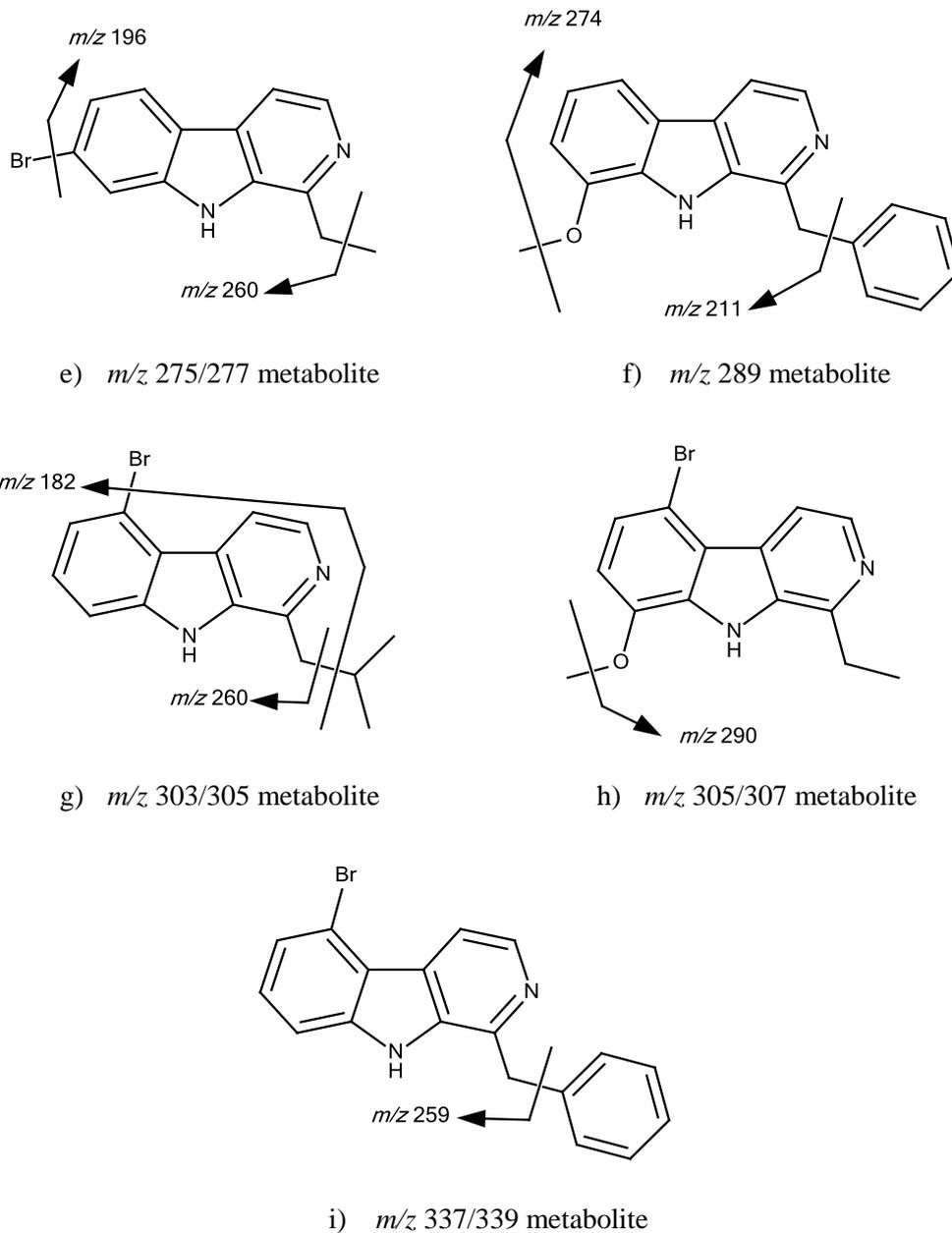


Figure 9: Hypothesised structures of β -carboline alkaloids with tandem MS fragmentations illustrated

Of the nine β -carboline alkaloids above, those illustrated in figures 9a,^{106, 109} 9b,¹¹⁰ 9d,¹⁰⁹ 9e¹¹⁰ and 9g¹¹⁰ had been previously reported from this species and those in figures 9c, 9f, 9h and 9i would appear to be novel natural products regardless of the position of their substituents, determined by an exact chemical structure search with the SciFinder database.¹²¹

2.4 Future Research

Due to time constraints associated with this project, a number of areas of interest remain unexplored and could form the basis of future research. Isolation of greater amounts of pterocellin H (**169**) and I (**170**), would still be a priority, along with isolation and characterisation of the β -carboline alkaloids. Additional information on the fragmentation patterns of the β -carboline alkaloids could also be easily obtained to further confirm theorised structures. By performing tandem MS on both isotopes simultaneously, confirmation could be made on the loss of bromine in relevant metabolites. Identification of new pterocellin compounds and the polar metabolites is another area of research which needs to be further explored. The structures of the m/z 345 and 379 ions also need further investigation to confirm their relationship to the pterocellins. Investigation of new pterocellin compounds would be greatly assisted with optimisation of the UV detector, in order get UV chromatograms with sufficient intensities to identify metabolites of lower concentrations.

Another area of potential future research is the isolation and characterisation of the minor monobrominated metabolites, yielding ions with m/z of 353/355, 362/364, 417/419, 455/457, 535/537, 552/554 and 582/584. Isolation of these metabolites however, may be problematic and would likely require an extract obtained from greater amounts of starting material, when considering their low intensities and the general ease of ionisation of brominated metabolites.

Chapter Three: *Plumatella repens*

3.1 Introduction

Plumatella repens is a species of bryozoan that belongs to the class Phylactolaemata, which consists of species that live exclusively in freshwater environments.⁹ There is no known information detailing the secondary metabolites of *P. repens* (or any other freshwater species). On account of this lack of information and the very high P388 activity in screening of the crude extract (22 443 ng mL⁻¹), this species was chosen as an ideal candidate for research. The particular specimen used for this research was obtained from the Dunedin City Council, where it was growing prolifically in a reservoir at a water treatment and storage plant.¹²²

Work on a methanol/dichloromethane extract of this specimen had been previously undertaken in a pilot study in the Chemistry Department at the University of Waikato by summer research student Ashleigh Richards. Initial bioassay of the crude extract indicated considerable activity against the murine P388 lymphocytic leukaemia cell line with an IC₅₀ value of 22 443 ng mL⁻¹. The original extract was separated into two proportions (Extracts A and B) as part of the pilot study. Extract A was further divided into two separate extracts (Extract A1 and Extract A2) for ease of handling. It was noted that Extract B had become contaminated by solvent which had come into contact with a rubber seal, so it was kept separate. Each of the extracts was fractionated further by C₁₈ chromatography and subsamples were prepared from each fraction for bioassay (Appendices: A2.3, A2.4 and A3.1) against the P388 cell line. In each case, the activity peaked in fractions seven to ten, eluting with methanol, methanol/dichloromethane. As bioassay facilities were not available at this stage of the pilot study, six Extract A1 fractions were fractionated further by size exclusion chromatography in an attempt to ensure the inclusion of bioactive fractions. This resulted in six series of fractions (Columns A-F), each of which were eventually assayed against the P388 cell line, the results of which indicated a range of activity (Appendix: A2.2). Of the six series of fractions, three (Columns D-F) showed significant biological activity. The three series that were active, in

each case, had activity which peaked at fraction three with IC₅₀ values of 1512, 1630, and 1916 ng mL⁻¹, from Columns D to F respectively. Ashleigh attempted to isolate the metabolites responsible for this activity, however due to the relatively short time period available, identification of specific metabolites responsible for the activity was not determined.

Isolation of the compounds responsible for the bioactivity of *P. repens* was the primary focus of the investigation, however determination of the sterol composition was also undertaken.

3.2 Investigation of Bioactive Metabolites

To isolate the active metabolites, fraction three from each of Columns E and F were combined for size exclusion chromatography. To prevent overloading of the LC column, fraction three from Column D was excluded from the combination procedure. The odd numbered fractions from size exclusion chromatography were assayed against the P388 cell line. These results appeared to indicate the presence of at least two active metabolites, as the bioactivity peaked twice, once at fraction three (IC₅₀ value of 866.2 ng mL⁻¹) and again at fraction eleven (IC₅₀ value of 133.2 ng mL⁻¹), as depicted in Table 7.

Table 7: IC₅₀ values of odd numbered AA1-16 fractions against the P388 cell line

Fraction	IC₅₀ values (ng mL⁻¹)
AA1-16.3	866.2
AA1-16.5	2537.3
AA1-16.7	>12500
AA1-16.9	285.6
AA1-16.11	133.2
AA1-16.13	634.0
AA1-16.15	>12500
AA1-16.17	>12500
AA1-16.19	>12500
AA1-16.20	>12500

Fractions from this column (AA1-16.2 to 15) were subjected to analysis by LCMS to determine whether a peak in activity would correlate with specific metabolites in the TIC. Over the fourteen fractions, nine major ions (Appendices: A2.6 to A2.8) were observed (Table 8). Unfortunately no ions appeared to correlate well with the observed P388 activity.

Table 8: Ions observed from MS analysis of AA1-16 fractions

Observed Ions (<i>m/z</i>)	Time of elution (minutes)
368.5	36.3
482.3	25.3
496.3	24.3
512.5	30.5
526.5	30.7
550.7	37.1
578.9	28.3
803.1	37.6

Ions with *m/z* of 512.5, 482.3 and 803.2 were chosen for tandem MS analysis, as they appeared to most closely correlate with P388 activity. It was concluded from the fragmentation patterns of the *m/z* 512.5 ion that it likely contained a long chain hydrocarbon, as it displayed sequential losses of 14 Da, typical of long chain organic molecules.¹²³ Results of tandem MS analysis of *m/z* 482.3 and 803.2 ions, including theorised mass fragments are illustrated in Table 9.

Table 9: Tandem MS analysis of m/z 482.3 and 803.2 ions from AA1-16 fractions

Observed ions (m/z)	MS/MS fragments (m/z)	Theorised Fragmentations [M – X + H] ⁺
482.3	464.3	[M – H ₂ O + H] ⁺
	405.3	[M – C ₆ H ₅ + H] ⁺
	184.0	
803.2	413.3	

It was concluded from the tandem MS analysis that the m/z 482.3 metabolite likely contained a hydroxyl group, indicated by the loss of 18 Da and also a phenyl group which would explain the loss of 77 Da. The m/z 184.0 and m/z 413.3 fragments elude explanation without further information on the metabolites. In an attempt to explain the m/z 413.3 ion derived from the m/z 803.2 metabolite, MS³ and MS⁴ experiments were undertaken, the results of which are summarised in Table 10.

Table 10: MSⁿ fragments from the m/z 803.2 ion

Observed ion (m/z)	MS/MS fragment (m/z)	MS ³ fragment (m/z)	MS ⁴ fragments (m/z)
803.2	413.3	301.1	189
			171

The MSⁿ analysis of m/z 803.2 indicated the presence of a repeating unit of 112.2 Da, with the m/z 413.3 and 301.1 fragments each losing this mass. This loss could be explained by an unsaturated ester side chain (Figure 10) with proton transfer, similar to the loss of the octa-2,4-dienoate side chains in the FAB mass spectra of bryostatin 1 (**4**) and 3 (**6**).³⁰ This however, is highly speculative and the observed ions could also be explained by the loss of many other structural combinations such as a δ -lactam, a proline-like group (Figure 10) or loss of both sequentially.

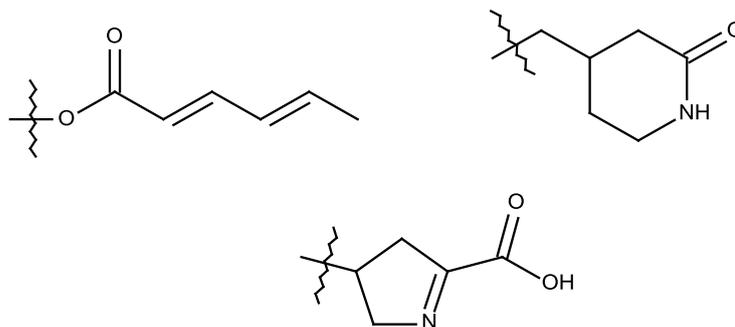


Figure 10: Substituents potentially responsible for the loss of 112.2 Da

Although this information goes some way in determining possible substructures of metabolites, none of these above ions correlated well with the observed biological activity. Due to the ambiguity in the structural analysis of the molecular ions, it was decided that the next step forward would be to assay the even numbered fractions. In contrast to the activity of the odd numbered fractions, results of these assays indicated only a single peak in activity, at fraction ten, with an IC_{50} value of 1531 ng mL^{-1} . The activity also seemed to have decreased significantly compared to that of the odd numbered fractions. Analysis of the assay results from the even numbered fractions revealed the possibility of a concentration error, as they seemed to mismatch the results from the odd numbered fractions by a factor of ten (Table 11). The preparation of fresh biological assay samples of the even numbered fractions was considered, however the facilities at the University of Canterbury to which the samples were sent for biological assay no longer processed such samples. Finding another laboratory which could assess the fractions was initially unsuccessful, so it was decided that given the earlier reproducible chromatographic behaviour of the bioactivity, continuation of the *P. repens* project would involve purification without the use of biological assay. Fractionation of more crude samples would proceed, guided by the methods used in previous LC columns.

Table 11: IC₅₀ values of AA1-16 fractions against the P388 cell line

Fraction	IC₅₀ values (ng mL⁻¹)
AA1-16.2	>12500
AA1-16.3	866.2
AA1-16.4	>12500
AA1-16.5	2537.3
AA1-16.6	>12500
AA1-16.7	>12500
AA1-16.8	>12500
AA1-16.9	285.6
AA1-16.10	1531
AA1-16.11	133.2
AA1-16.12	2223
AA1-16.13	634.0
AA1-16.14	>12500
AA1-16.15	>12500
AA1-16.17	>12500
AA1-16.19	>12500
AA1-16.20	>12500

A series of C₁₈ fractions from Extract A2 were obtained. Three fractions from the reversed phase column were subjected to separation by size exclusion LC. This resulted in three series of fractions (Columns G-I; Appendix: A2.4) which, by comparison to separation procedures, were equivalents of the earlier run Columns D, E and F. As activity peaked in fraction three of each of Columns D, E and F, it was decided to combine fractions two, fractions three, and fractions four, from columns G to I. This was in an effort to ensure the inclusion of the active proportions of each column. A subsample of each of these combined fractions was prepared for future bioassay analysis. The three combined samples were then subjected to further separation by size exclusion chromatography, resulting in three series of fractions (Columns J-L) (Appendix: A2.5). The fractions from

these columns were now method equivalents of the even and odd numbered fractions that were assayed previously.

At this stage, it was essential to get bioassay results of the fractions to determine the location of the original activity and to see if it was comparable to that of the fractions assayed earlier. After considerable effort to find facilities to process the *P. repens* samples, it was arranged with AgResearch, Hamilton for the author of this thesis to use their facilities to assay the samples, under the supervision of Megan Callaghan.

The assay protocol that was used for analysis of the samples at the University of Canterbury was acquired and used as a reference method for the first samples run at the AgResearch facilities. The first samples run were AR1-9.6 and AR1-16.7, chosen as they had previously been tested at the University of Canterbury and had known IC₅₀ values of 43 034 ng mL⁻¹ and 10 064 ng mL⁻¹ respectively. It was intended to assay these samples as a practice run, as well as to compare the results with those previously obtained, to determine the efficiency of the method used at AgResearch. Each of the samples was run following the method outlined in Chapter One, subsection 1.4.

The results from the assay indicated that activity was relative to increasing concentration, however in each of the samples, the activity was much lower than expected. Each sample had percentage inhibitions greater than 50 % at 500 000 ng mL⁻¹, but each fraction was significantly less active than the IC₅₀ values determined in Christchurch for the same samples, with AR1-9.6 having an IC₅₀ value of 315 500 ng mL⁻¹ and AR1-16.7 an IC₅₀ value of 379 315 ng mL⁻¹, compared to 43 034 ng mL⁻¹ and 10 064 ng mL⁻¹, respectively. The reason for the unexpected results was put down to errors in plate preparation and degradation of the triton X-100 standard. The time constraints associated with this project meant that reanalysis of the same samples was impractical. Rather than reanalysing the previous samples, fractions that were more significant to the continuation of the project would be assayed and the triton X-100 standard would be used to determine the effectiveness of the assay procedure.

The next samples to be tested were the combined samples from Columns G-I, as well as two other fractions (AA1-62.2 and AA1-62.3, Appendix: A2.5) from

Column J. Before preparation of the microtitre plate, it was noted that the growth of the P388 cells was much less than what would be expected. The decision was made to continue with the assay but with a smaller number of cells per well (2 000, rather than the 5 000 cells which was used at the University of Canterbury). This assay gave inconsistent results, with some survival rates of the most concentrated dilutions being more than the corresponding less concentrated dilutions, including those of the triton X-100 standard. This was attributed to the low cell count per well of 2 000.¹²⁴

It was a little over a month before the AgResearch facilities were again available and the P388 cell lines were at an acceptable concentration for use. Over a period of a month, a bioassay method was developed, as test plates were run using triton X-100 and pterocellin A (**162**) as reference standards. The method developed required 10 000 cells per well in order to obtain accurate results. This new method was verified by triton X-100 and (**162**) reference samples. Using the new method, the IC₅₀ value over four repetitions of (**162**) was between 391 and 195 ng mL⁻¹, which is not too dissimilar to its previously reported IC₅₀ value of 477 ng mL⁻¹ (Table 12). Also noted in this experiment was the presence of an edge effect in the least concentrated row, this led to higher than expected percentage inhibition results.¹²⁴ The edge effect is a common phenomenon, typically occurs on 96 well microtitre plates and can affect the reliability of results from the outermost wells.¹²⁵

Table 12: Growth inhibition of the P388 cell line by pterocellin A

Concentration (ng mL ⁻¹)	Pterocellin A			
	(% inhibition, values >100 rounded to 100.0)			
12 500	100.0	100.0	100.0	100.0
6 250	100.0	100.0	100.0	100.0
3 125	100.0	100.0	100.0	100.0
1 563	100.0	100.0	100.0	100.0
781	93.0	95.8	97.3	96.6
391	61.7	56.4	69.9	68.0
195	43.3	40.8	42.1	41.9
98	45.6*	44.1*	48.1*	47.5*

* = edge effect affected results

The first samples to be analysed using the new method were the three combined samples from Columns G-I and fractions AA1-16.12 and AA1-16.13 (Appendix: A2.3) from Extract A1. The results from this assay indicated that all combined samples from Extract A2 were inactive and there was very little activity for Extract A1 fractions. Sample AA1-16.12 displayed an IC₅₀ value between 12 500 and 6 250 ng mL⁻¹; this was much less active than the value obtained in Christchurch (2 223 ng mL⁻¹). The subsample of AA1-16.13 displayed 22.6 % inhibition at 12 500 ng mL⁻¹ also much less active than the results from the Christchurch assay (634.0 ng mL⁻¹). A large edge effect was also seen in this assay along the less concentrated row, as well as down the AA1-50.4 sample, rendering AA1-50.4 results meaningless. It should also be noted that Extract A2 fractions were prepared three months prior and had been stored frozen in cell media, which likely had an effect on the activity of the samples.

The results of this assay suggest that the bioactive metabolites of *P. repens* may slowly break down over time which would result in inactivity. However, the problem may also lie with the assay method or more likely, with the storage method of the samples. Further investigation into the active metabolites would be necessary to determine this, unfortunately due to time constraints and inconclusive LCMS results, it was not possible to determine the metabolites responsible for the bioactivity or their stability.

3.3 Identification of Sterols

Sterols are a subtype of steroids, containing an hydroxyl substituent on C3 of the steroid skeleton (Figure 11). They are a common constituent of plants, fungi and animals and help to maintain cell structure and fluidity.¹²⁶ Sterols are either synthesised by the organism or can be acquired via dietary intake.¹²⁶ One previous study on the sterol composition of marine organisms undertaken at the University of Waikato, found a wide range of sterols present, with one unidentified marine organism containing thirteen different sterols.¹⁰⁹ Another earlier investigation into sterols of marine bryozoans at the University of Waikato also found a wide range of sterols present, with up to 15 sterols in extracts of both *Watersipora subtorquata* and *Amathia wilsoni*.¹²⁷ This, along with the little known about

freshwater metabolites, in particular freshwater bryozoa, prompted the investigation into the sterol composition of *P. repens*.

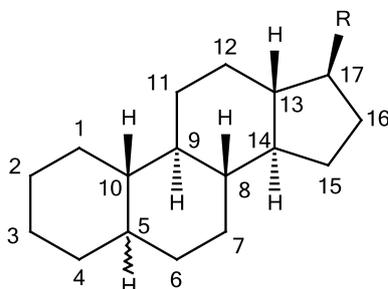


Figure 11: Core structure of steroids

TLC analysis of *P. repens* fractions (AR1-16.09 to 12; Appendix: A3.1) from a size exclusion LC column, against a cholesterol standard and visualisation with iodine vapour, led to the identification of three fractions which contained cholesterol and or similar compounds. Subsamples of these fractions were derivatised with trimethylsilyl (TMS) imidazole and were analysed by gas chromatography mass spectrometry (GCMS). The GCMS data indicated that each of the fractions contained the same sterols in similar compositions. A total of five predominant sterols were observed (Appendices: A3.2 and A3.3). Comparison of the fragmentation patterns with those of the NIST/EPA/NIH Mass Spectral Library (Appendices: A3.4 to A3.8) revealed the presence of cholesterol, β -sitosterol and stigmasterol. Fragmentation patterns also suggested the presence of sterols similar to (3 β ,22 E)-ergosta-7,22-dien-3-ol and (3 β ,24 R)-ergosta-5-en-3-ol. Also present in the chromatogram were four small peaks eluting at similar times to the sterols, however their concentrations were too low to determine their possible structures via fragmentation pattern analysis. The sterol composition of *P. repens* is summarised in Table 13.

Table 13: Sterol composition of fraction AR1-16.10, determined by GCMS

Sterol (as TMS ether)	Composition (%)	Time of elution (minutes)
cholesterol	68.53	17.14
β -sitosterol	11.46	18.78
stigmasterol	8.25	18.25
(3 β ,22 <i>E</i>)-ergosta-7,22-dien-3-ol	6.54	17.46
(3 β ,24 <i>R</i>)-ergosta-5-en-3-ol	3.73	17.96
unidentified sterol	0.89	16.83
unidentified sterol	0.27	17.57
unidentified sterol	0.20	16.89
unidentified sterol	0.12	17.86

There were surprisingly few sterols observed from GCMS analysis of *P. repens* in comparison to previous work on sterol identification from marine bryozoa. This may be due to differences between freshwater and marine bryozoa or it may be a specimen specific effect when considering the unique environment from which the *P. repens* sample was obtained.¹²²

3.4 Future Research

Identification of the metabolites that were responsible for the original bioactivity still needs to be determined. This may require the preparation of a fresh extract, as the compounds which exhibit the bioactivity may be unstable. Although this may be the case, reassay of the active fraction AR1-29.03 (Appendix: A2.2) would give a credible indication of the stability of the active metabolites. In addition to this, for future bioassay guided isolation, testing of fractions at two different facilities, while unavoidable in this case, should be avoided in the interest of consistent, reliable bioassay results.

Chapter Four: Experimental

4.1 General Experimental Methods

LCMS data was generated on a Bruker amaZon X™ using electrospray ionisation in positive ion mode. All LCMS experiments were performed using a standard solvent gradient (Table 14) with two solvents (Solvent A, water (H₂O); Solvent B, acetonitrile (CH₃CN) – Honeywell, Burdick & Jackson ACS/HPLC Certified Solvent) each with 0.05 % trifluoroacetic acid (TFA; Merck, > 99 %) with a DIONEX UltiMate 3000 diode array detector. LCMS experiments used a reversed phase column (Phenomenex, Luna 5 μ C₁₈₍₂₎ 100 A, 150 x 4.60 mm, stored in CH₃CN:H₂O (9:1) when not in use) on a DIONEX UltiMate 3 000 pump and autosampler column compartment. Samples were dissolved in HPLC grade methanol (MeOH; Ajax Finechem Pty Ltd, Unichrom), filtered (0.2 μm LabServ millipore filters) and made up to concentrations of approximately 150 to 250 mg mL⁻¹. H₂O was obtained from a Barnstead E-pure® water system.

Table 14: Standard solvent gradient used for LCMS experiments

Time (min)	Solvent A (% of total volume)	Solvent B (% of total volume)
0	95	5
2	95	5
27	0	100
39	0	100
45	95	5
50	95	5

All HRESIMS data was obtained on a Bruker Daltonics MicrOTOF™ which uses electro-spray ionisation and time of flight mass separation. Samples were dissolved in HPLC grade MeOH at a concentration of approximately 250 mg mL⁻¹ and analysed using varying cone voltages, to obtain optimum ionisation in positive ion mode.

GCMS results were obtained from a Hewlett Packard 5973 Mass Selective Detector with a Hewlett Packard 6890 series GC System.

Reversed phase chromatography utilised C₁₈ YMC Gel ODS-A (120 Å) I-230/70 mesh, slurry packed into glass columns in MeOH and equilibrated back to H₂O. Samples were loaded as solids and were run with a steep stepped gradient from H₂O to MeOH to dichloromethane (DCM) and back to H₂O (Table 15) under applied nitrogen gas (N_{2 (g)}) pressure.

Table 15: Solvent system used for reversed phase chromatography

Solvent	Volume (mL)
H ₂ O	150
H ₂ O:MeOH (1:1)	340
H ₂ O:MeOH (3:7)	150
H ₂ O:MeOH (1:9)	150
MeOH	300
MeOH:DCM (9:1)	150
MeOH:DCM (1:1)	150
DCM	150
MeOH	225
H ₂ O:MeOH (1:1)	150
H ₂ O	225

Size exclusion chromatography utilised Sephadex LH-20 packed into glass columns and eluted with MeOH. Samples were filtered with cotton wool in glass pipettes and loaded in minimum MeOH.

Column fractions were dried under a stream of N_{2 (g)} on a Lab-Line[®] Multi-Blok[®] heating block set to 36°C degrees or evaporated to dryness with a rotary evaporator (BÜCHI Rotavapor[®] R-110) in a water bath (BÜCHI 461, 40°C) under a vacuum (BÜCHI Vacuum Pump V-700) then transferred to glass vials for drying under applied N_{2 (g)} on the Lab-Line heating block.

TLC analyses were performed on Merck (60 F₂₅₄ plates) TLC aluminium backed plates coated with silica gel (0.2 millimetres thick). The standard solvent system

employed was ethyl acetate (EtOAc):MeOH (5:1). Samples were dissolved in MeOH and applied to plates using drawn glass capillaries.

Preparation of all biological microtitre plates at AgResearch, Hamilton was completed in an Email Westinghouse Pty Ltd Laminar Flow Work Station, made sterile by washing with 70 % ethanol (EtOH).

Microtitre plates were incubated in a Forma Scientific Water-Jacket Incubator 3250 (37°C, 72 hours) and analysed on a BioTek Synergy 2 plate reader (at 490 nm and 360 nm).

Cells used in biological assays were murine P388D1 lymphocytic leukaemia cells, strain DBA2, grown in suspension.

Cell media used in biological assays was a mixture of Dulbecco's Modified Eagle Media (90 %, high D-glucose formula with L-glutamine) and fetal bovine serum (10 %, sourced in New Zealand by Sigma Chemical Co.) with penicillin-streptomycin added (1 mL per 100 mL, penicillin G salt and streptomycin sulphate in 0.85 % saline).

Sterol samples were derivatised with *N*-trimethylsilyl imidazole (Thermo Scientific; product number: TS-88625), sonicated on an Astrason[®] Ultrasonic Cleaner and centrifuged in an Eppendorf Centrifuge 5702.

Positive control, triton X-100 (*t*-octylphenoxy polyethoxy ethanol) (Figure 12) was obtained from Sigma Chemical Co.

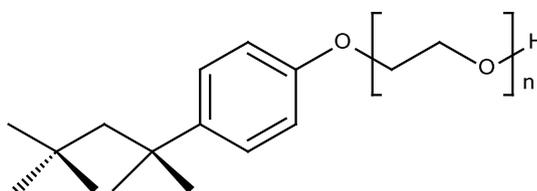


Figure 12: Structure of Triton X-100

The cholesterol reference sample (95-98 % pure ash-free, precipitated from alcohol) was obtained from the Sigma Chemical Co.

All organic solvents were either HPLC grade or distilled from drum grade prior to use.

4.2 Work Described in Chapter Two

The *P. vesiculosa* sample used in this project was collected from Sump Bay, Alderman Islands in 2001 by SCUBA during a recreational dive. A voucher (AI201-03) is kept in the Department of Chemistry at the University of Waikato. The specimen was split in two (Specimen A and Specimen B). Specimen A was used in the initial research performed by Marisa Till.

4.2.1 Initial Research on Specimen B

Katie Moore undertook initial investigations on Specimen B. Specimen B (833.57 g) was exhaustively extracted (DCM) resulting in a crude extract (4.71 g) which was analysed by LCMS (Appendix: A1.1). The crude extract was separated into two (Extract A, 1.30 g; Extract B, 2.64 g) for ease of handling, then Extract A was separated using reversed phase chromatography (30 g, C₁₈ packing) resulting in a series of fractions (KM1-7.1 to 17; Appendix: A1.2). One fraction (KM1-7.14) was further separated by size exclusion chromatography resulting in eleven fractions (KM1-14).

4.2.2 Work on *P. vesiculosa* Outlined in this Thesis

Extract B (2.64 g) from the initial study was obtained. Following the same procedure used to fractionate Extract A, Extract B was separated by reversed phase chromatography to yield seventeen fractions (AA1.5.1 to 17; Appendix: A1.2). Fractions from each extract were analysed by TLC (EtOAc:MeOH, 5:1).

4.2.2.1 Isolation of Pterocellin Alkaloids

Fractions containing pterocellin-like compounds by TLC analysis were combined as appropriate and further separated using size exclusion chromatography (150 g packing).

TLC analysis of the size exclusion fractions (AA1-42.1 to 15; Appendix: A1.2) indicated the presence of late eluting, pterocellin-like compounds (pink spot on TLC). The fractions of interest (AA1-42.8 to 10) were combined and subjected to size exclusion chromatography (30 g packing). The resulting fractions (AA1-58.1 to 25; Appendix: A1.2) were analysed by LCMS and HRESIMS.

TLC analysis of size exclusion fractions (AA1-38.1 to 15, AA1-40.1 to 15 and AA1-42.1 to 15; Appendix: A1.3) indicated the presence of polar, pterocellin-like metabolite/s (pink spot on the baseline of silica gel TLC plate, EtOAc:MeOH, 5:1) across six fractions (AA1-38.3 to 4, AA1-40.3 to 4 and AA1-42.3 to 4). Increased resolution of the polar metabolite/s was achieved with MeOH:EtOH (1:1), which indicated the presence of three polar metabolites (R_f values ~ 0.13 , ~ 0.1 , < 0.1). Each fraction was analysed by LCMS then combined appropriately and fractionated with size exclusion chromatography (30 g packing). The resulting fractions (AA1-75.1 to 29, AA1-78.1 to 28; Appendix: A1.4) were analysed by tandem MS and HRESIMS.

4.2.2.2 Isolation of β -Carboline Alkaloids

TLC analysis of the size exclusion fractions (AA1-34.1 to 17 and AA1-36.1 to 16; Appendix: A1.5) displayed a series of yellow/orange compounds with relatively high R_f values (~ 0.85). The fractions containing these yellow/orange metabolites (AA1-34.8 to 10 and AA1-38.8 to 10) were analysed by LCMS and were then combined appropriately based on mass and LCMS results before further fractionation by size exclusion chromatography (30 g packing; Appendix: A1.5). LCMS analysis of the resulting fractions (AA1-94.1 to 32 and AA1-114.1 to 37; Appendix: A1.5) indicated the presence of β -carboline alkaloids based on analysis of UV chromatograms. Metabolites of interest were then analysed by tandem MS and HRESIMS.

4.3 Work Described in Chapter Three

P. repens (2.89 kg) was collected by hand from a water reservoir at a treatment and storage plant in Dunedin, South Island, New Zealand in 2003. The sample was stored frozen. A voucher sample (03DC-01) is stored in the Chemistry Department of the University of Waikato.

4.3.1 Bioassay Procedure

Prepared cell media (500 μL) is added to bioassay subsamples (0.1 mg) which are reconstituted (0.2 mg mL^{-1}). A proportion (200 μL) is then removed and diluted into additional cell media (1 400 μL). A subsample (100 μL) of the resulting mixture (25 000 ng mL^{-1}) is added into wells A3 and A4 (Figure 13). Cell media (50 μL) is then added to wells B3-H4. Sample solution (50 μL) is then removed from wells A3 and A4 and added to wells B3 and B4 respectively, creating a two-fold dilution. This process is repeated from B-H, discarding the final additional media from H wells, creating a series of eight two-fold dilutions (from 25 000 ng mL^{-1} to 195 ng mL^{-1} , 50 μL in each well). This process is repeated in wells A5-H12 for multiple samples on a microtitre plate. Samples were run in duplicate rather than triplicate or greater because of the cost of assay consumables. A similar process is then used to prepare the positive control (triton X100) wells (one series of eight ten-fold dilutions, wells A2-H2). Cell media is added to wells A1 (100 μL) and C1-F1 (50 μL) for the negative controls, blank without cells and blank with cells respectively.

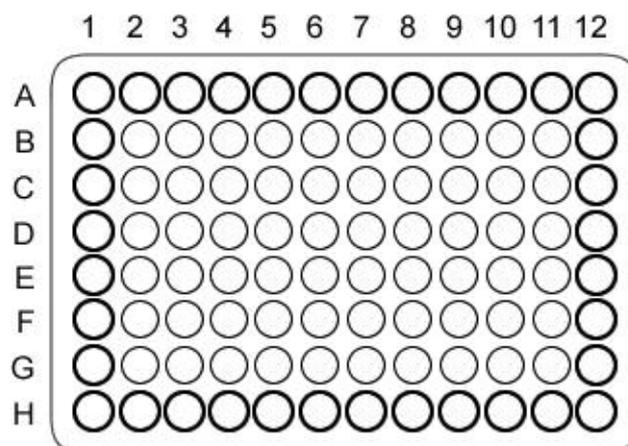


Figure 13: Diagram of a 96 well microtitre plate. Outside wells are prone to edge effects

P388 cell concentration is determined by centrifuging (5 min, 1 000 rpm; MS6 Mistral 1000 centrifuge) cells (9 mL) and decanting half the supernatant. The cells are resuspended and a proportion removed (100 μ L) into a centrifuge tube (1.5 mL) followed by addition of crystal violet solution (900 μ L) made up of crystal violet (0.008 g), citric acid (2 mL, 1 M) and deionised H₂O (18 mL). The centrifuge tube is agitated (10 sec; Cenco Vortex Mixer), a proportion is placed on a haemocytometer and cells are counted. A known amount of cells are diluted (10 000 cells per 50 μ L) in cell media. Cells (10 000 in 50 μ L) are then added to all wells containing 50 μ L (all but the blank without cells well) and incubated (72 hours).

After incubation, MTS tetrazolium is added (20 μ L) to each of the wells on the plate. The plate is incubated (4 hours) to allow the MTS tetrazolium to metabolise, then analysed on the plate reader (absorbance readings at 490 nm and at 360 nm).

To determine the percentage of inhibition of a particular concentration, the turbidity adjusted measurement (TAM) of the blank with no cells is subtracted from the TAM of all wells to give the baseline TAM for each. The baseline TAM of the sample is then divided by the baseline TAM of the blank with cells. This value is then multiplied by 100 and subtracted from 100 to give the percent inhibition of P388 cell lines for a given sample concentration.

4.3.2 Pilot Study

Exhaustive extraction (MeOH:DCM, 3:1; 17th of December 2009) on a proportion (500.23 g) of the *P. repens* sample resulted in two crude extracts (Extract A, 5.93 g and Extract B, 1.98 g) which were analysed by LCMS (Appendix: A2.1). Extract A was divided into two proportions (Extract A1, 2.56 g and Extract A2, 2.55 g) for ease of handling. Each extract was then separated by reversed phase chromatography (30 g packing) resulting in three series of fractions (Extract A1, AR1-5.1-15; Extract A2, AR1-9.1-14; Extract B, AR1-16.1-15; Appendices: A2.3, A2.4 and A3.1 respectively). Six fractions (AR1-5.4-9) were separated by size exclusion chromatography (150 g packing) and resulting fractions (AR1-21.1 to 11, AR1-29.1 to 13, AR1-32.1 to 13, AR1-36.1 to 10, AR1-40.1 to 13 and AR1-46.1 to 13; Appendix: A2.2) were assayed against the P388 cell line.

4.3.3 Work on *P. repens* Outlined in this Thesis

The following details the work undertaken on the *P. repens* extracts A1, A2 and B.

4.3.3.1 Isolation of Bioactive Metabolites

Fractions (AR1-21.1-11 and AR1-46.1-13) from Extract A1 prepared in the pilot study were obtained and fractionated by size exclusion chromatography (150 g packing). Subsamples (0.1 mg) of the resulting fractions (AA1-16.1 to 23; Appendix: A2.3) were prepared and sent to the University of Canterbury for bioassay analysis. Fractions (AA1-16.2 to 15) were analysed by LCMS, including tandem MS experiments.

Three fractions (AR1-9.7 to 9) prepared in the pilot study were fractionated by size exclusion chromatography (150 g packing) following the same solvent system used to separate similar fractions (AR1-5.4 to 9 fractionations). Similar eluting fractions (AA1-50.2, 52.2 and 54.2; AA1-50.3, 52.3 and 54.3; AA1-50.4, 52.4 and 54.4; Appendix: A2.4) were combined and then separated by size exclusion chromatography (30 g packing; Appendix: A2.5). Bioassay of a number of these fractions was undertaken at AgResearch, Hamilton, following the assay procedure outlined in section 4.3.1.

4.3.3.2 Sterol Identification

TLC of relevant fractions (AR1-16.9-12; Appendix: A3.1) against a cholesterol reference standard, indicated that fraction AR1-16.10 (169.4 mg) contained significant amounts of sterols. TMS imidazole (250 μ L; Thermo Scientific, Prod # TS-88625) was added to a subsample (8.3 mg). The mixture was sonicated (2 min; Astrason[®] Ultrasonic Cleaner) and left to react on a heating block (60°C, 1 hour). Heptane (1 mL) was added and the sample shaken on a vortex mixer (10 sec) followed by centrifuging (5 min, 3000 rpm; Eppendorf centrifuge 5702). The top heptane layer was removed by Pasteur pipette and placed in a 1.5 mL GC vial, while the lower layer was discarded.

The GC method used a splitless injection (1 μ L) with a temperature ramp (increasing from 150°C at 10°C per minute for 15 min, to a maximum temperature of 300°C) outlined in Table 16. This resulted in elution of sterol derivatives between 16.5 and 19.0 minutes (Appendices: A3.2 and A3.3).

Table 16: Temperature gradient used for GCMS experiments

Time (min)	Temperature (°C)
0.0	100
0.5	100
1.5	150
16.5	300
26.5	300

The fragmentation patterns of the sterols were computer matched to those of the NIST/EPA/NIH Mass Spectral Library, Version 2.0 d, build Dec 2 2005 to determine the sterol composition (Appendices: A3.4 to A3.8).

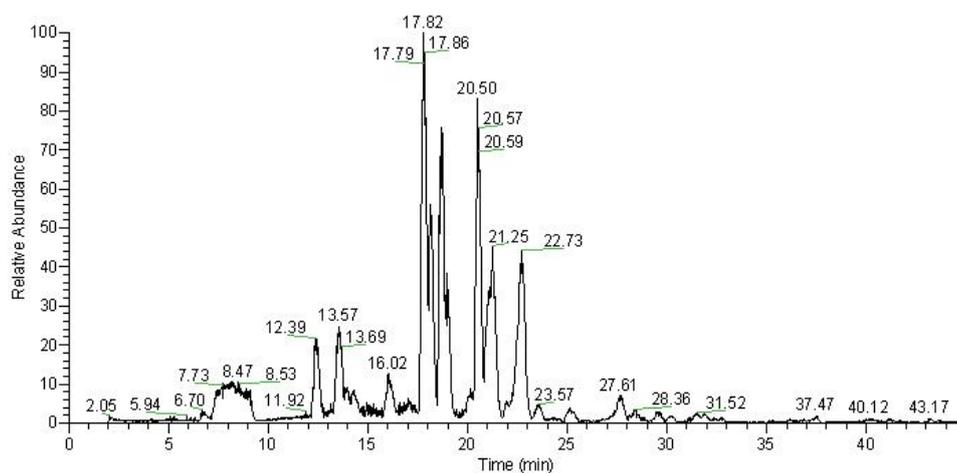
Appendices

Appendix One: Investigation of Natural Products from

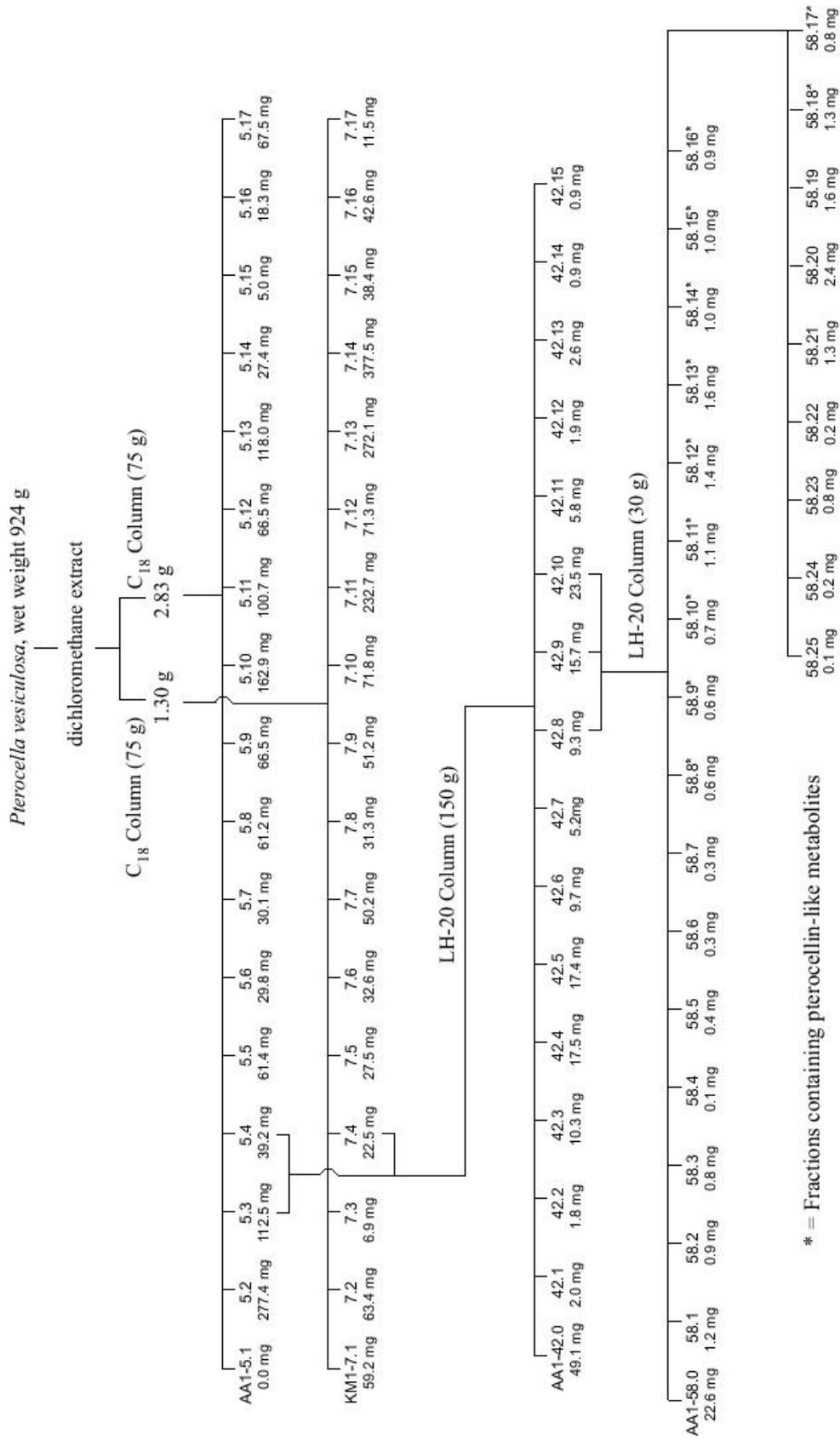
P. vesiculosa

- A1.1** Base peak chromatogram from LCMS analysis of *P. vesiculosa* crude extract
- A1.2** Separation tree for the isolation of pterocellin-like metabolites from *Pterocella vesiculosa*
- A1.3** Separation tree for the isolation of polar metabolites from *Pterocella vesiculosa*
- A1.4** Separation tree for the isolation of polar metabolites from combined samples AA1-38.3 and AA1-38.4 of *Pterocella vesiculosa*
- A1.5** Separation tree for the isolation of β -carboline alkaloids from *Pterocella vesiculosa*
- A1.6** Base peak chromatogram from LCMS analysis of fraction AA1-58.5 and mass spectra of significant metabolites
- A1.7** Base peak chromatogram from LCMS analysis of fraction AA1-58.9 and mass spectra of significant metabolites
- A1.8** Base peak chromatogram from LCMS analysis of fraction AA1-58.20 and mass spectra of significant metabolites
- A1.9** Base peak chromatogram from LCMS analysis of fraction AA1-78.11 and mass spectra of significant metabolites
- A1.10** Base peak chromatogram from LCMS analysis of fraction AA1-78.18 and mass spectra of significant metabolites
- A1.11** Base peak chromatogram from LCMS analysis of fraction AA1-38.5 and mass spectra of significant metabolites

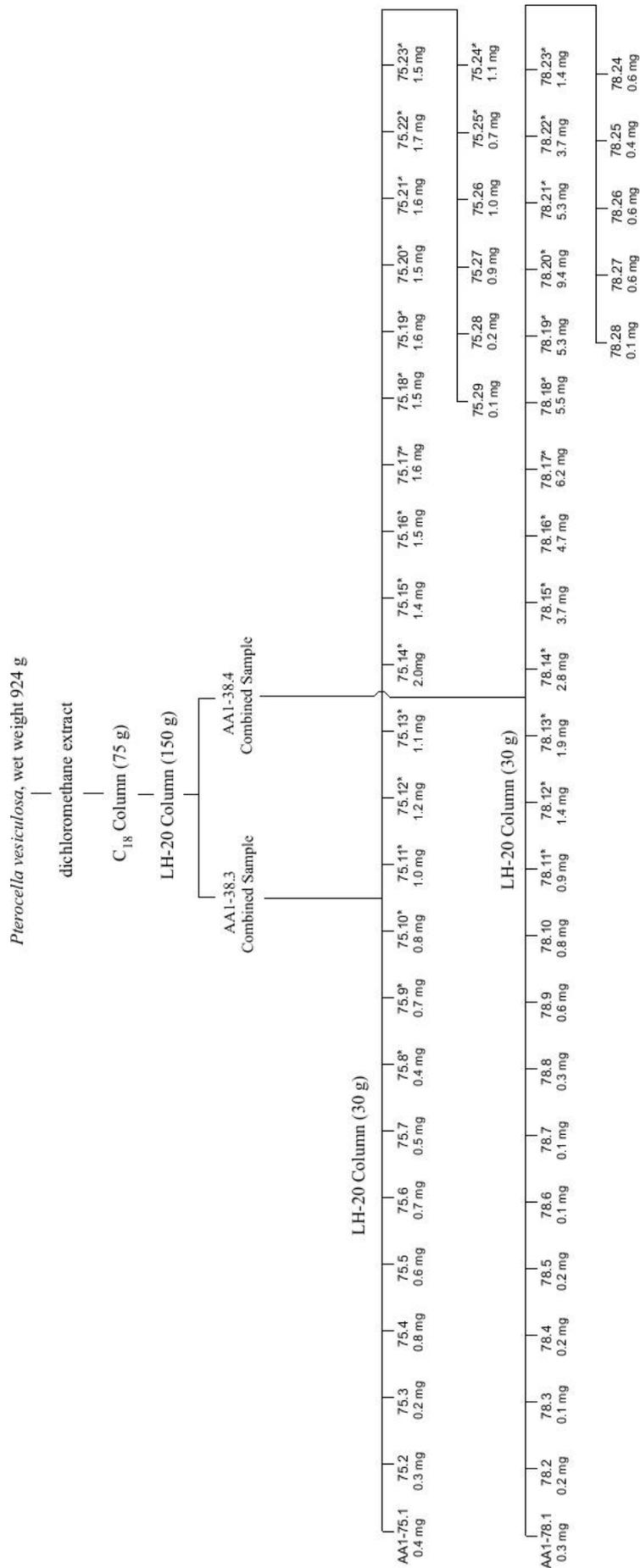
- A1.12** Base peak chromatogram from LCMS analysis of fraction AA1-38.6
and mass spectra of significant metabolites
- A1.13** Base peak chromatogram from LCMS analysis of fraction AA1-94.10
and mass spectra of significant metabolites
- A1.14** Base peak chromatogram from LCMS analysis of fraction AA1-114.13
and mass spectra of significant metabolites
- A1.15** Base peak chromatogram from LCMS analysis of fraction AA1-94.29
and mass spectra of minor brominated metabolites



A1.1: Base peak chromatogram from LCMS analysis of *P. vesiculosa* crude extract



A1.2: Separation tree for the isolation of pterocellin-like metabolites from *Pterocella vesiculosa*

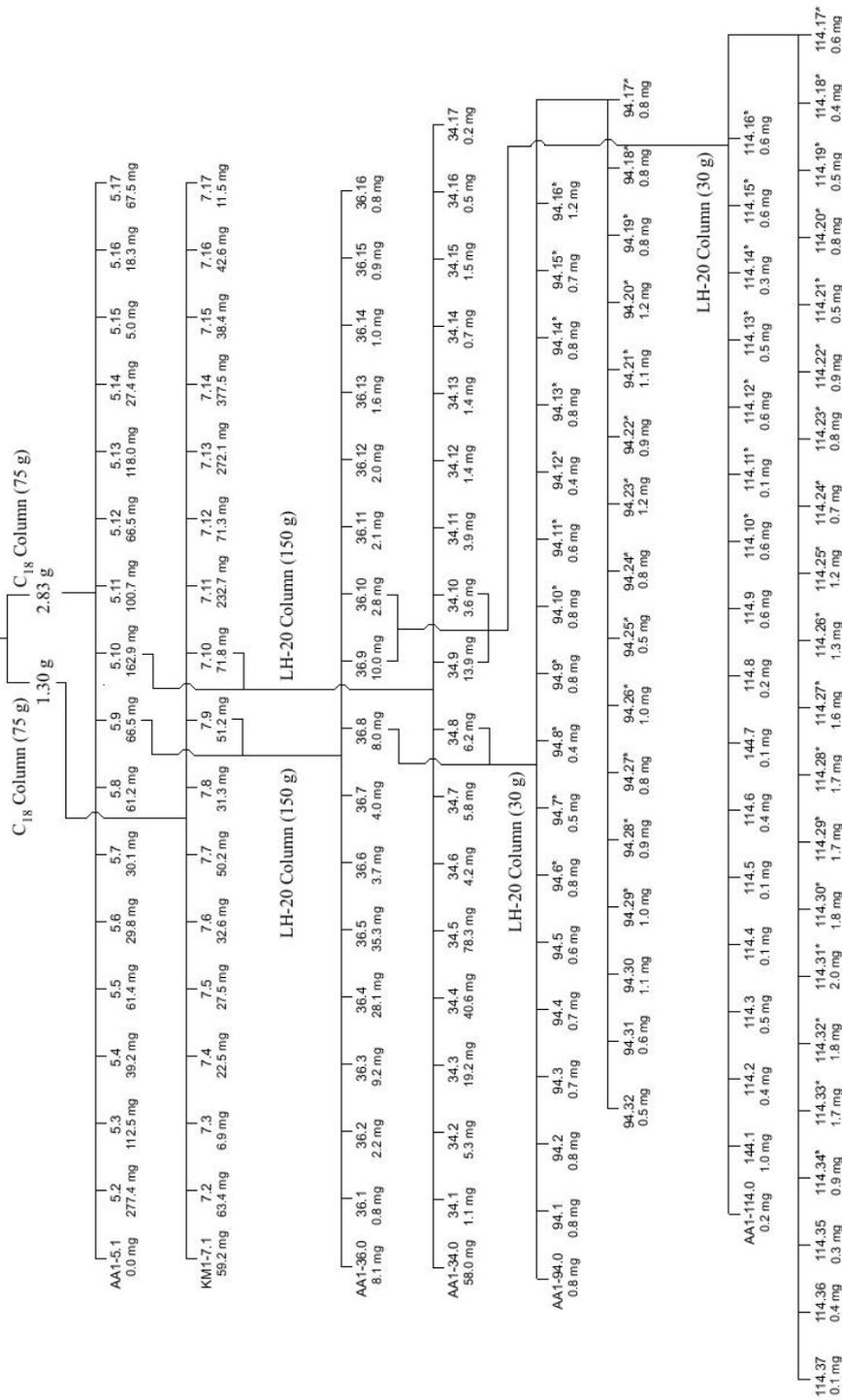


* = Fractions containing polar, pterocellin-like metabolites

A1.4: Separation tree for the isolation of polar metabolites from combined samples AA1-38.3 and AA1-38.4 of *Pterocella vesiculosa*

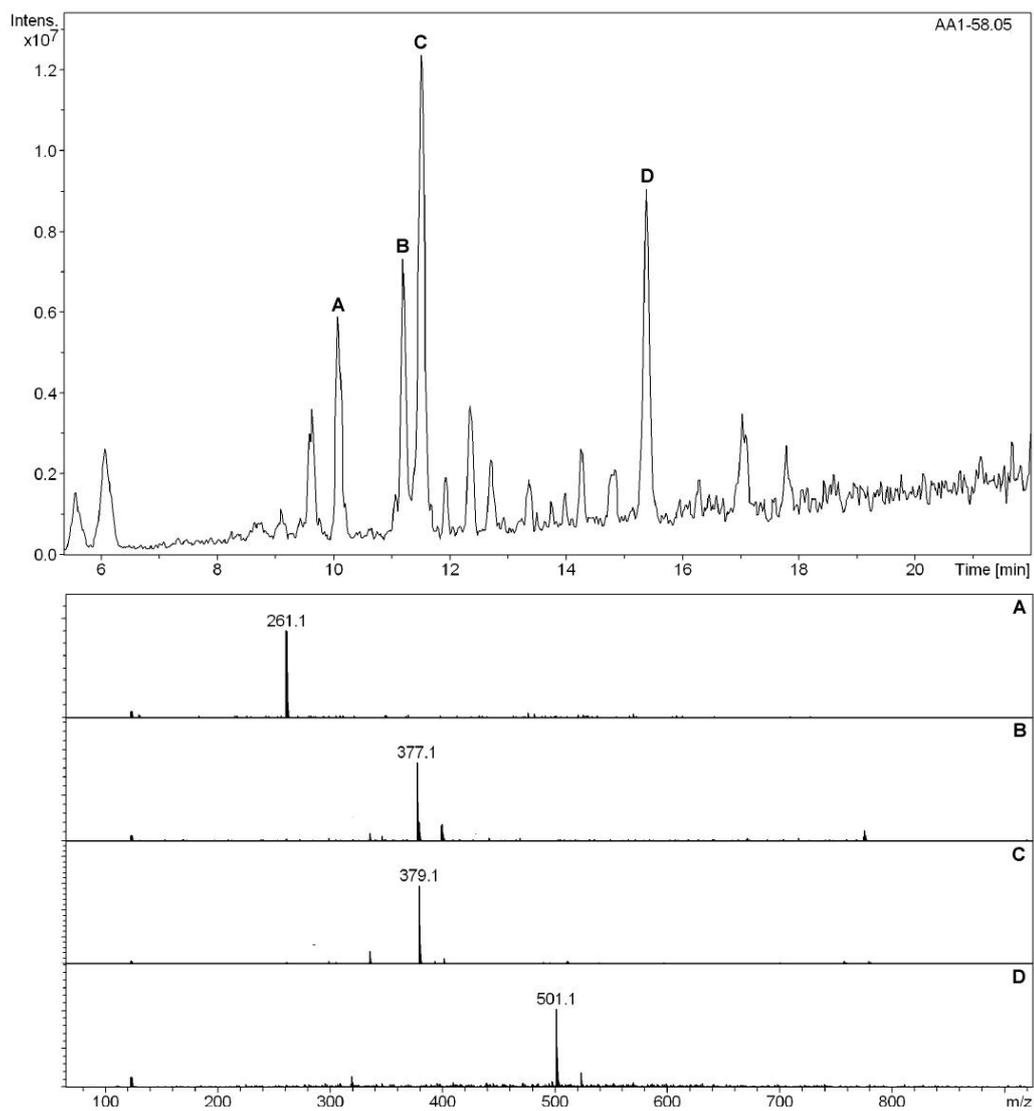
Pterocella vesiculosa, wet weight 924 g

dichloromethane extract

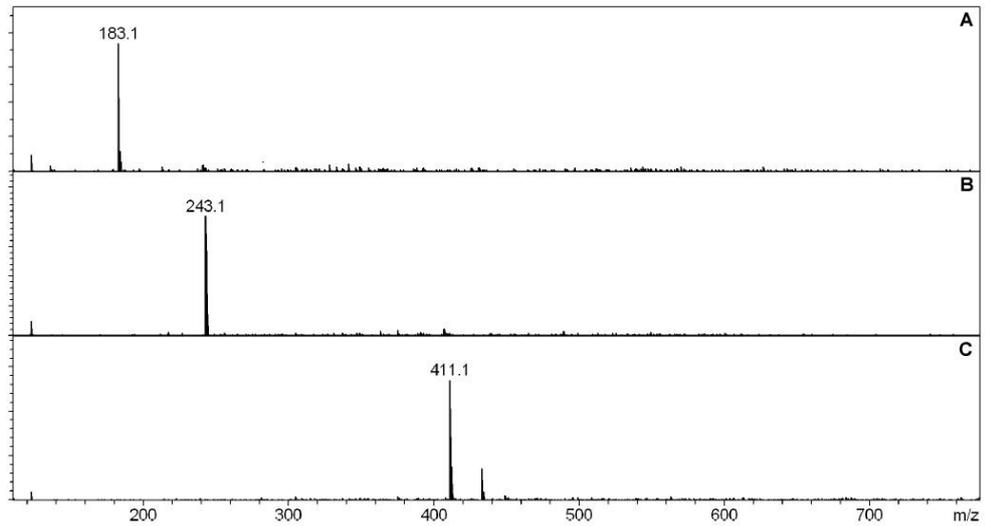
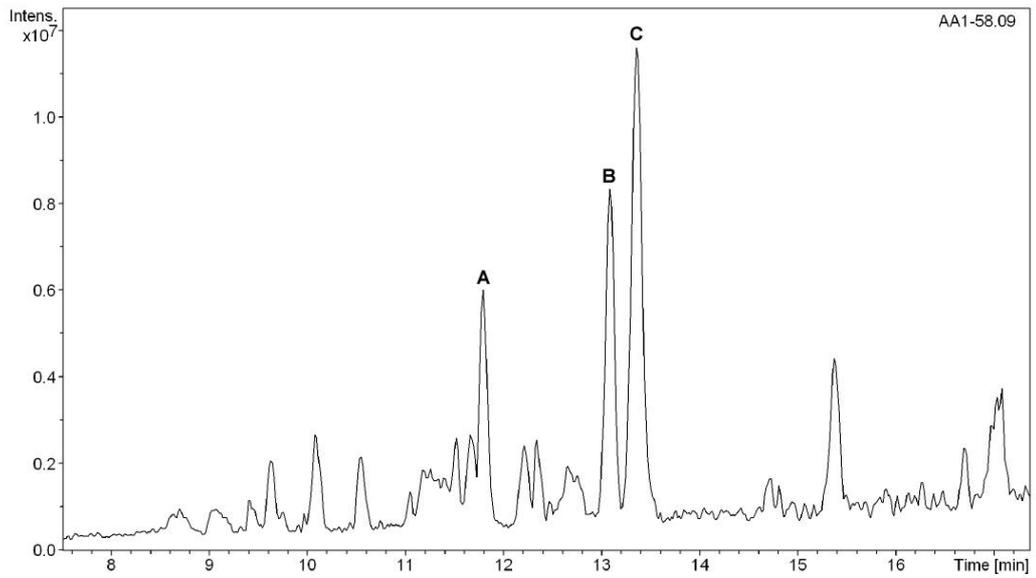


* = Fractions containing β-carboline alkaloids

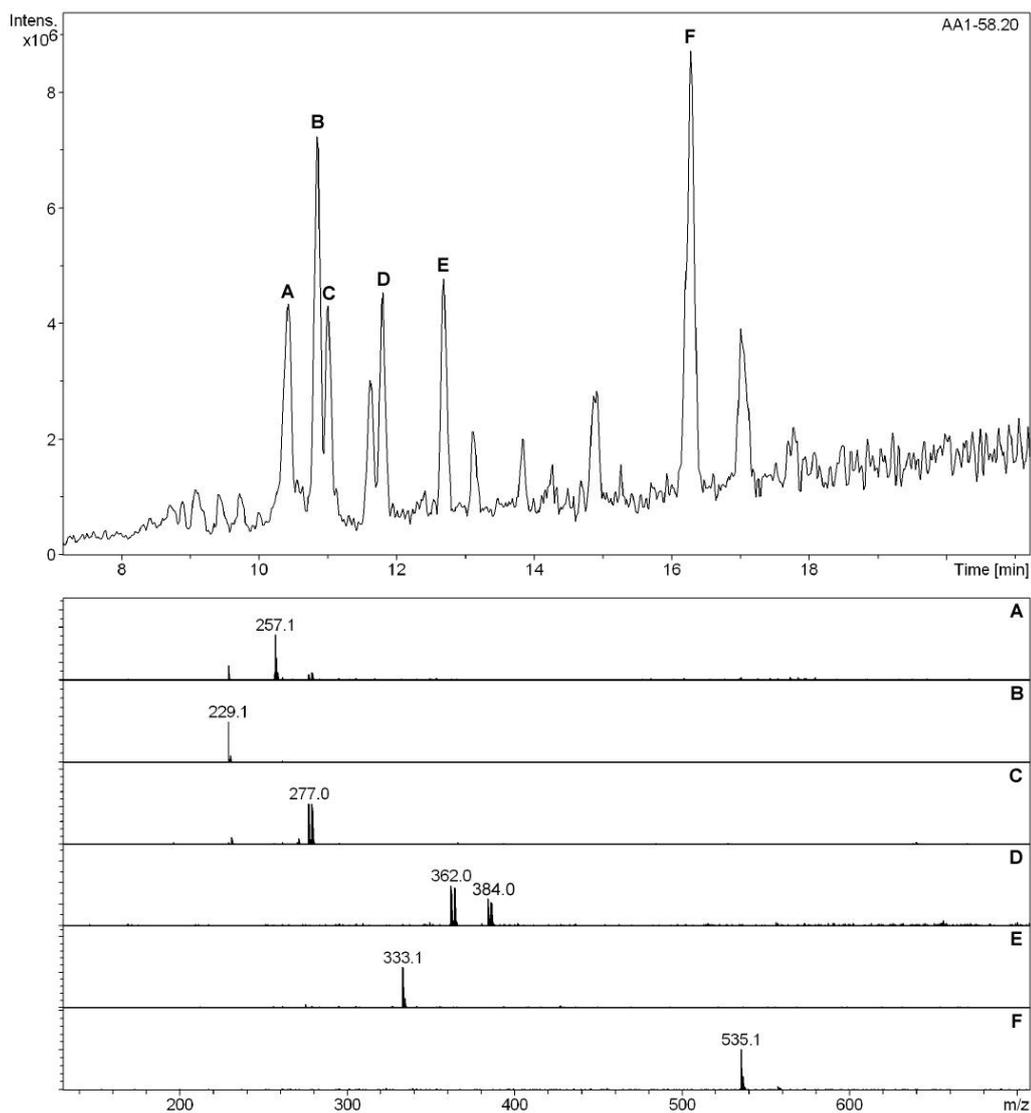
A1.5: Separation tree for the isolation of β-carboline alkaloids from *Pterocella vesiculosa*



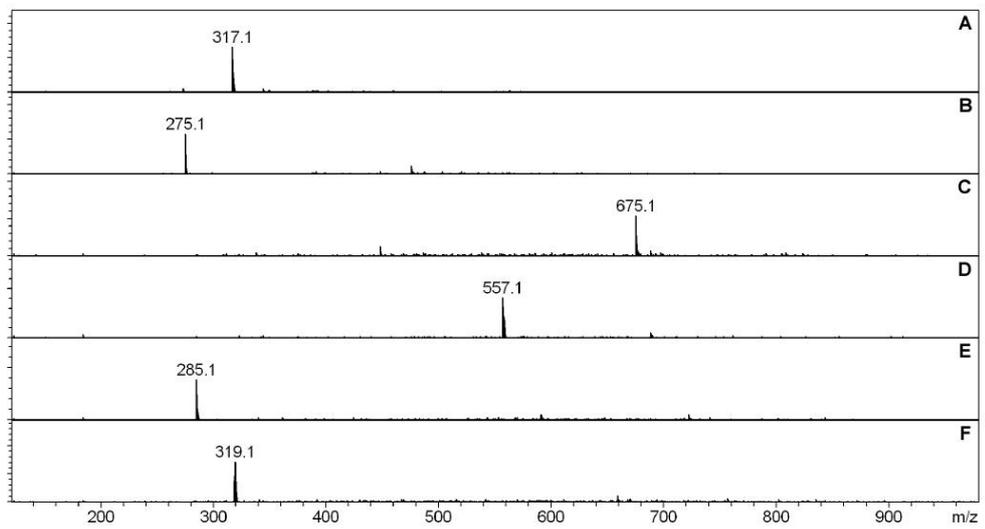
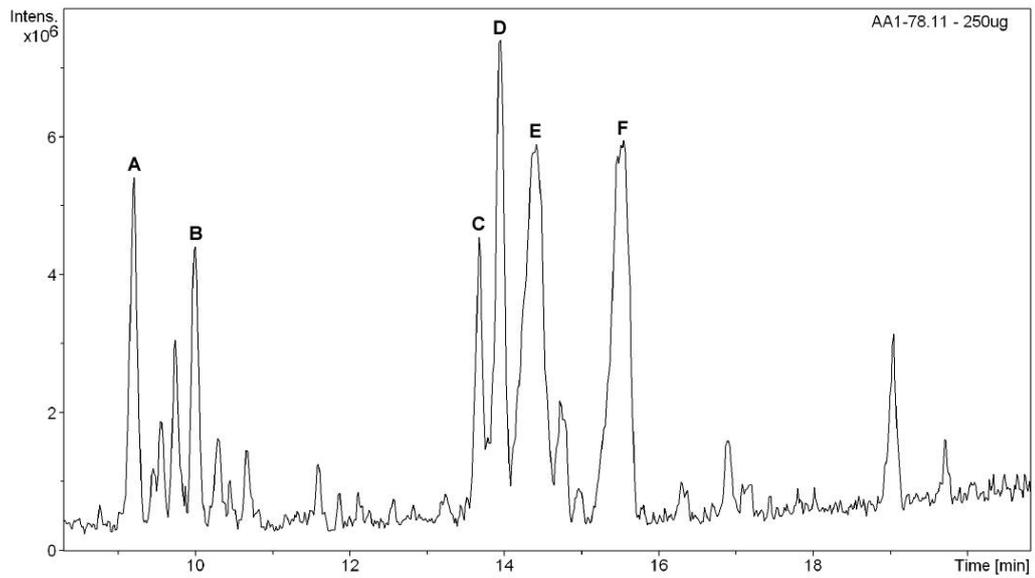
A1.6: Base peak chromatogram from LCMS analysis of fraction AA1-58.5 and mass spectra of significant metabolites



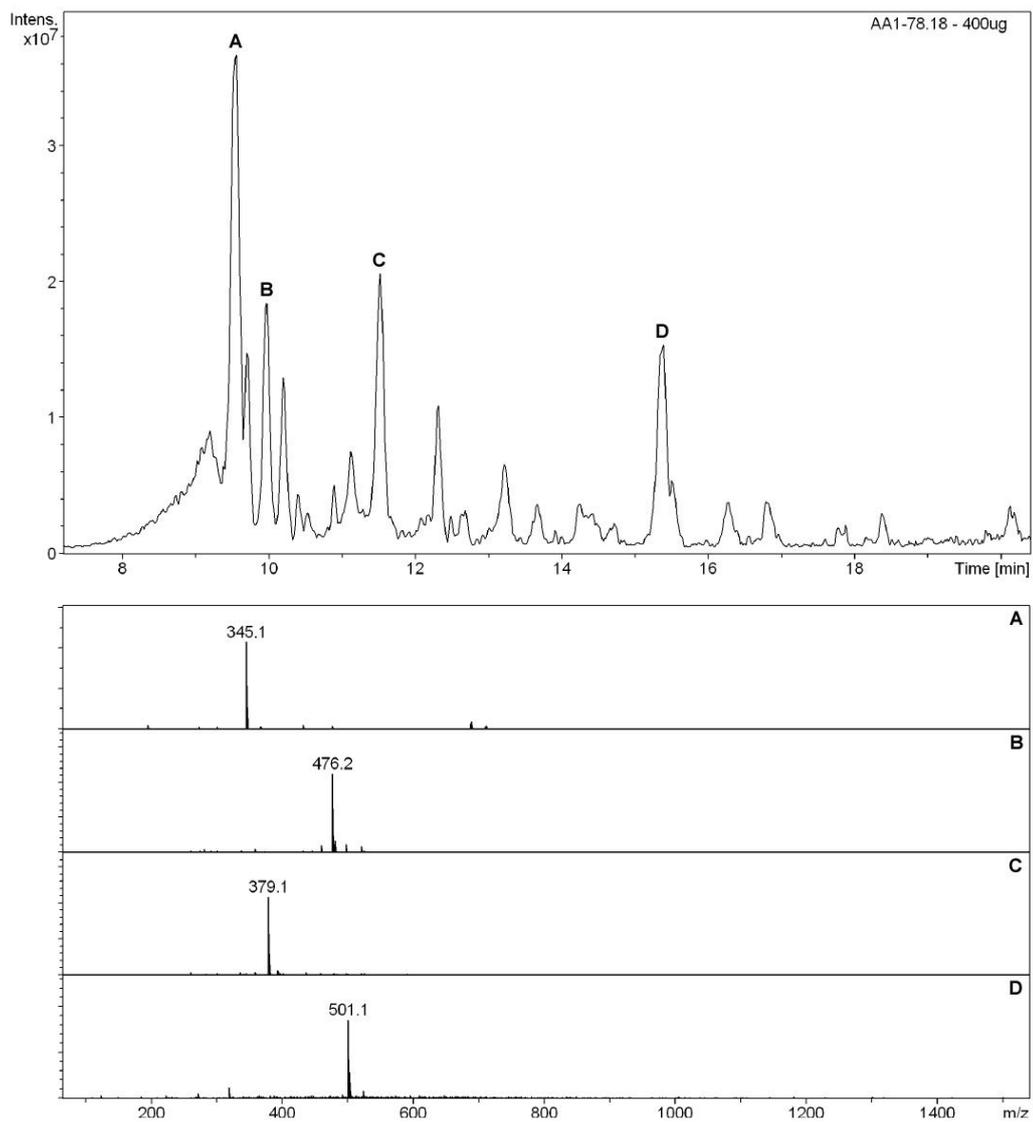
A1.7: Base peak chromatogram from LCMS analysis of fraction AA1-58.9 and mass spectra of significant metabolites



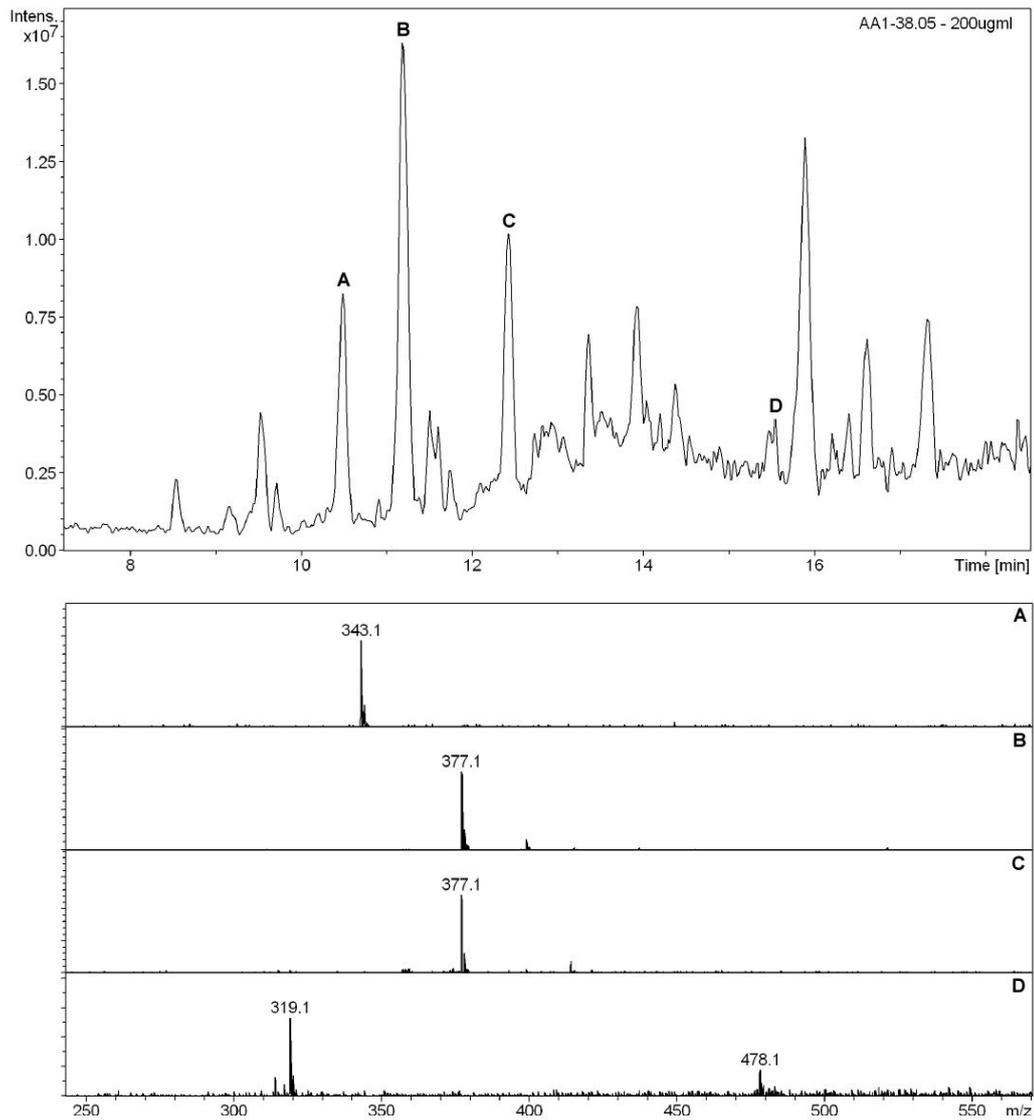
A1.8: Base peak chromatogram from LCMS analysis of fraction AA1-58.20 and mass spectra of significant metabolites



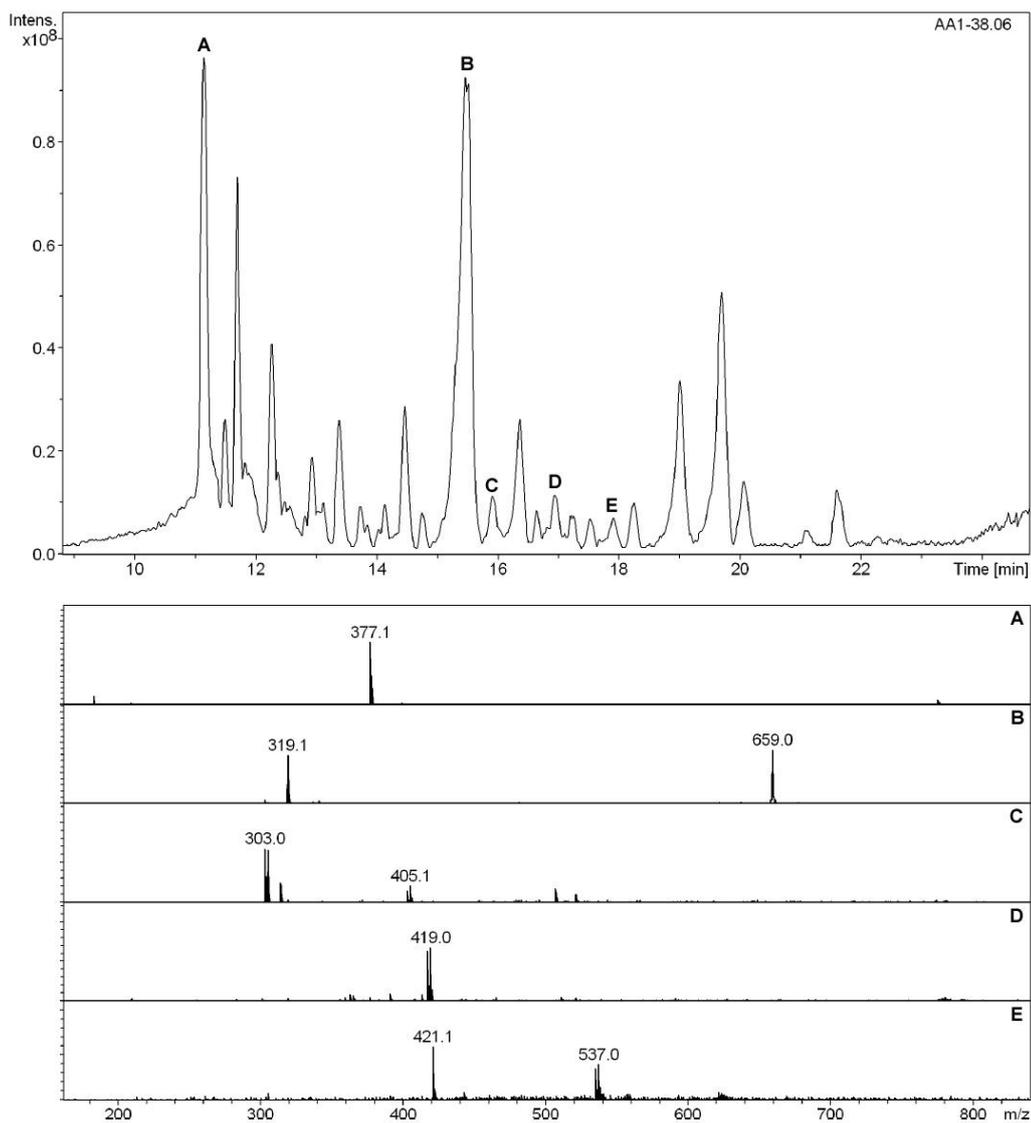
A1.9: Base peak chromatogram from LCMS analysis of fraction AA1-78.11 and mass spectra of significant metabolites



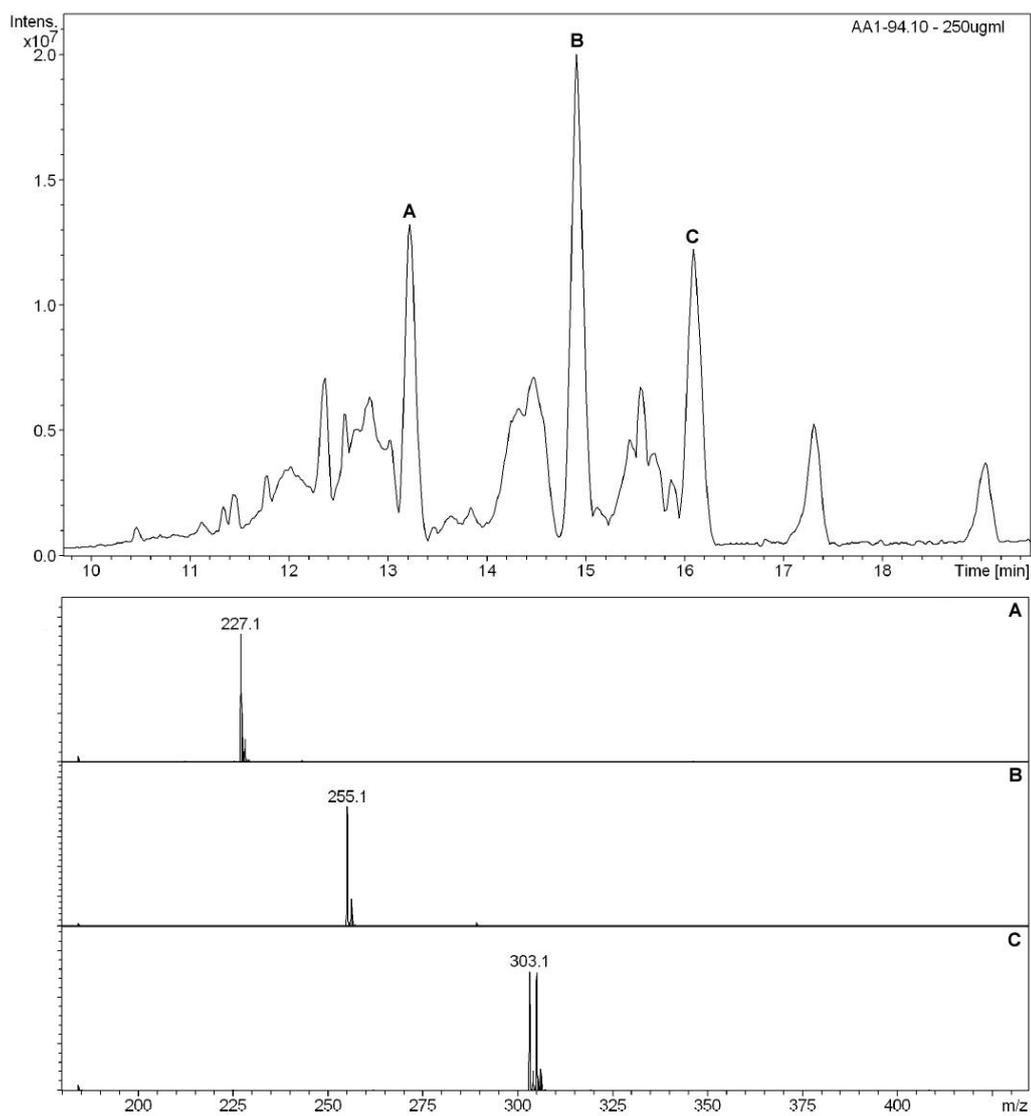
A1.10: Base peak chromatogram from LCMS analysis of fraction AA1-78.18 and mass spectra of significant metabolites



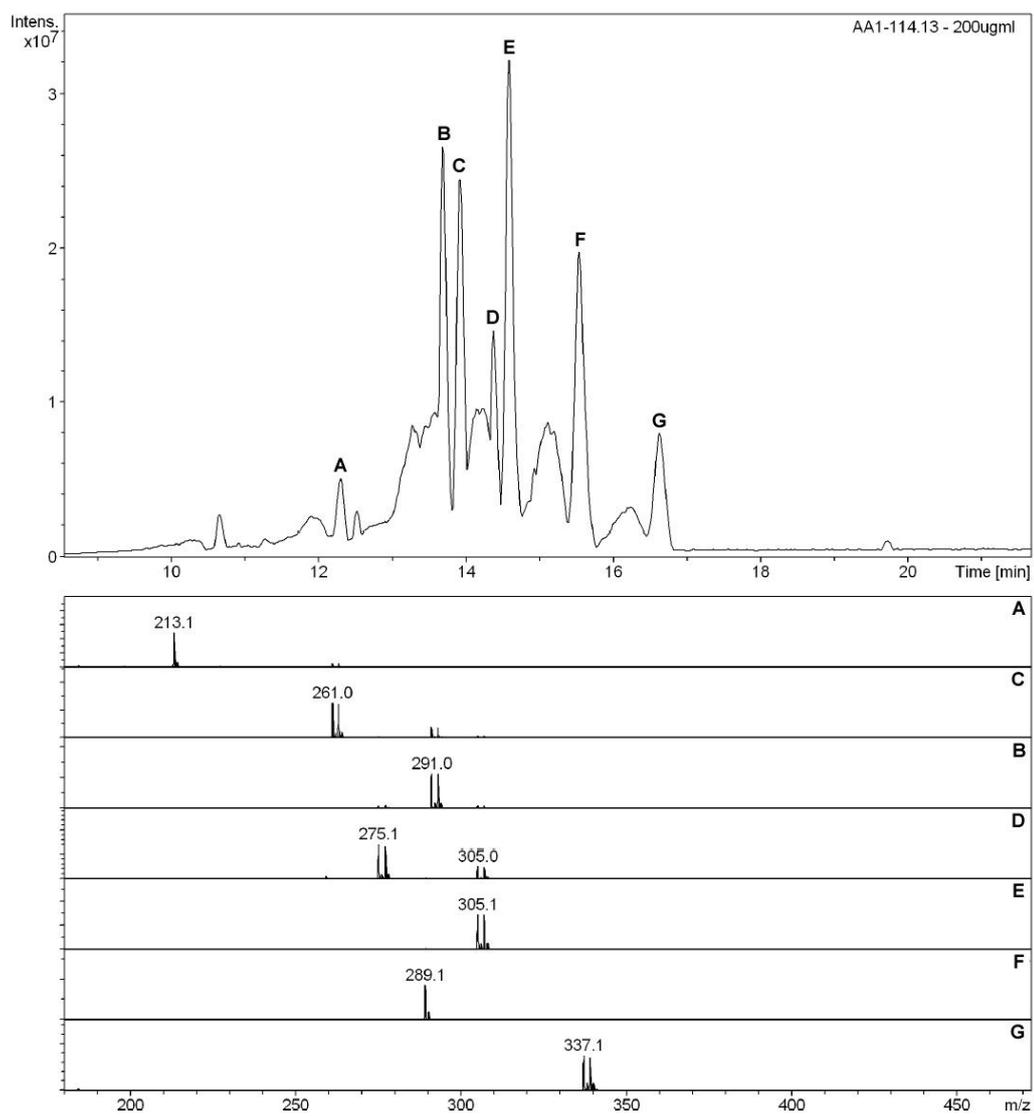
A1.11: Base peak chromatogram from LCMS analysis of fraction AA1-38.5 and mass spectra of significant metabolites



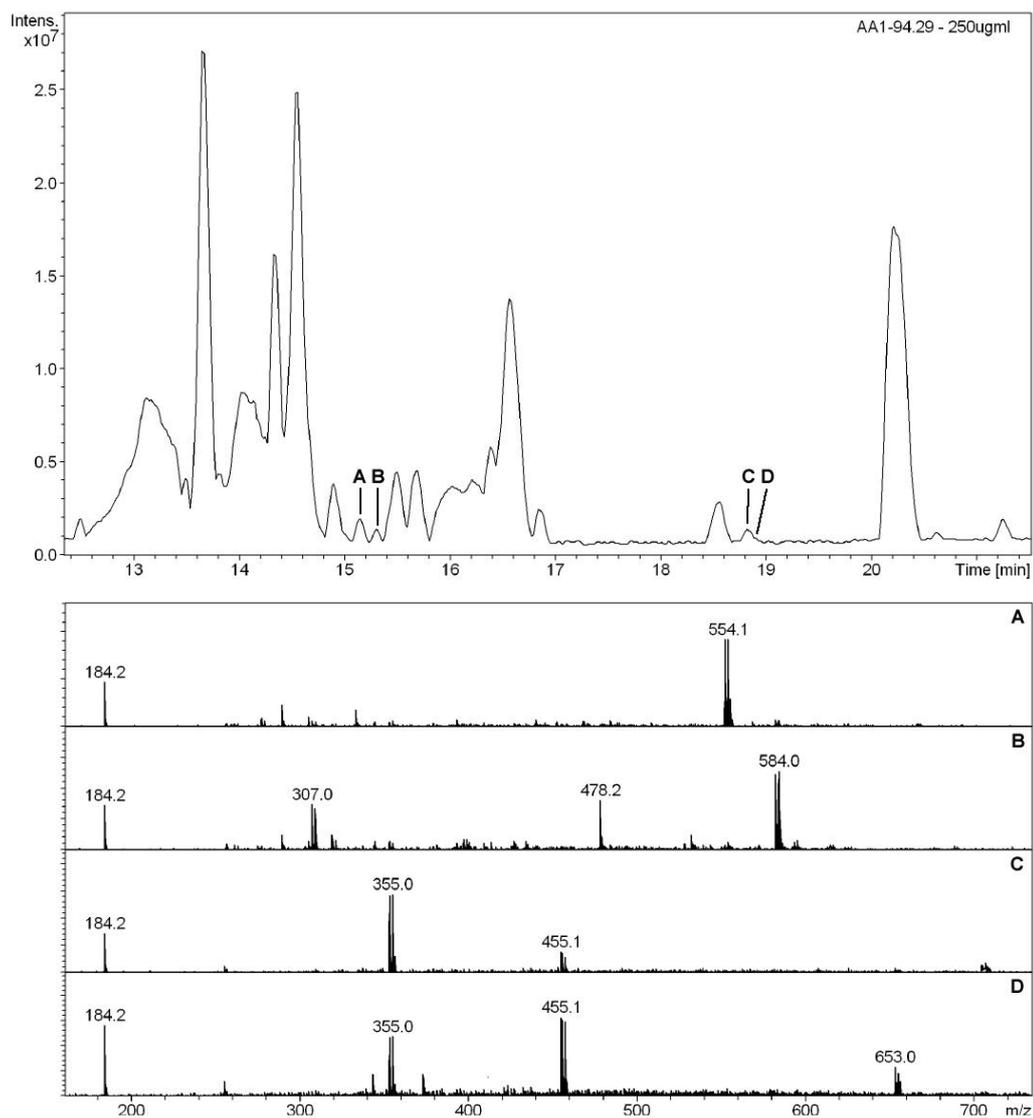
A1.12: Base peak chromatogram from LCMS analysis of fraction AA1-38.6 and mass spectra of significant metabolites



A1.13: Base peak chromatogram from LCMS analysis of fraction AA1-94.10 and mass spectra of significant metabolites



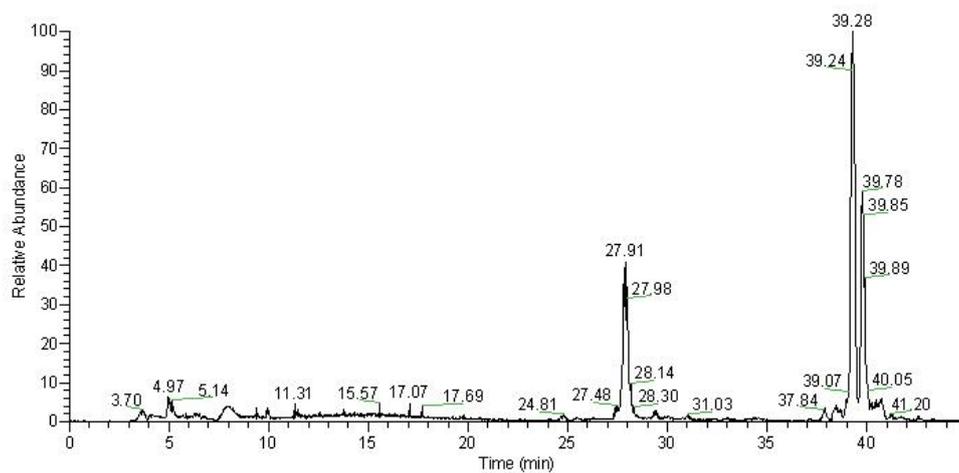
A1.14: Base peak chromatogram from LCMS analysis of fraction AA1-114.13 and mass spectra of significant metabolites



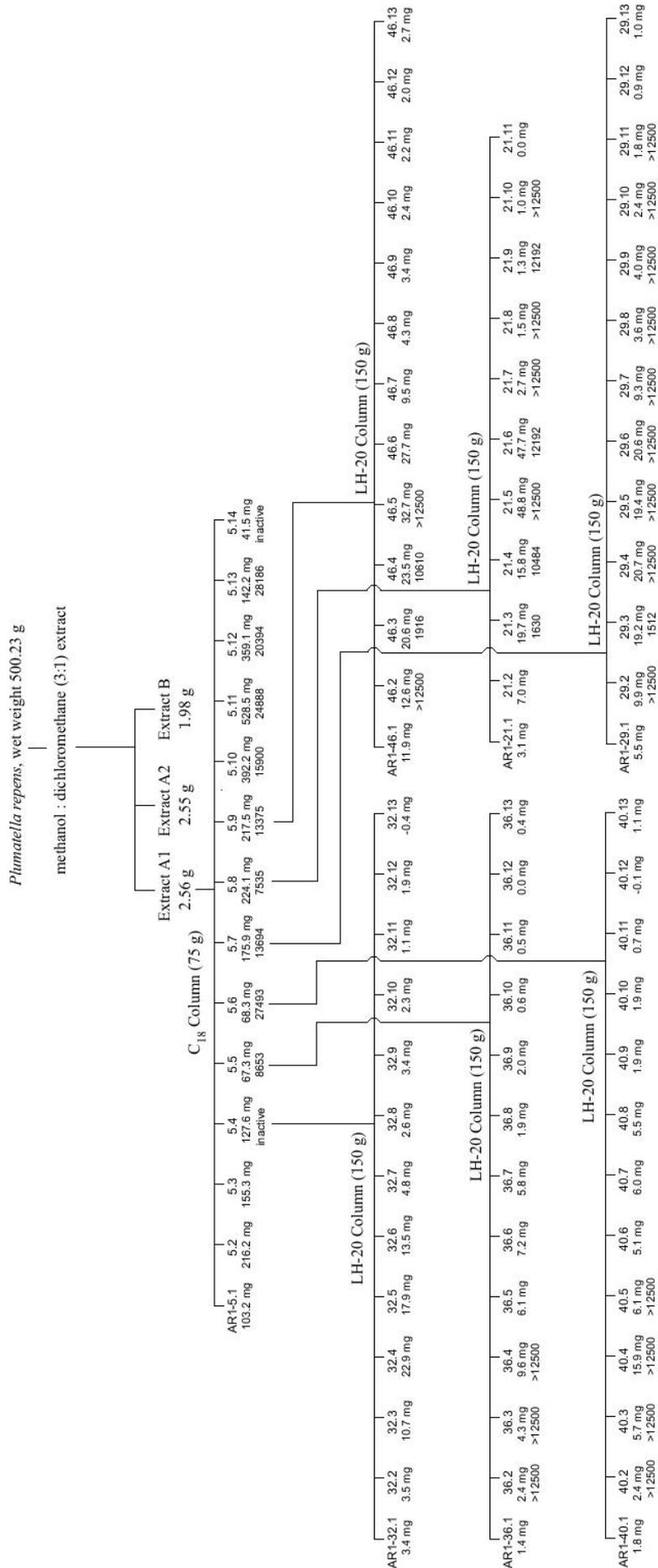
A1.15: Base peak chromatogram from LCMS analysis of fraction AA1-94.29 and mass spectra of minor brominated metabolites

Appendix Two: Investigation of Natural Products from *P. repens*

- A2.1** Base peak chromatogram from LCMS analysis of *Plumatella repens* crude extract
- A2.2** Separation tree for *Plumatella repens*, Extract A1 from the pilot study
- A2.3** Separation tree for the isolation of bioactive metabolites from Extract A1 of *Plumatella repens*
- A2.4** Separation tree for the isolation of bioactive metabolites from Extract A2 of *Plumatella repens*
- A2.5** Separation tree for the isolation of bioactive metabolites from combined samples AA1-50.2 to AA1-50.4 of *Plumatella repens*
- A2.6** Base peak chromatogram from LCMS analysis of fraction AA1-16.2 and mass spectra of significant metabolites
- A2.7** Base peak chromatogram from LCMS analysis of fraction AA1-16.8 and mass spectra of significant metabolites
- A2.8** Base peak chromatogram from LCMS analysis of fraction AA1-16.14 and mass spectra of significant metabolites



A2.1: Base peak chromatogram from LCMS analysis of *Plumatella repens* crude extract

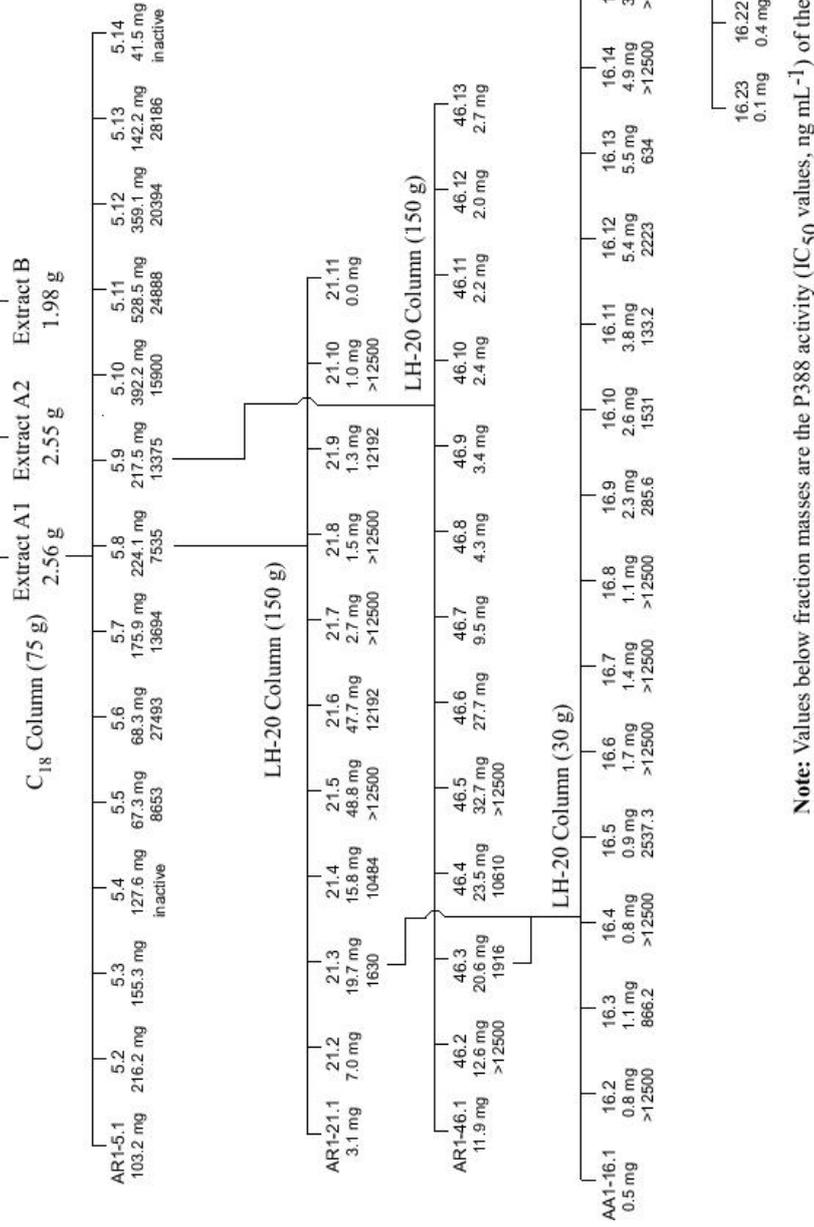


Note: Values below fraction masses are the P388 activity (IC₅₀ values, ng mL⁻¹) of the above fraction

A2.2: Separation tree for *Plumatella repens*, Extract A1 from the pilot study

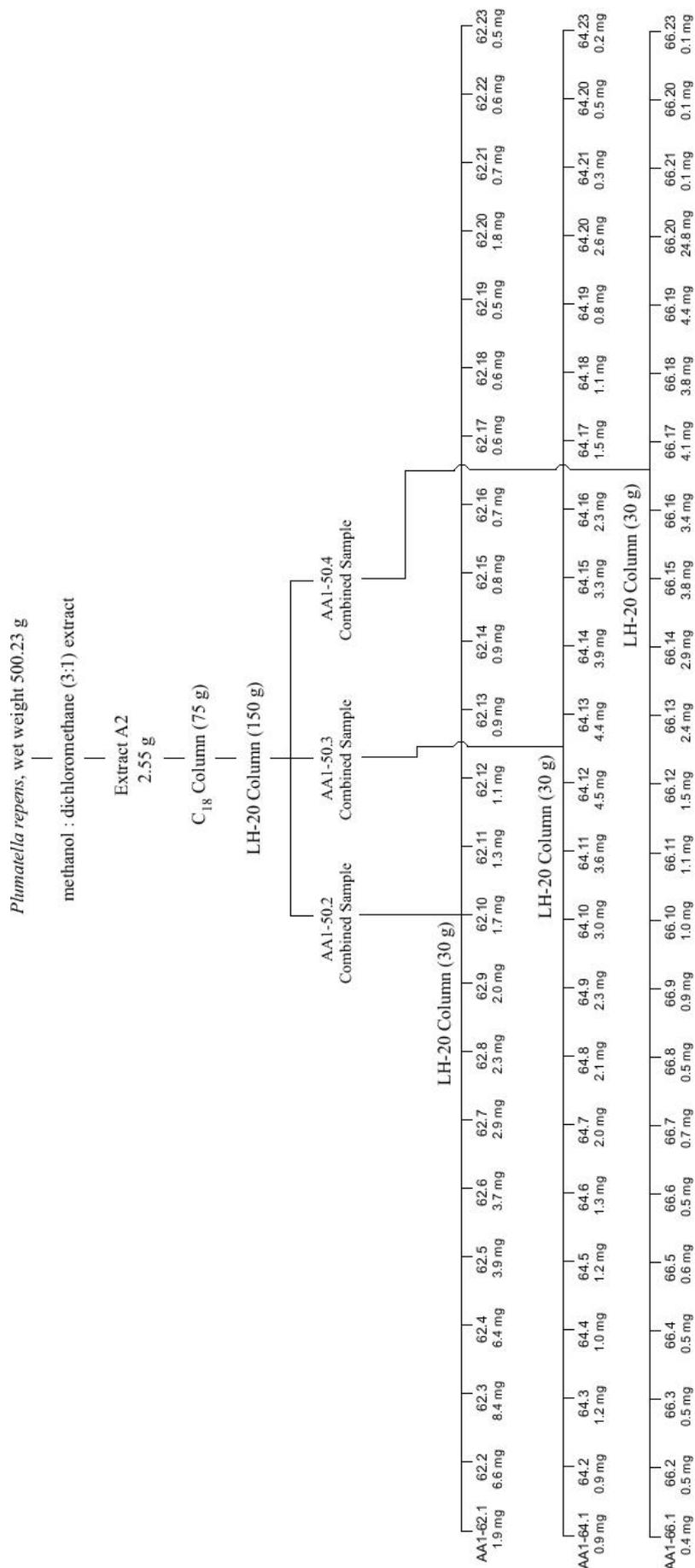
Plumatella repens, wet weight 500.23 g

methanol : dichloromethane (3:1) extract

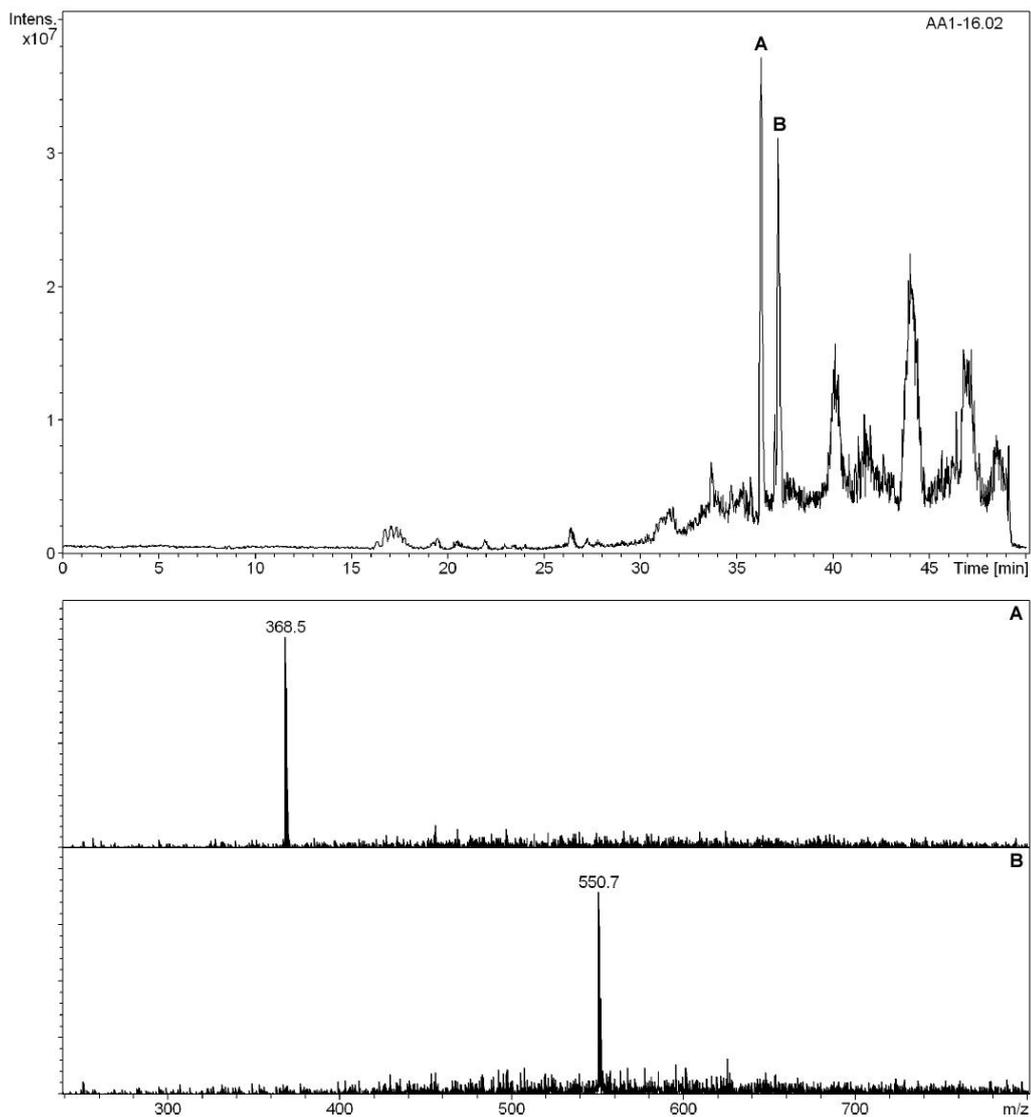


Note: Values below fraction masses are the P388 activity (IC₅₀ values, ng mL⁻¹) of the above fraction

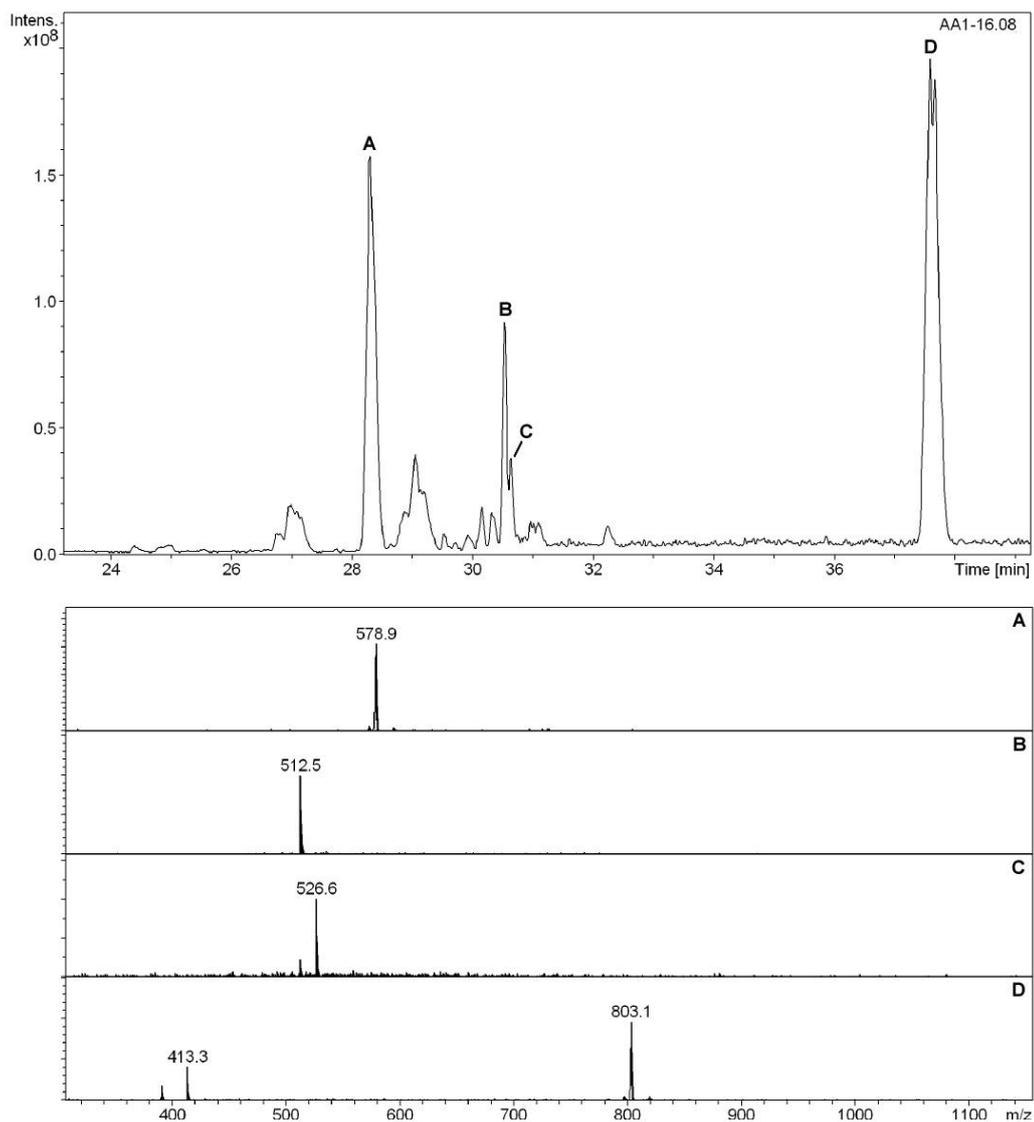
A2.3: Separation tree for the isolation of bioactive metabolites from Extract A1 of *Plumatella repens*



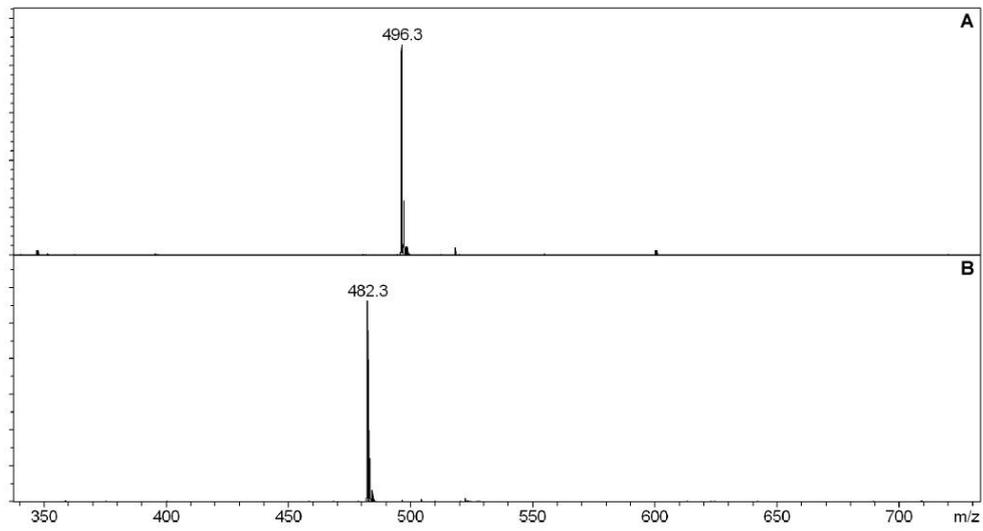
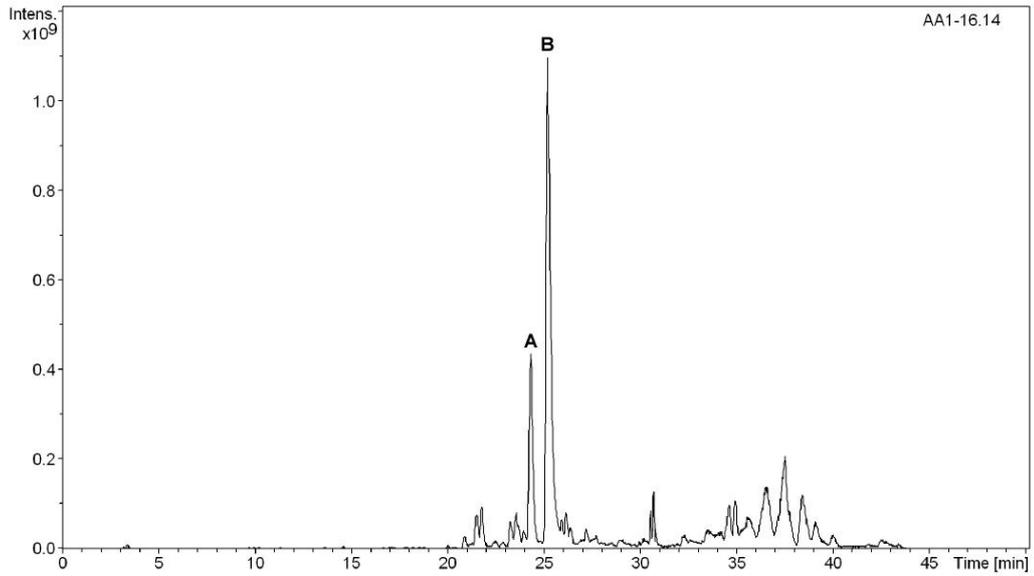
A2.5: Separation tree for the isolation of bioactive metabolites from combined samples AA1-50.2 to AA1-50.4 of *Plumatella repens*



A2.6: Base peak chromatogram from LCMS analysis of fraction AA1-16.2 and mass spectra of significant metabolites



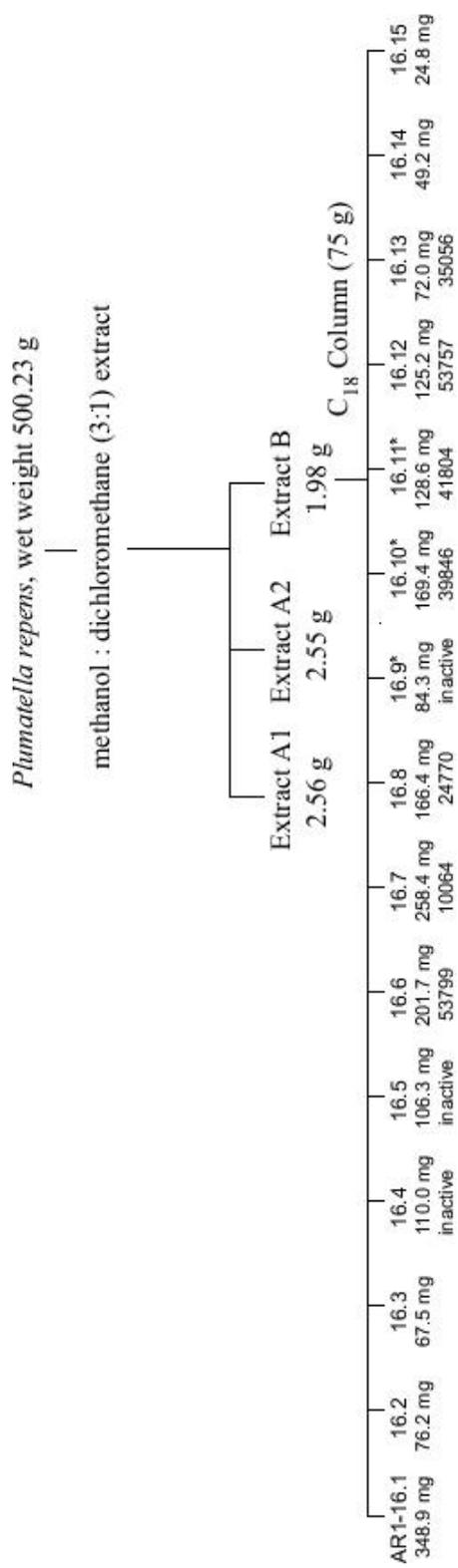
A2.7: Base peak chromatogram from LCMS analysis of fraction AA1-16.8 and mass spectra of significant metabolites



A2.8: Base peak chromatogram from LCMS analysis of fraction AA1-16.14 and mass spectra of significant metabolites

Appendix Three: Investigation of Sterols from *P. repens*

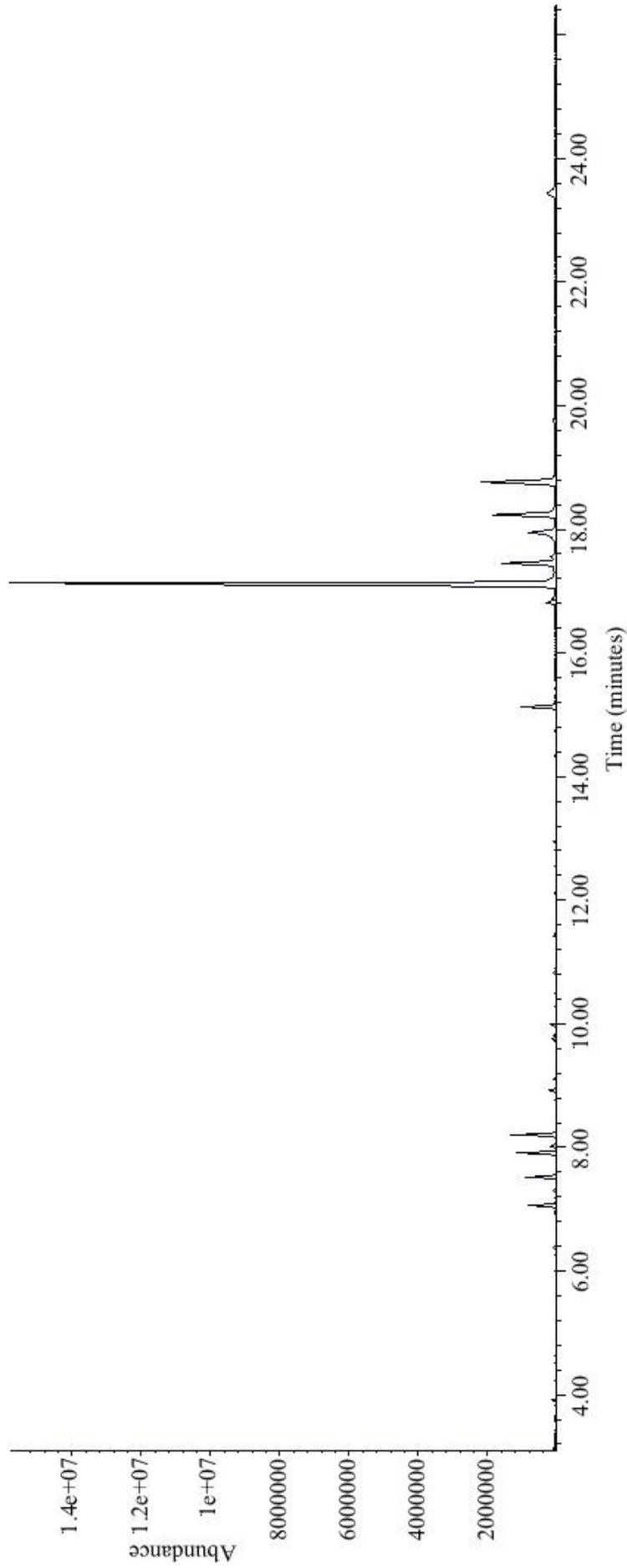
- A3.1** Separation tree for the identification of sterol containing fractions of *Plumatella repens*
- A3.2** GCMS spectrum of *Plumatella repens* Extract B, fraction AR1-16.10
- A3.3** Sterol eluting section of GCMS spectrum from *Plumatella repens* Extract B, fraction AR1-16.10
- A3.4** Fragmentation pattern from TMS ether of sterol eluting at 17.14 min by GCMS, compared with the library spectrum of cholesterol trimethylsilyl ether
- A3.5** Fragmentation pattern from TMS ether of sterol eluting at 17.46 min by GCMS, compared with the library spectrum of (3 β ,22*E*)-ergosta-7,22-dien-3-ol trimethylsilyl ether
- A3.6** Fragmentation pattern from TMS ether of sterol eluting at 17.96 min by GCMS, compared with the library spectrum of (3 β ,24*R*)-ergosta-5-en-3-ol trimethylsilyl ether
- A3.7** Fragmentation pattern from TMS ether of sterol eluting at 18.25 min by GCMS, compared with the library spectrum of stigmasterol trimethylsilyl ether
- A3.8** Fragmentation pattern from TMS ether of sterol eluting at 18.78 min by GCMS, compared with the library spectrum of β -sitosterol trimethylsilyl ether



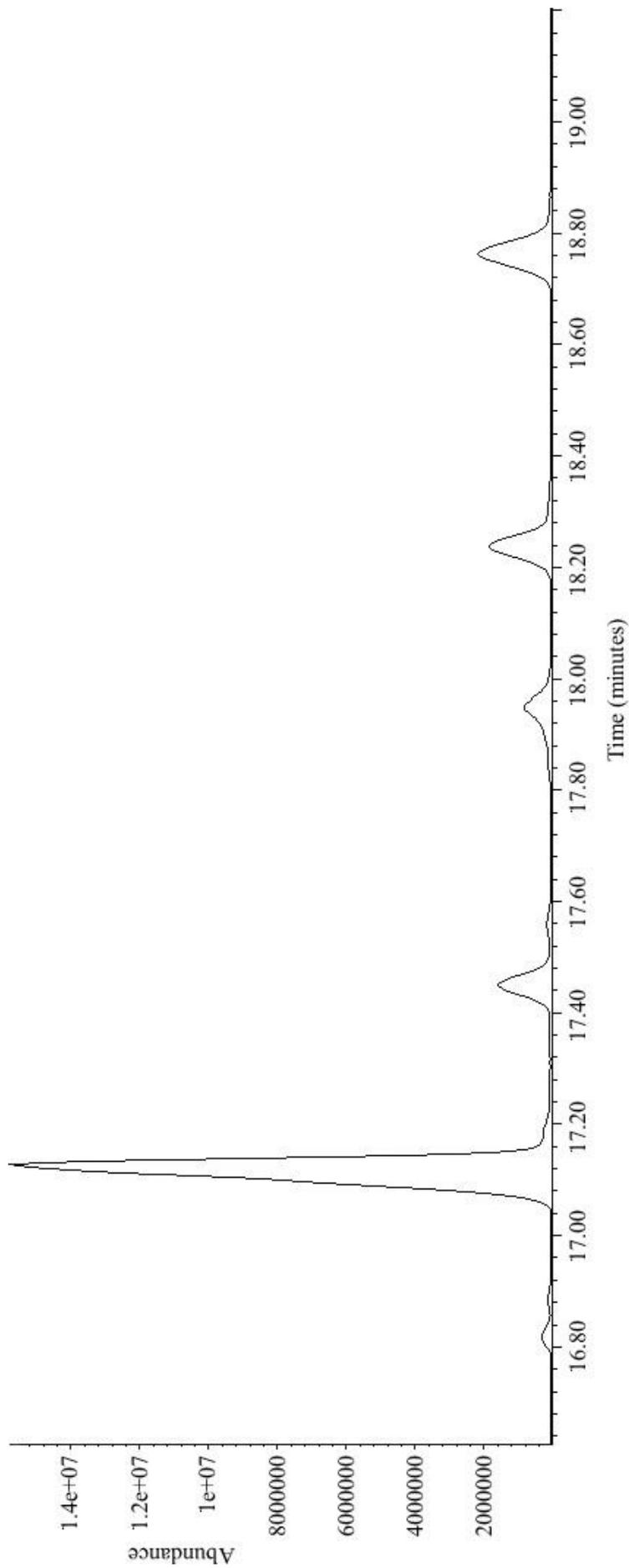
* = Fractions analysed for sterol composition

Note: Values below fraction masses are the P388 activity (IC₅₀ values, ng mL⁻¹) of the above fraction

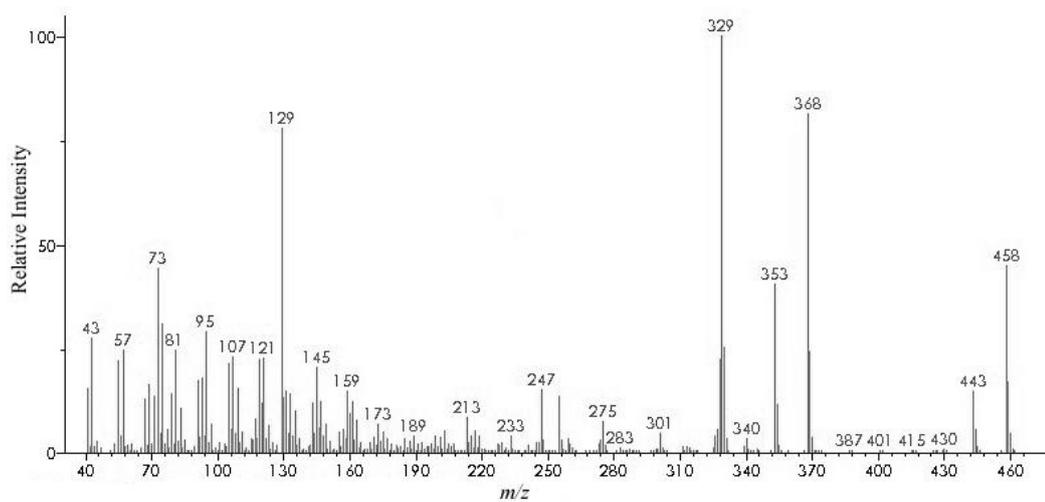
A3.1: Separation tree for the fractionation of Extract B from *Plumatella repens*



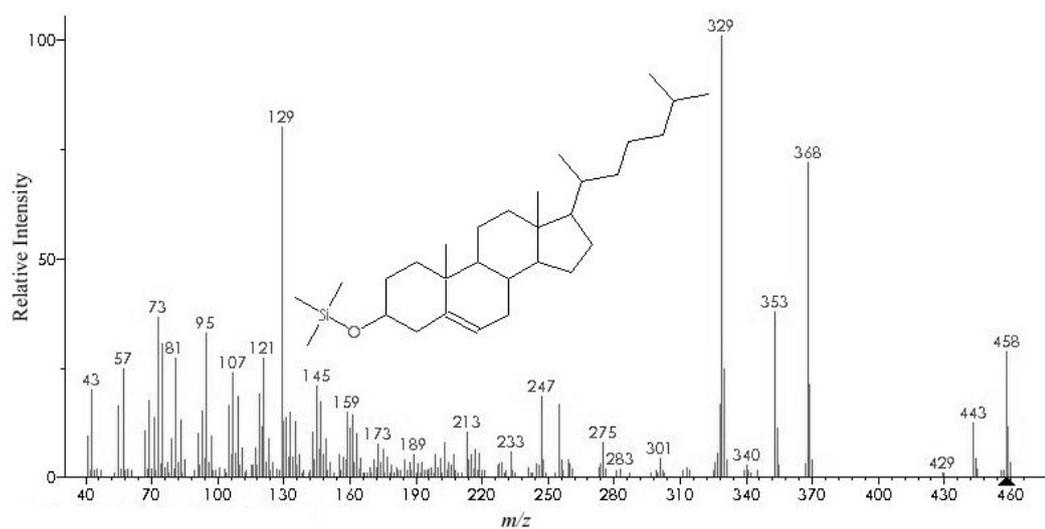
A.3.2: GCMS spectrum of *Plumatella repens* Extract B, fraction AR1-16.10



A.3.3: Sterol eluting section of GCMS spectrum from *Plumatella repens* Extract B, fraction AR1-16.10

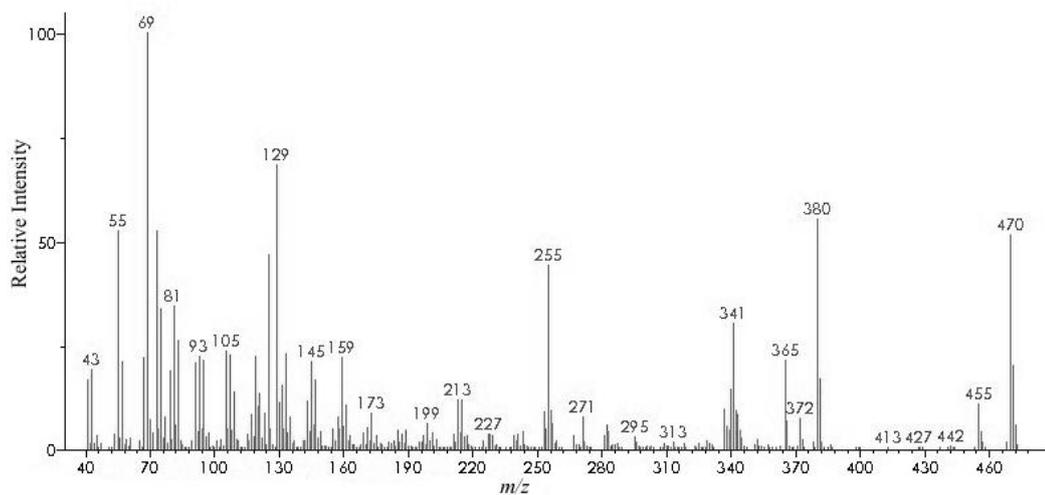


Background subtracted spectrum of AR1-16.10 (averaged from 17.080 to 17.138 minutes)

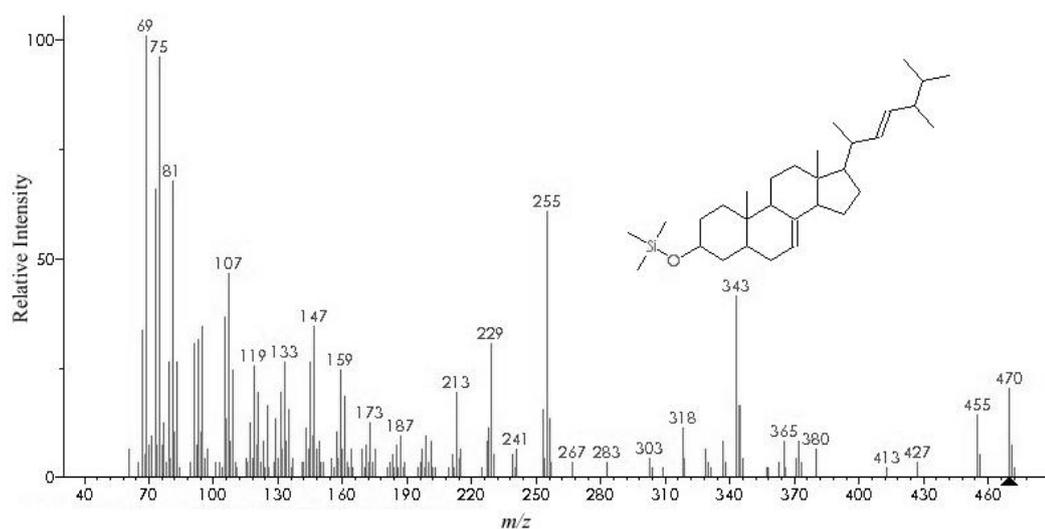


Library spectrum for cholesteroltrimethylsilylether

A3.4: Fragmentation pattern from TMS ether of sterol eluting at 17.14 min by GCMS, compared with the library spectrum of cholesterol trimethylsilyl ether

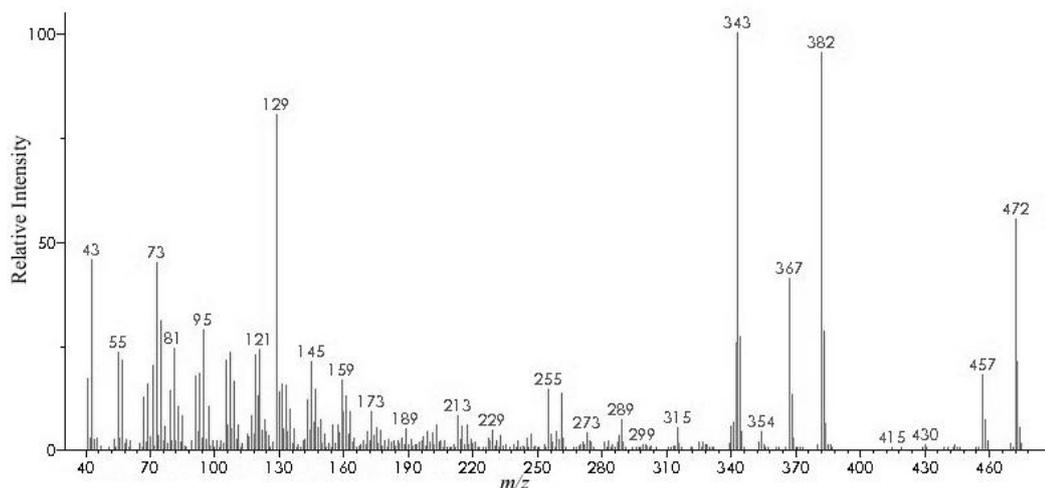


Background subtracted spectrum of AR1-16.10 (averaged from 17.439 to 17.465 minutes)

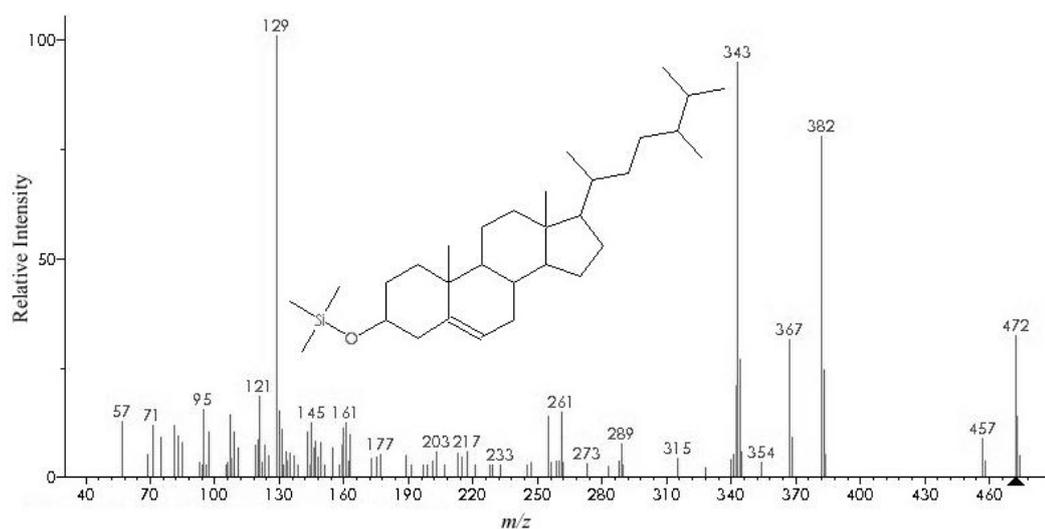


Library spectrum for $(3\beta,22E)$ -ergosta-7,22-dien-3-yltrimethylsilane

A3.5: Fragmentation pattern from TMS ether of sterol eluting at 17.46 min by GCMS, compared with the library spectrum of $(3\beta,22E)$ -ergosta-7,22-dien-3-ol trimethylsilyl ether

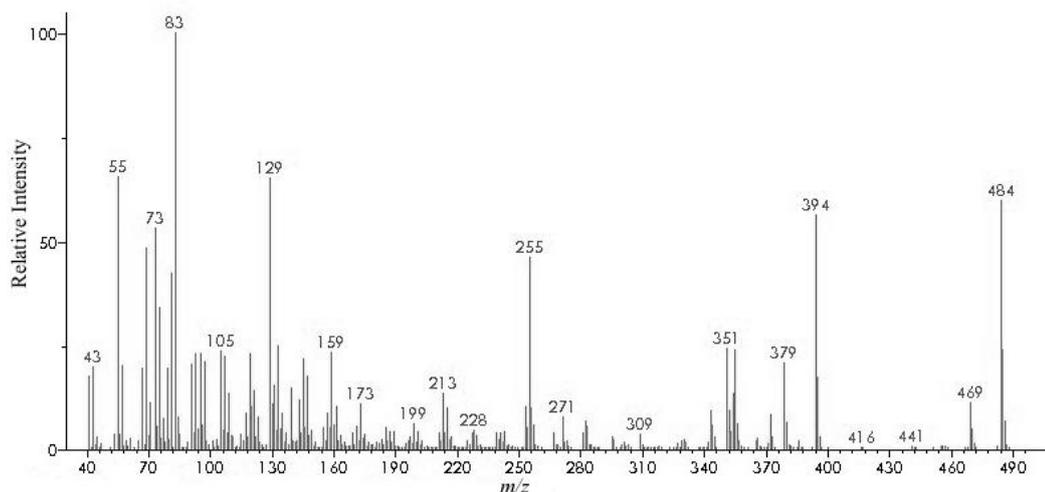


Background subtracted spectrum of AR1-16.10 (averaged from 17.945 to 17.982 minutes)

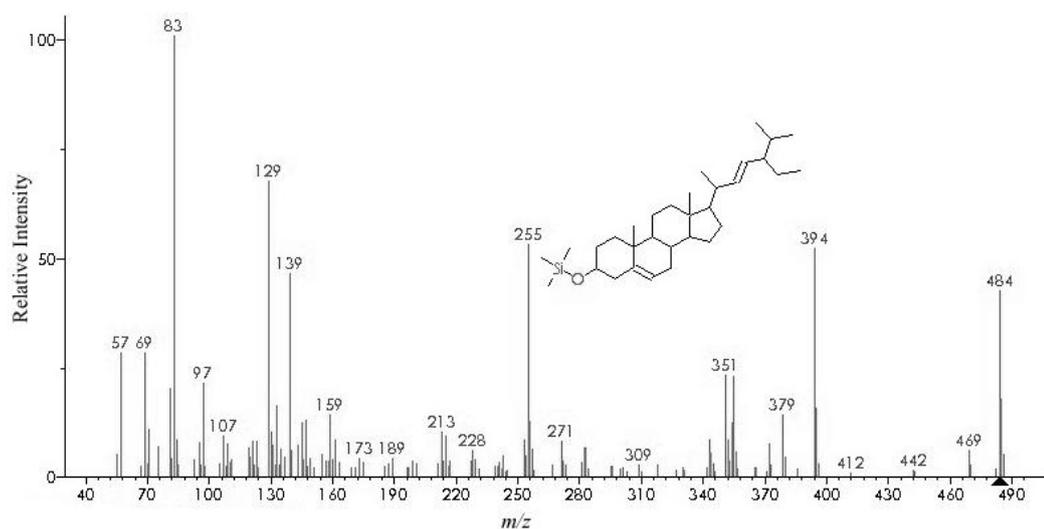


Library spectrum for $(3\beta,24R)$ -ergosta-5-en-3-yltrimethylsilane

A3.6: Fragmentation pattern from TMS ether of sterol eluting at 17.96 min by GCMS, compared with the library spectrum of $(3\beta,24R)$ -ergosta-5-en-3-ol trimethylsilyl ether

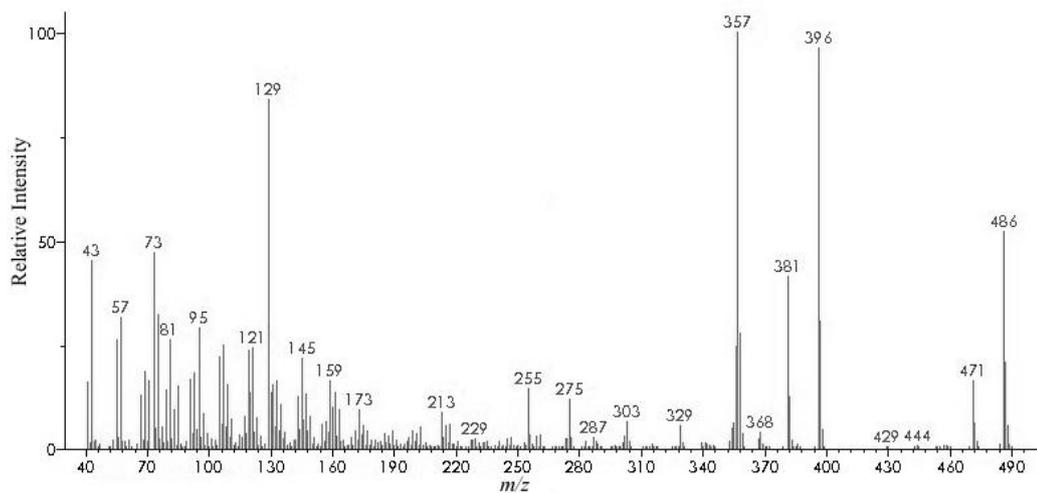


Background subtracted spectrum of AR1-16.10 (averaged from 18.214 to 18.262 minutes)

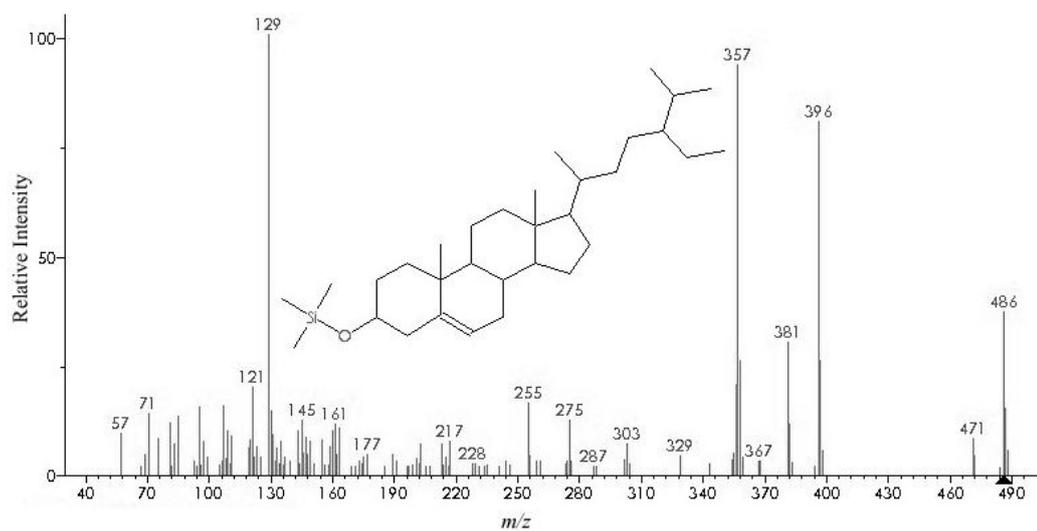


Library spectrum for stigmasteroltrimethylsilyl ether

A3.7: Fragmentation pattern from TMS ether of sterol eluting at 18.25 min by GCMS, compared with the library spectrum of stigmasterol trimethylsilyl ether



Background subtracted spectrum of AR1-16.10 (averaged from 18.748 to 18.785 minutes)



Library spectrum for β -sitosteroltrimethylsilylether

A3.8: Fragmentation pattern from TMS ether of sterol eluting at 18.78 min by GCMS, compared with the library spectrum of β -sitosterol trimethylsilyl ether

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