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Natural Product Analysis of Coastal Marine Species of New Zealand

A thesis submitted in partial fulfilment

of the requirements for the degree

of

Master of Science

at

The University of Waikato

by

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____

The University of Waikato

2013

THE UNIVERSITY OF

WAIKATO

Te Whare Wānanga o Waikato

Abstract

A chemical survey of 58 marine samples obtained from three sites around Tauranga New Zealand was undertaken in an effort to identify new metabolites. MTT assays using HeLa and P388 cell lines were carried out to determine if any of the samples contained bioactive compounds.

For each sample, a crude extract was obtained and analysed by Liquid Chromatography Mass Spectrometry (LCMS). The chromatogram traces were examined and fives samples containing potential metabolites of interest were chosen for further investigation. Of the five samples two were identified from preliminary taxonomic analysis as *Lyngbya sp.* (cyanobacterium) and *Cliona celata* (a sponge). The compound targeted in *Lyngbya sp.* was thought to potentially be a new peptide and the *Cliona celata* compounds contained bromine atoms. These two samples were extracted in bulk and fractionated by reversed phase and size exclusion chromatography. From the mass spectral data and the taxonomy there were no identified samples on the MarinLit database. The separation yielded several fractions and these were analysed by LCMS to identify which ones contained the targeted compounds. Nuclear magnetic resonance (NMR) spectroscopy and tandem mass spectrometry was then used on the most concentrated (targeted compound) fractions to get more structural information. NMR spectroscopy of *Cliona celata* was found contain many impurities and the target compounds concentrations were not sufficient enough to give any structural information. The compound from *Lyngbya sp.* appeared to be a fatty acid. The sterol composition of *Cliona celata* was also analysed by gas chromatography mass spectrometry (GCMS). The three other samples analysed in more detail were *Aplidium sp.* (RI 2-13), *Alcyonaria sp.* (RI 2-14) and *Ircinia sp.* (RI 2-17). These samples were fractionated by small scale chromatography and then subjected to tandem mass spectrometry with some structural features being identified.

All samples from the chemical survey were tested against the HeLa and P388 cell lines using the MTT assay. After inconclusive results from initial tests, the method was modified to give more consistent results. Even with the modifications to the method, there were still inconsistencies in the results. Only one sample, the brown alga *Xiphophora sp.* gave an expected trend of decreasing cell metabolism with increasing sample concentration which would indicate cytotoxic activity. The experiment would need to be repeated in independent tests to confirm the result.

Acknowledgements

Firstly I would like to thank my supervisor Dr Michèle Prinsep without whose advice and assistance I would not have been able to complete this thesis. I would also like to thank staff of the Chemistry Department of the University of Waikato, in particular Wendy Jackson and Jenny Stockdill for the assistance, training and technical expertise they have given me.

For the biological aspects of the research I would like to thank Dr Ryan Martinus for his help on cell culturing, Kerry Allen for technical assistance and Megan Callahan from AgResearch for her advice on P388 cell growth.

Thank you to Professor Chris Battershill and Nikki Webb for the collection of the samples, without which this research would not have been possible.

Table of Contents

Abstract ii

Acknowledgements....................................iv

List of Figures vii

List of Tables....................................x

List of Abbreviations xi

1 Natural Products Overview 1

1.1 What are natural products and why are they of interest? 1

1.2 Why Study marine samples? 2

1.2.1 Cnidaria 3

1.2.2 Molluscs 6

1.2.3 Tunicates 10

1.2.4 Algae 13

1.2.5 Sponges (Porifera) 14

1.2.6 Cyanobacteria 21

1.3 Bioassays and Cell Lines Used in Natural Product Research 28

1.3.1.1 HeLa Cells....................................28

1.3.2 P388 Cells 30

1.3.3 The MTT assay 30

2 Chemical Analysis of Coastal Marine Samples 33

2.1 Introduction 33

2.2 Initial Survey of the Marine Samples 34

2.2.1 Common High Mass Ions 35

2.2.2 Analysis of the Mangrove Samples and More Common Ions 37

2.3 Analysis of the Halogenated Compounds 38

3 Analysis of *Lyngbya sp.* (WE 2-20) 40

4 Analysis of *Cliona celata* (RI 1-11) 44

5 Analysis of Three Additional Extracts 47

6 Bioassay Screening and Method Development 50

7 Experimental Methods 56

7.1 General Experimental Methods 56
7.1.1 Solution Preparations.. 58
7.2 Chemical Methods.. 58
7.2.1 Crude Extraction of Marine Samples 58
7.2.2 Bulk Extraction Method 59
7.2.3 Reversed Phase Chromatography of *Cliona celata* (RI 1-11) and *Lyngbya* sp. (WE 2-20).. 60
7.2.4 Small Scale Reversed Phase Chromatography............................... 61
7.2.5 Size Exclusion Chromatography 61
7.3 Cell Culturing and Bioassay Methods.. 62
7.3.1 Splitting of HeLa Cells.. 62
7.3.2 Splitting of P388 Cells.. 62
7.3.3 Preparation of the Cell Media... 62
7.3.4 MTT Assay Methods... 63
7.3.5 Sodium Azide (NaN_3) Cell Treatments 65
8 Appendices... 66
8.1 Appendix One: Initial Survey of the Marine Samples 66
8.2 Appendix Two:Separation Trees and Diagrams of Marine Species Analysed in Chapters 3-5 89
References.. 101

List of Figures

Figure 1: (A) Metabolisation of MTT to Formazan and (B) absorbance spectra of MTT and MTT Formazan 31
Figure 2: (A) Media (B) acidified isopropanol added (C) acidified isopropanol+10% triton X 51
Figure 3: (A) Acidified isopropanol+DMSO, (B) acidified isopropanol+1hr incubation, ... 52
Figure 4: Sodium azide treatments of cells 53
Figure 5: P388 LI 2-3 MTT Response curve 54
Figure 6: HeLa LI 2-3 MTT Response curve 54
Figure 7: LCMS C_{18} reversed phase chromatography solvent gradient profile 56
Figure 8: Serial dilution concentration layout of one row of a 96 well plate. 63
Figure 9: Diagram of a 96 well plate 64
Figure 10: LCMS Chromatogram of crude extract LI 1-1 69
Figure 11: LCMS Chromatogram of crude extract LI 1-2 69
Figure 12: LCMS Chromatogram of crude extract LI 2-3 69
Figure 13: LCMS Chromatogram of crude extract LI 2-4 70
Figure 14: LCMS Chromatogram of crude extract LI 2-5 70
Figure 15: LCMS Chromatogram of crude extract LI 2-6 70
Figure 16: LCMS Chromatogram of crude extract LI 2-7 71
Figure 17: LCMS Chromatogram of crude extract LI 2-8 71
Figure 18: LCMS Chromatogram of crude extract LI 2-9 71
Figure 19: LCMS Chromatogram of crude extract LI 2-10 72
Figure 20: LCMS Chromatogram of crude extract LI 2-11 72
Figure 21: LCMS Chromatogram of crude extract LI 2-12 72
Figure 22: LCMS Chromatogram of crude extract LI 3-13 73
Figure 23: LCMS Chromatogram of crude extract LI 3-14 73
Figure 24: LCMS Chromatogram of crude extract LI 3-15 73
Figure 25: LCMS Chromatogram of crude extract LI 3-16 74
Figure 26: LCMS Chromatogram of crude extract LI 3-17 74
Figure 27: LCMS Chromatogram of crude extract LI 4-18 74

Figure 28: LCMS Chromatogram of crude extract RI 1-1 75
Figure 29: LCMS Chromatogram of crude extract RI 1-2 75
Figure 30: LCMS Chromatogram of crude extract RI 1-3 75
Figure 31: LCMS Chromatogram of crude extract RI 1-4 76
Figure 32: LCMS Chromatogram of crude extract RI 1-5 76
Figure 33: LCMS Chromatogram of crude extract RI 1-6 76
Figure 34: LCMS Chromatogram of crude extract RI 1-7 77
Figure 35: LCMS Chromatogram of crude extract RI 1-8 77
Figure 36: LCMS Chromatogram of crude extract RI 1-9 77
Figure 37: LCMS Chromatogram of crude extract RI 1-10 78
Figure 38: LCMS Chromatogram of crude extract RI 1-11 78
Figure 39: LCMS Chromatogram of crude extract RI 2-12 78
Figure 40: LCMS Chromatogram of crude extract RI 2-13 79
Figure 41: LCMS Chromatogram of crude extract RI 2-14 79
Figure 42: LCMS Chromatogram of crude extract RI 2-15 79
Figure 43: LCMS Chromatogram of crude extract RI 2-16 80
Figure 44: LCMS Chromatogram of crude extract RI 2-17 80
Figure 45: LCMS Chromatogram of crude extract RI 2-18 80
Figure 46: LCMS Chromatogram of crude extract RI 2-19 81
Figure 47: LCMS Chromatogram of crude extract RI 2-20 81
Figure 48: LCMS Chromatogram of crude extract WE 1-1.................................. 82
Figure 49: LCMS Chromatogram of crude extract WE 1-2.................................. 82
Figure 50: LCMS Chromatogram of crude extract WE 1-3.................................. 82
Figure 51: LCMS Chromatogram of crude extract WE 1-4.................................. 83
Figure 52: LCMS Chromatogram of crude extract WE 1-5.................................. 83
Figure 53: LCMS Chromatogram of crude extract WE 1-6.................................. 83
Figure 54: LCMS Chromatogram of crude extract WE 1-7.................................. 84
Figure 55: LCMS Chromatogram of crude extract WE 1-8.................................. 84
Figure 56: LCMS Chromatogram of crude extract WE 1-9.................................. 84
Figure 57: LCMS Chromatogram of crude extract WE 2-10................................ 85
Figure 58: LCMS Chromatogram of crude extract WE 2-11................................ 85
Figure 59: LCMS Chromatogram of crude extract WE 2-12................................ 85

Figure 60: LCMS Chromatogram of crude extract WE 2-13................................ 86
Figure 61: LCMS Chromatogram of crude extract WE 2-14................................ 86
Figure 62: LCMS Chromatogram of crude extract WE 2-15................................ 86
Figure 63: LCMS Chromatogram of crude extract WE 2-16................................ 87
Figure 64: LCMS Chromatogram of crude extract WE 2-17................................ 87
Figure 65: LCMS Chromatogram of crude extract WE 2-18................................ 87
Figure 66: LCMS Chromatogram of crude extract WE 2-19................................ 88
Figure 67: LCMS Chromatogram of crude extract WE 2-20................................ 88
Figure 68: Separation tree of *Cliona celata* (RI 1-11)... 90
Figure 69: Separation tree of *Lyngbya sp.*(WE 2-20) 91
Figure 70: Base peak chromatogram of RI 1-11 *Cliona celata* with mass spectra of metabolites of interest.. 92
Figure 71: Base peak chromatogram of RI 1-11.6.6 with mass spectra of metabolites of interest.. 93
Figure 72: Base peak chromatogram of WE 2-20 *Lyngbya sp.* with mass spectra of metabolites of interest.. 94
Figure 73: Base peak chromatogram of WE 2-20.7.3 with mass spectra of metabolites of interest.. 95
Figure 74: Base peak chromatogram of WE 2-20.8.7 with mass spectra of metabolites of interest.. 96
Figure 75: Base peak chromatogram of WE 2-20.9.3 with mass spectra of metabolites of interest.. 97
Figure 76: Base peak chromatogram of RI 2-13 *Aplidium sp.* with mass spectra of metabolites of interest.. 98
Figure 77: Base peak chromatogram of RI 2-14 *Alcyonaria sp.* with mass spectra of metabolites of interest 99
Figure 78: Base peak chromatogram of RI 2-17 *Ircinia sp.* (horny sponge) with mass spectra of the metabolite of interest 100

List of Tables

Table 1: Common high mass ions observed in Algae Species Surveyed.............. 36

Table 2: Compounds of interest in Mangrove samples....................................... 37

Table 3: 656.5 *m/z* tandem mass spectrometry. 43

Table 4: Dibrominated and monobrominated tandem mass spectrometry. 45

Table 5: RI 2-13 452 and 524 ions tandem mass spectrometry. 47

Table 6: RI 2-14 324/326 ions tandem mass spectrometry. 48

Table 7: RI 2-17 606 *m/z* ion tandem mass spectrometry. 48

Table 8: Absorbance values for different solubilisation conditions (5000 HeLa cells/well) 52

Table 9: Reversed phase chromatography solvent gradient of RI 1-11............... 60

Table 10: Reversed phase chromatography solvent gradient of WE 2-20 61

Table A1.1: Leisure Island Sample Taxonomy... 66

Table A1.2: Rabbit Island Sample Taxonomy... 67

Table A1.3: Waikareao Estuary Sample Taxonomy 68

List of Abbreviations

COSY	Correlation Spectroscopy
COLOC	Correlation through Long-range Coupling
ED_{50}	Effective dose for 50% of test subjects
EIMS	Electron Impact Mass Spectrometry
ESMS	Electrospray Mass Spectrometry
GCMS	Gas Chromatography Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HPLC	High-Performance Liquid Chromatography
HRESIMS	High Resolution Electrospray Ionisation Mass Spectrometry
IC_{50}	Concentration for 50 % of Growth Inhibition
IR	Infrared
LCMS	Liquid Chromatography Mass Spectrometry
NMR	Nuclear Magnetic Resonance
SCUBA	Self Contained Underwater Breathing Apparatus

1 Natural Products Overview

1.1 What are natural products and why are they of interest?

Living species produce two types of compounds, primary metabolites and secondary metabolites. Primary metabolites sustain cell metabolism and support the functioning of the organism.[1] These molecules generally only have a biological effect within the cell or organism producing them. Secondary metabolites (which are also referred to as natural products) tend to have bioactive effects on a range of species rather than just the one that produces them and often their purpose is not readily apparent. [2, 5] Once their range of bioactivity is known the compounds can then be utilised for research and commercial applications.

Generally the role of natural products is to assist in the protection and maintenance of homeostasis of the organism.[3] To humans they are largely of interest due to their technological applications. Natural products have been used as flavours, fragrances and pharmaceuticals along with many more rich and varied applications.[4] By isolating and solving the structures of these molecules scientists have been able to learn about their biosynthesis and have applied combined knowledge of the structural information with therapeutic biochemistry to develop pharmaceuticals.[5] For example the discovery of penicillin in 1929 led to its large scale manufacture as an antibiotic.[6]

Natural Product structures have already been “screened” by evolution for their usefulness and can act as a starting point for drug development.[7] Also by isolating and studying these molecules information about their larger ecological role can be obtained.[8] The need to find new and useful substances for continued medical development is one important aspect natural products play in modern life. A major problem with current antibacterial drugs is that bacteria gradually become more resistant to treatments. Natural products have already proven their effectiveness and have contributed well to this effort, with two thirds of clinical antibacterial drugs we use today either being directly sourced natural

compounds or altered semi-synthetic versions of them.[9] However, current antibiotics being relied upon are based on only a handful of frameworks and their synthetic derivations. Therefore, varied compounds usually found in diverse natural sources are needed.[9]

1.2 Why Study marine samples?

Marine samples are a rich but largely unexplored (in part because marine organisms have been harder to collect and identify) source of biodiversity.[10] As a chemical environment the ocean is considered to be quite constant but in fact nutrient levels in the ocean vary greatly as do trace metals and vitamins.[4] On account of the differing environment both chemically and biologically it follows that marine species will produce different types of natural products from terrestrial species.[4] This has already been proven with many marine natural product structures that have no precedent from the terrestrial environment and show new toxicological properties.[3] The peptides and proteins are also of interest because their high site specificity can help determine biological functions.[11]

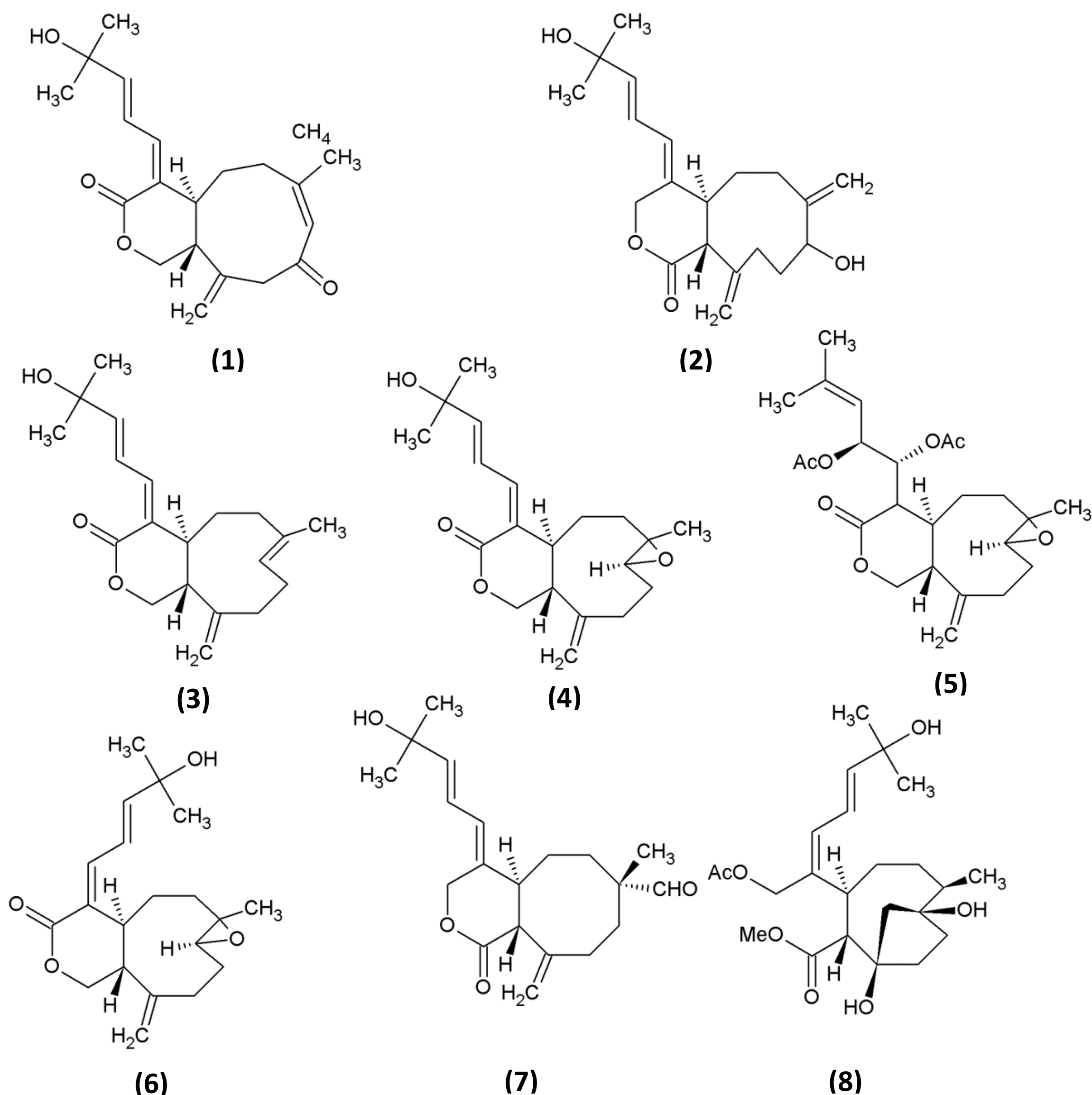
The collection of biologically varied samples of marine species is relatively easy as a large amount of marine biodiversity is found in areas along the shoreline. Estimations have put these areas along with deep sea ocean vents as the most species rich areas in the world.[12] These concentrations of species in one environment drives competition and particularly in the sessile organisms forces chemical adaptations to evolve that act as feeding deterrents or inhibit the growth of other species.[12] For example, the cone snail *Conus purpurascens* uses a mixture of peptides (conotoxins) injected via a harpoon like structure that disrupts the voltage gated ion channels and immobilises its prey.[13] Some species also produce feeding deterrents. For example, the green alga *Pseudochlorodesmis furcellata* produces two diterpenoids that reduce predation by herbivorous fish.[14] Modern advances in technology such as SCUBA gear has enabled access to new areas and facilitated extensive sampling of marine life.[15]

Accessing this new diverse biota increases the chances of finding new molecular frameworks. When the function of a molecule is understood such as finding a selectively reactive functional group or how a specific framework is utilised, it can provide a platform for new drugs to be synthesised.[5]

The journal *Natural Product Reports* has annually reviewed and documented the recently discovered compounds of a wide variety of marine phyla. Some examples representative of the natural products found in species within six phyla discussed in *Natural Products Reports* are presented here to provide an overview of the diversity and application of marine natural products.[16, 17]

1.2.1 Cnidaria

Cnidaria is a phylum of marine invertebrates containing a diverse range of over 11,000 species.[18] They are primitive multi-celled organisms with a basic nervous system and have an important role in large ecosystems such as coral reefs.[18, 19] The hard coral *Scleractinia* forms the basis of most coral reefs.[20] Cnidarians have two known main methods of defences; nematocysts which are stinging structures and chemicals.[21] A study of 18 cnidarians found that the species using both defensive methods to live in habitats with high predation pressure such as coral reefs.[21] There have already been compounds with potential chemotherapeutic uses discovered in this phylum, mainly with anti-tumour activity but also compounds with anti-bacterial, anti-HIV (Human immunodeficiency virus) and anti-inflammatory properties as well as molecules to support nervous system health. Several different families of natural products have been isolated from cnidarians such as prostanoids, steroids and polyketides. Of more interest are the terpenoids which are found to have the most biotechnological potential of the compounds isolated from cnidarians.[18] The diterpenoid blumiolide C (**1**) isolated from the cnidarian soft coral *Xenia blumi* showed strong cytotoxic effect against the P388 (mouse leukaemia) and HT-29 (human colon adenocarcinoma) cell lines.[18]



Seven other related diterpenoids (**2-8**) as well as blumiolide C (**1**) were isolated from *Xenia blumi* and were subjected to bioassay guided fractionation. Their structures were elucidated by 1- and 2-D proton and carbon nuclear magnetic resonance (NMR) spectroscopy. Blumiolide C (**1**) was very cytotoxic to both cell lines with ED_{50} (effective dose to inhibit 50% of the population) values of 0.5 µg/mL and 0.2 µg/mL against the P388 and HT-29 cell lines respectively.[22]

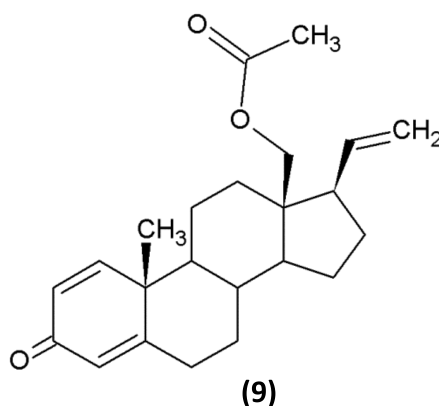
In the nervous systems of cnidarians there are many peptides that are important for neurotransmission and some that are neurohormones.[19] Isolation and characterisation of these peptides can lead to information about their functions, the roles they play in the nervous system and their biosynthesis. The separation of neuropeptides can be difficult as in extracts there can be several other

proteins.[19] A Japanese group, in conjunction with German and American colleagues, fractionated a cnidarian extract by HPLC and analysed a chromatogram containing over 10,000 peaks. From this exhaustive process, several C-terminally amidated peptides were isolated.[19]

Cnidarians have also been shown to produce many severe toxins.[23] Two cnidarians *Stichodactyla helianthus* and *Bunodosoma granulifera* were collected from the sea around Cuba and their crude extracts injected into mice. After neurotoxic symptoms were observed, another experiment was carried out to test the extracts inhibitory effects of the extracts on the activity of cholinesterase. It was concluded that the crude extracts contained cholinesterase inhibitors that were able to pass through the blood brain barrier. The crude material was fractionated by gel filtration and reversed phase chromatography with two inhibitors being isolated, BgK a 37 amino acid peptide from *Bunodosoma granulifera* and ShK, a 35 amino acid peptide from *Stichodactyla helianthus*. Both peptides blocked potassium channels and each contained three disulphide bridges. The importance of knowing the details about these structures can assist in the analysis of similar peptides which have almost the same amino acid numbers but several differences in activity. In the case of potassium channel blockers, BgK and ShK block different subtypes of the channels but due to their similar amino acid number, it is likely a structural difference causing the selectivity.[24]

A study from Brazil tested eight cnidarian species to look for natural products for use as therapeutic agents against the protozoan diseases Leishmaniasis and Chagas disease.[25, 26] Current treatments for both diseases are expensive, have severe side effects and resistance to them is increasing. Current Chagas chemotherapy can cause vomiting and skin disorders. [27] Leishmaniasis has partially become resistant to the pentavalent antimonial class of drugs, a treatment used for sixty years.[28] Of the nine crude extracts that were screened, four species: *Carijoa riisei, Heterogorgia uatumani, Leptogorgia punicea,* and *Macrorhynchia philippina* were found to have antiprotozoal properties. Based on

the IC_{50} values of the four species, the steroid 18-acetoxipregna-1, 4, 20-trien-3-one (**9**) from the octocoral *C. riisei* was further investigated and found to have good selectivity. Its cytotoxic effects targeted *Leishmania sp.* with much more potency than macrophage cells.[25]



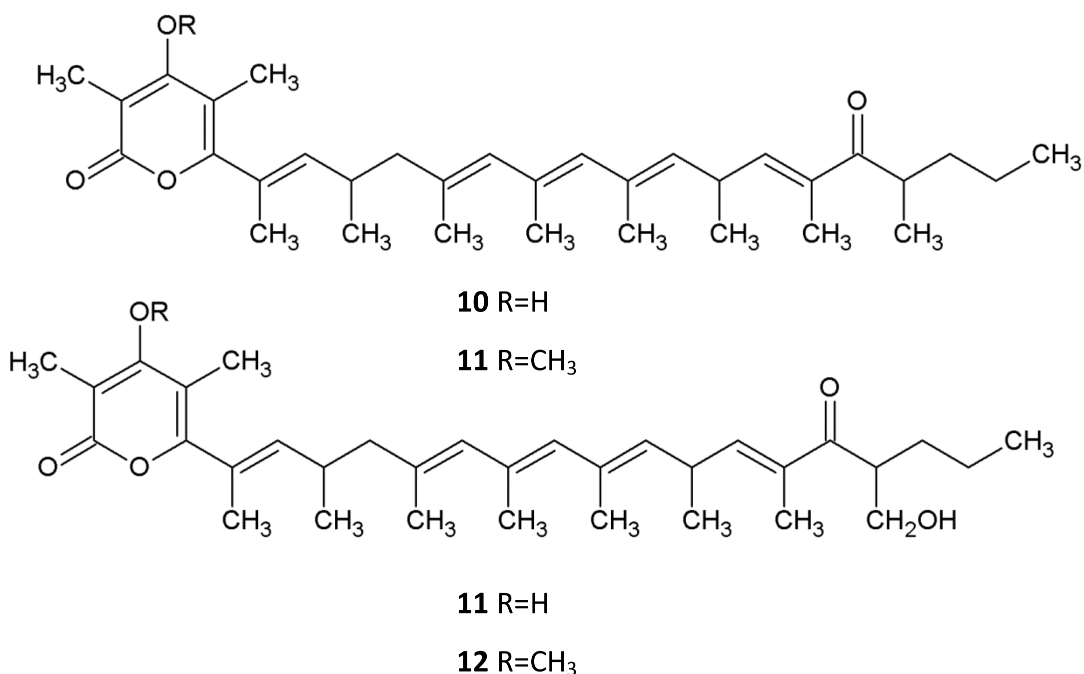
(9)

1.2.2 Molluscs

With over 80,000 species divided into six diverse classes Mollusca is another phylum from which a variety of useful and interesting natural products have been procured. The molluscs' body structure consists of three parts: a head, foot and visceral mass containing the organs. Most molluscs are also protected by a shell.[29]

Molluscs that are not protected by a shell however have adapted chemical and other defences for survival. For example, the subclass Opistobranchia does not grow a shell and produces several toxic anti-feedants to deter predators.[30] Even the molluscs whose shells protects them from predatory danger still filter feed which can lead to the uptake of viruses and bacteria.[31] In response to this species such as *Cerstoderma edule* and *Ostrea edulis* produce anti-viral and anti-bacterial compounds.[32]

Two polypropionates with methylated variants named Fusaripyrones A (**10** and **11**) and B (**11** and **12**) have been isolated and characterised from the opistobranch mollusc *Haminoea fusari*.[33]



10 R=H

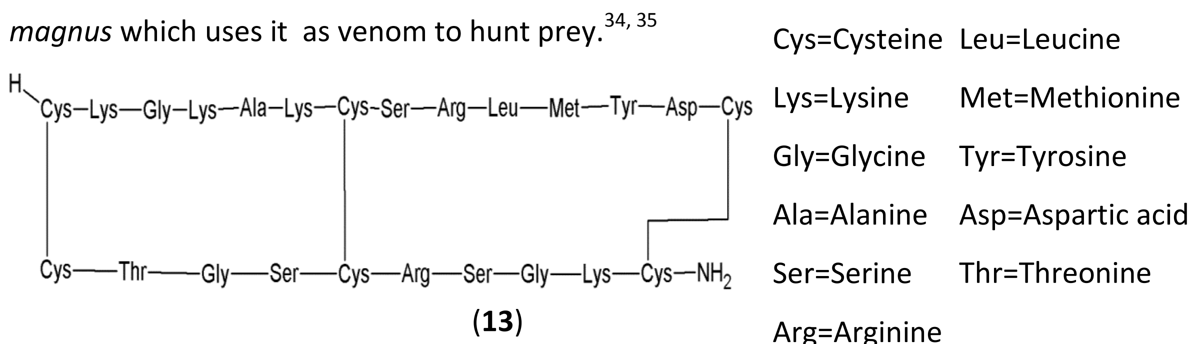
11 R=CH_3

11 R=H

12 R=CH_3

The electrospray ionisation mass spectra contained the pseudo-molecular ions ($M+Na^+$) of these compounds and combined with various types of NMR spectroscopy, enabled the structures to be elucidated. On account of the unstable nature of these molecules, they were methylated which allowed them to be purified for analysis. The underivatised compounds were isolated later. These structures may assist with solving the evolutionary relationships of *Haminoea* as there are other related species which produce molecules with similar structures and therefore probably share at least some of the same genes driving that biosynthetic pathway.[33]

The natural product ziconotide (**13**) was isolated from the cone snail *Conus magnus* which uses it as venom to hunt prey.[34, 35]

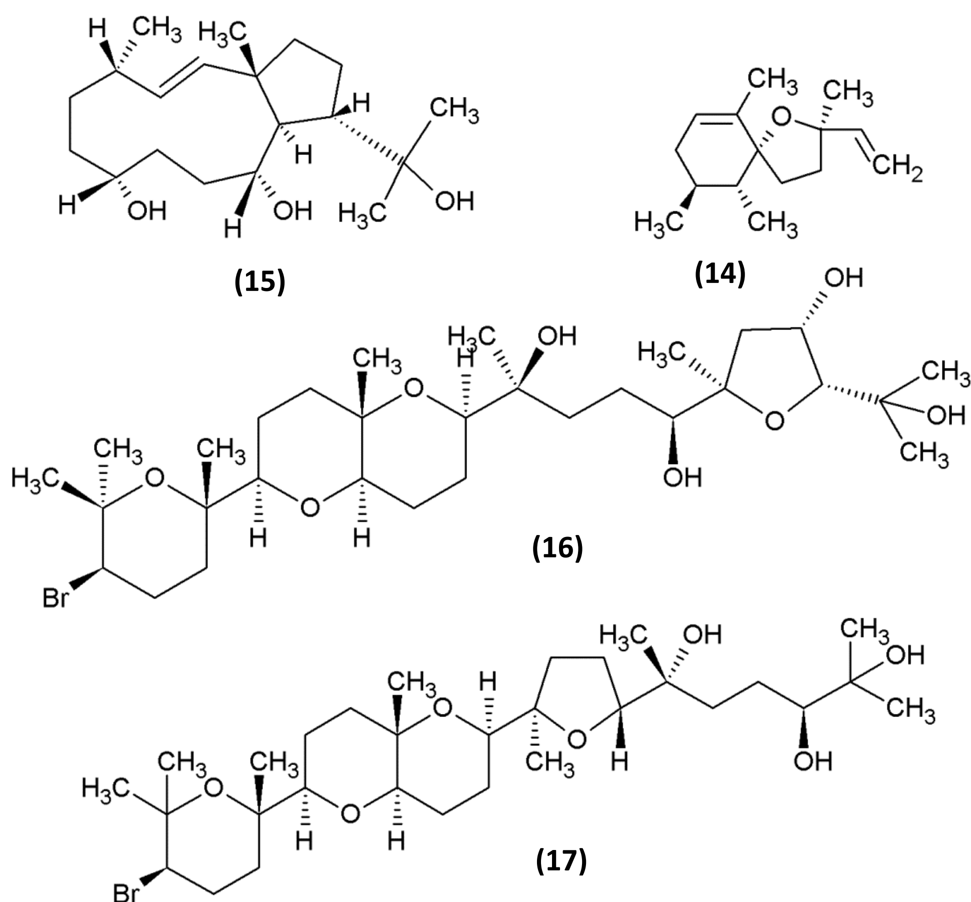


(**13**)

Ziconotide's structure is a 25 amino acid chain with three disulphide bridges to form four asymmetrical loops. The rigid structure of the molecule allows it to bind selectively to N-type calcium channels. Its mechanism of action is to block

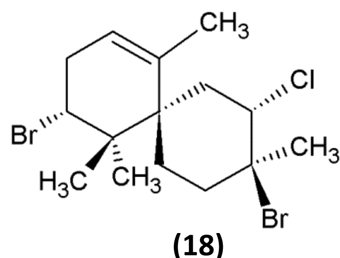
the release of N-type calcium ion channels that facilitate neuronal excitability and neurotransmission. It has advantages over current opiate based medication as it does not have the potential to form a mental or physical addiction and there has been no observed respiratory depression.[36] Ziconotide (marketed under the name Prialt) has been approved for clinical use in both the U.S.A and Europe, it is 1000 times more potent than morphine and therefore a much more effective treatment for neuropathic pain.[35]

The sea hare *Aplysia dactylomela* has been found to be a rich source of novel compounds, particularly terpenoids. *Aplysia* feeds on red algae containing a large number of terpenoids, some of which it sequesters for its own use and can chemically alter to reduce their toxicity.[37, 38] *Aplysia dactylomela* has been a source of natural products over a long period of time, Dactoxylene-B (**14**) is one of three sesquiterpene ethers that were isolated from a sample in 1974,[39] a diterpene (**15**) was reported from a sample off the Canary Islands from another sample of *A. dactylomela* in 1983[40] and Aplysiols A (**16**) and B (**17**) in 2007.[41]



The unnamed diterpene (**15**) structure was solved by X-ray crystallography.[39, 40]

Three new chamigrene compounds one unnamed (**18**), Acetyldeschloroelatol (**19**) and Acetylelatol (**20**) were isolated from a sample of *Aplysia dactylomela*. Some known related compounds were also isolated (**21-24**).



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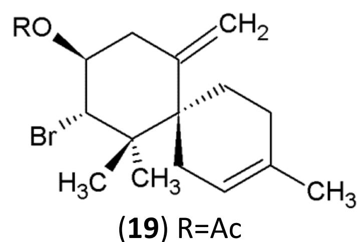
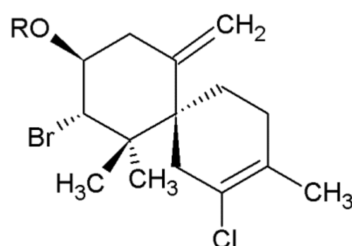
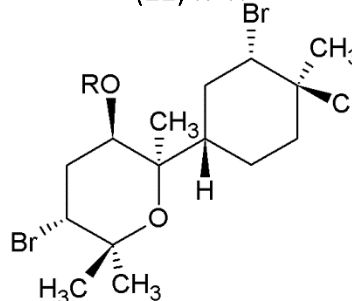


(**19**) R=Ac

(**21**) R=H





(**20**) R=Ac

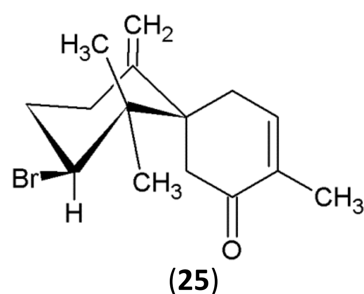
(**22**) R=H

(**23**) R=Ac

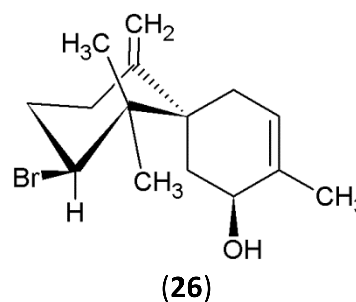
(**24**) R=H

The crude extracts were separated by LH-20 chromatography and then high performance liquid chromatography (HPLC) used to obtain pure samples. Electron impact mass spectrometry (EIMS) spectrum data allowed the identifications of the halogens in the molecules. The structures were solved by combining this information with high resolution EIMS along with proton and carbon NMR spectroscopic techniques. The compounds were tested against two cancer cell lines (HeLa and Hep-2) and Vero (monkey kidney cells) to see if they had any bioactivity. The acetyl derived compound acetylelatol showed much less cytotoxicity than elatol (**22**) indicating under these conditions acetylation reduces toxicity of elatol. It has been proposed that *Aplysia dactylomela* performs this structural change itself after it ingests the algae containing elatol.[38]

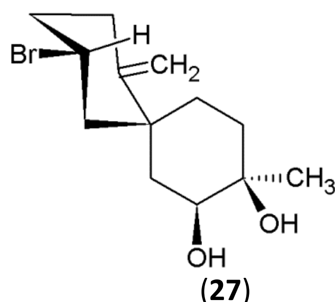
Four more chamigrene sesquiterpenoids (**25-28**) were isolated from *Aplysia dactylomela* and tested against HL-60 and THP-1 leukaemia cells.



(**25**)



(**26**)



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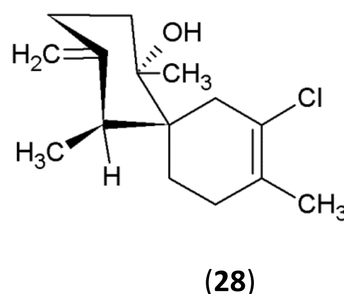


(**28**)

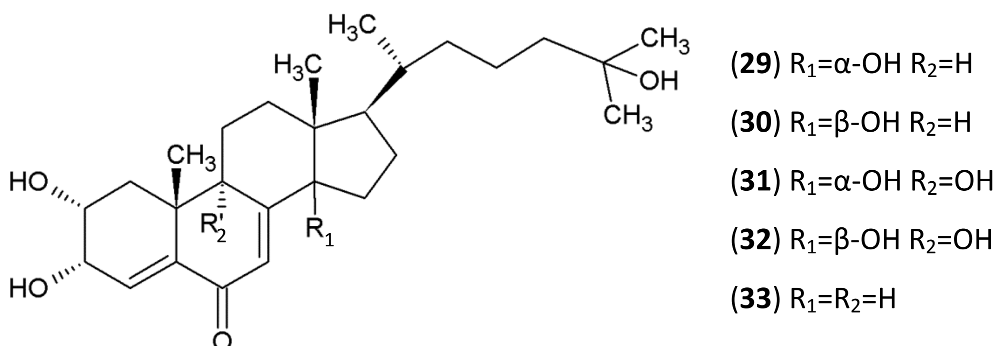
Extensive 1- and 2-D NMR spectroscopy combined with mass spectral data were used to solve the structures. Sesquiterpene (**28**) showed some cytotoxic activity against both THP-1 and HL-60 cell lines with IC_{50} values of 152 and 102 µmol L^{-1} respectively. By comparing (**28**) to the non-cytotoxic dactylone (a related analogue), it was determined that the replacement of the carbonyl group and double bond in dactylone with the diol structure of (**28**) plays an important role in the cytotoxicity.[37]

1.2.3 Tunicates

Tunicates (Ascidians), also known as sea squirts, are a group of sedentary filter feeding animals that consist of around 3000 species and have been found to contain many interesting natural products.[42] Tunicates spread their populations by releasing swimming larvae that eventually settle onto hard surfaces, where they undergo metamorphosis to their adult form and develop a protective cellulose layer. Once they have attached themselves to a surface they can asexually reproduce to establish colonies.[43] There have been over 1000

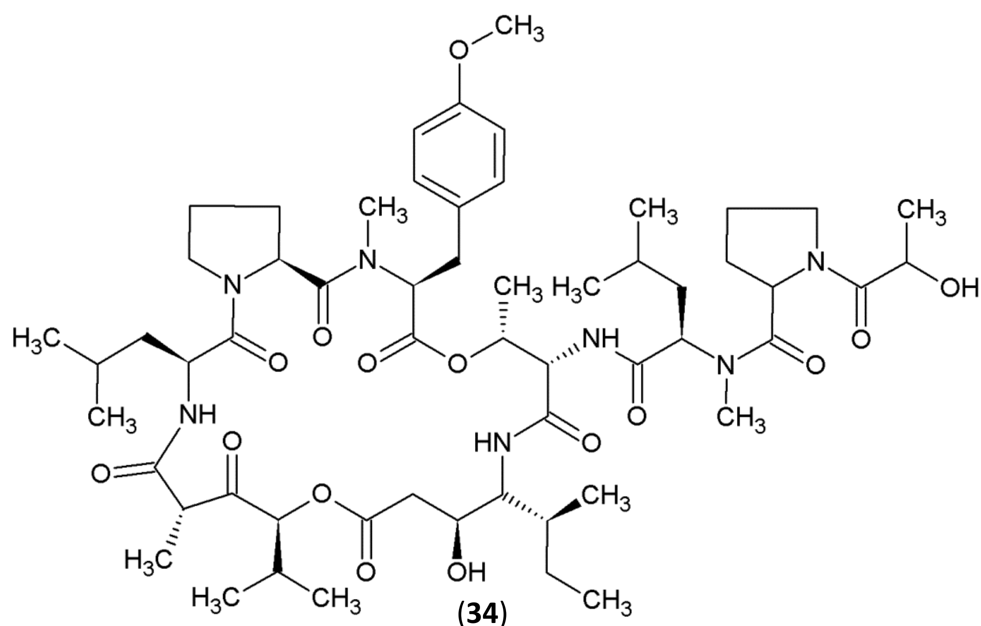
compounds isolated from tunicates with some in clinical use, others used as anti-tumour leads and some acting as multidrug resistance blockers.[44]

Five new ecdysteroids, hyousterone A-D (**29-32**) and dialusterol B (**33**) were isolated from a sample of an Antarctic tunicate *Synoicum adareanum*.



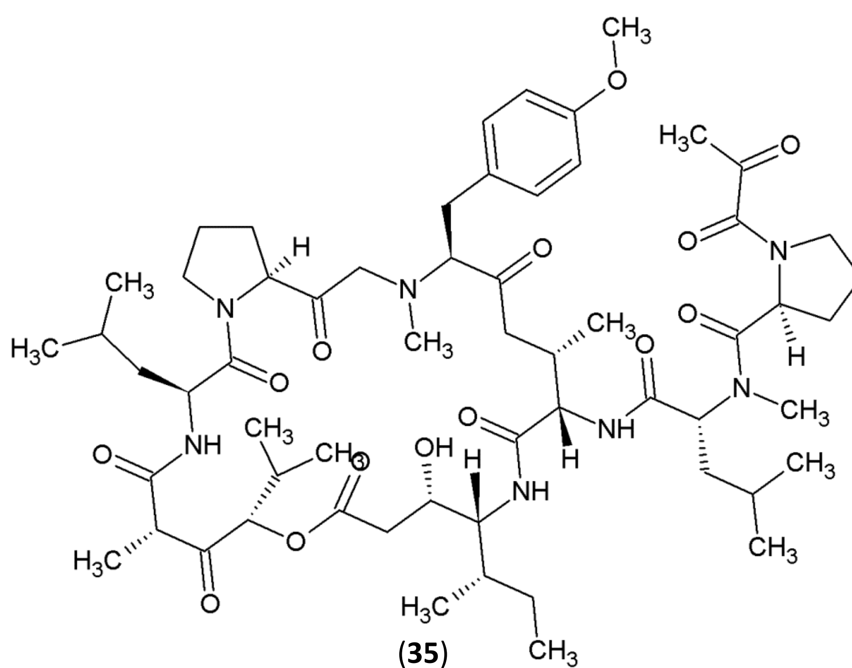
The sample was extracted in methanol: dichloromethane (1:1) and HPLC was used to separate the compounds. Their structures were elucidated using a variety of techniques including HRESIMS and 1- and 2-D NMR spectroscopy. The hyousterones structures (**29-32**) are very similar differing only by the α- or β-position of the hydroxyl group or a hydrogen atom substitution. The hyousterones B (**30**) and D (**32**) with β-hydroxyl groups and dialusterol B (**33**) showed little cytotoxicity but the hyousterones with α-hydroxyl groups (A and C **29**, **32**) showed significant cytotoxicity. Hyousterones A (**29**) and C (**32**) had IC_{50} values of 10.7 and 3.7 µmol/L respectively. The cytotoxicity assays were carried out against the cell lines HCT-116 (colon), H-125M (lung), L1210 (leukaemia) and CFU-GM (bone marrow) to see if any of the compounds had antitumour selectivity.[45, 46]

One of the first tunicate natural products that had promise as a potential drug was Didemnin B (**34**) which was isolated from *Tridemnum solidium* in 1981.[47]



(**34**)

After showing cytotoxic and antiviral properties, it was the first marine anticancer compound to enter Phase One and Two clinical trials. These trials were stopped due to anaphylaxis and neuromuscular side effects being observed.[47] However the precedent was set and the potential of marine natural products as a source of pharmaceuticals had been established. After this, structurally similar compounds were tested for their antitumour potency and Aplidin which was isolated from *Aplidium albicans* showed similar antitumour activity to Didemnin B.[47, 48]

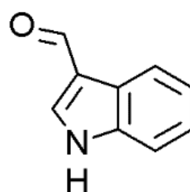


(**35**)

Aplidine (**35**) is a structural analogue of Didemnin B with a pyruvyl group at the N-lactyl side chain. It kills cells in a variety of ways, including two different mechanisms to induce apoptosis and inhibit receptor proteins. The structures of secondary metabolites are sometimes altered with the intention to change their properties and tailor them to be more suitable for drug use. During the synthesis of aplidine, modifications were made to keep it active for longer before being metabolised by enzymes.[47] If Didemnin B (**34**) and Aplidine (**35**) are found to be suitable drug candidates, they may be limited by supply, as collection and aquaculture of the source organisms is difficult. Currently they are being produced by a multistep synthesis which may not be viable for large scale production.[48]

1.2.4 Algae

Algae are photosynthetic organisms with tens of thousands of species that can be either unicellular or multicellular.[49] Their diversity and the extreme and changeable environment they live in means that they have adapted various chemical solutions by way of natural products to survive. In terms of a source of natural products beyond that of experimental work, algae can generally be grown quickly and easily to produce larger quantities of the compound(s) of interest.[49]

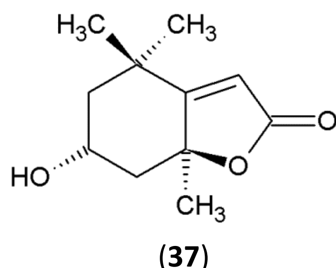


(**36**)

The red alga *Botrycladia leptopoda* was collected near Karachi and the compound 3-formylindole (**36**) was extracted. One of the fractions eluted from chromatography of the extract contained a crystalline compound. After structural elucidation by ^{1}H and ^{13}C NMR and mass spectrometry it was determined to be 3-Formylindole (**36**).[50] The molecule is a basic indole structure with an aldehyde side chain at carbon three on the pyrrole ring.

Interestingly 3-formylindole was also found in the plant species *Murraya exotica* potentially indicating a link in the chemoevolutionary history of both species.[51]

Another natural product found in both algae and terrestrial plants is loliolide (**37**).



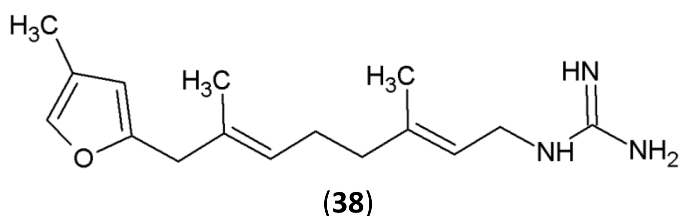
(**37**)

One study on thirteen species of algae found that they all contained loliolide (**37**) with other studies finding it in the plants *Lolium perenne* (ryegrass) and *Salvia divinorum*.[52-54] Identification of loliolide both quantitatively and qualitatively in the algae species was done by comparing extracts run through GC-MS to a calibration curve generated from known concentrations of loliolide. Of the thirteen algae species, seven were red, five brown and one green. This marked the first time loliolide had been found in a species of green algae.[54]

1.2.5 Sponges (Porifera)

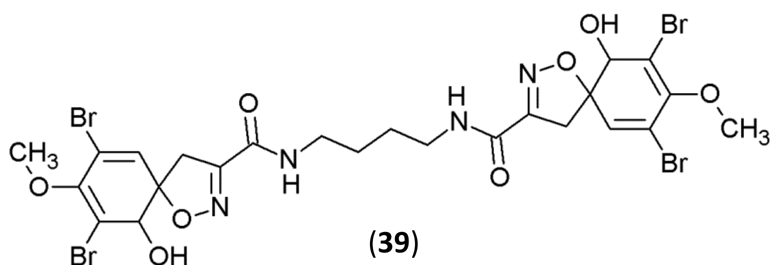
Sponges are sessile, filter-feeding animals with most species inhabiting the marine environment. They are essentially an assemblage of cells, forming no organs and with few cell specialisations.[55] Some sponges with soft bodies are internally supported by a skeleton of calcium carbonate, silica or spongin.[55] The pores (ostia) of the sponge intake water where it moves through the central cavity and out the osculum. During this process, the hairs of the collar cells (which maintain water flow through the sponge) trap any food contained in the water.[55] There are over 8000 species of sponges, varying greatly in size and shape from tubes only an inch long to large bulbs. In 2005, over 5000 different compounds had been isolated from 500 species of sponges.[55]

Care must be taken when analysing other marine organisms as their natural products may be sourced from sponges (or anything else lower in the food chain). For example, the red sponge *Latrunculia magnifica* produces a toxin (latrunculin) as a feeding deterrent that causes fish to immediately move away from it.[56] It is eaten by a nudibranch (sea slug) that sequesters this toxin as its own feeding deterrent.[55] Analysis of the nudibranch might conclude that it produces the toxin when actually it is synthesised by the sponge. Another two more proactive ways that marine sponges utilise their natural products is to inhibit the growth of other marine species and to release compounds to stop fouling organisms from settling on and potentially overgrowing the sponge.[56] The sponge *Siphonodictyon coralliphagum* uses the compound siphonodictidin (**38**) to inhibit corals from growing around its osculum.[56]



(**38**)

Aplysina fistularis releases aerothionin (**39**) into the sea surrounding it to stop fouling organisms from growing on and potentially smothering the sponge.[56]



(**39**)

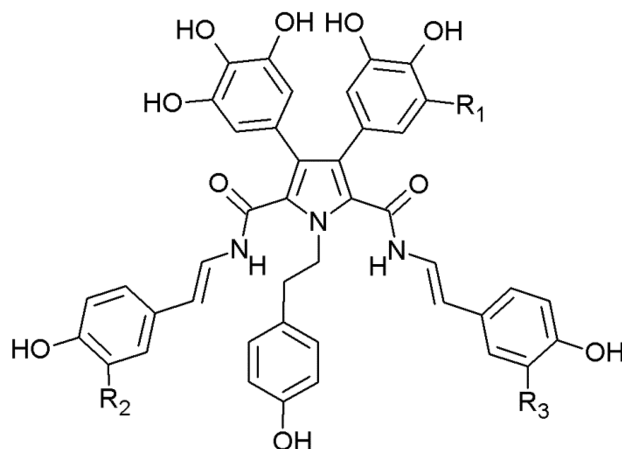
Sponges have remained almost identical morphologically since the Superior Cambrian 509 million years ago and are the simplest multicellular life forms on earth.[57] On account of their limited complexity and their evolutionary age, sponges have had a long time to diversify and develop a wide variety of chemical survival solutions. These chemical defences allow sponges to deter predators, gain space in overgrown environments and protect themselves from infection.

Natural products isolated from sponges have wide applications as treatments for a variety of viral diseases, cancers and inflammatory ailments. There is still much

to learn about how these compounds work at the biochemical level but the general mechanisms by which they inhibit the spread of many diseases are understood. This understanding allows for drugs to be developed to selectively attack specific targets.[58]

Many of these natural products exhibit cytotoxic or inhibitory effects indicating potential medicinal benefits and some are antifouling agents which slow the growth of organisms on the surface they are applied to.[3] A study of the sponges *Spongia* and *Ircinia* revealed that they contain fourteen compounds with varying levels of antiprotozoal properties, measured by bioassaying and obtaining IC_{50} values. The structures of these molecules were determined by 1- and 2-D NMR spectroscopic techniques.[59] The usefulness of chemicals with these properties is to kill protozoan species such as *Plasmodium falciparum* which causes Malaria. There is a need for new chemotherapeutic agents to combat this and other protozoan caused diseases. The current chemotherapeutics used were mostly discovered in the 1950s before marine natural products research was common. The treatments also have unwanted side effects and parasitic protozoa are developing resistance.[60]

Species from the genus *Cliona,* a sponge which grows in massive colonies on hard substrates have been examined from different areas around the world for their natural products.[61] Storniamides A-D (**40-43**), a group of pyrrole alkaloids were isolated from an unknown *Cliona* species found on the coast of Punta Verda, Argentina.[61, 62]



(**40**) R_1=OH R_2=R_3=H

(**41**) R_1= R_3=OH R_2=H

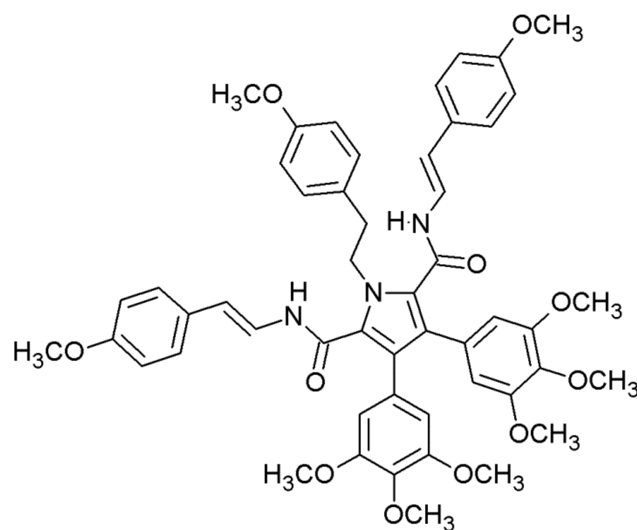
(**42**) R_1=H R_2=R_3=OH

(**43**) R_1=R_2= R_3=OH

The crude extract was separated into hexane and methanol: water (9:1) partitions. From bioassay, the bioactive compounds were seen to be in the methanol/water layer and this was further fractionated and pure compounds obtained by HPLC. By using high resolution fast atom bombardment mass spectrometry (HRFABMS) and many different kinds of 1- and 2-D NMR spectroscopic techniques, the structures of all four compounds were solved. The HRFABMS gave molecular formulae and indicated that the molecules were very similar, differing only in the number of oxygen atoms. Initially the structure of storniamide A was solved to give the major structure which the other storniamides were determined from. Analysis of FABMS fragmentation enabled determination of which substituent groups were place on what rings and NMR spectroscopy was used to confirm the structural analogues. The location of the hydroxyl groups was solved by FABMS and COSY and localised correlation spectroscopy (LCOSY) and confirmed by COrrelation through LOng range Coupling (COLOC) spectra. FABMS gave information on the hydroxyl groups

(from R_1, R_2 and R_3) locations on the structure. For stormiamide A (**40**), a 135 Da loss was seen twice indicating that both hydrogen atoms are on the enamide aromatics. Storniamide B (**41**) showed both 135 and 151 Da losses from the molecular ion, indicating one enamide aromatic ring has a hydrogen atom attached and the other a hydroxyl group attached. This in contrast to Storniamide C which only shows two consecutive 151 Da losses, indicating both hydroxyl groups are on the enamide aromatic rings. Storniamide D (**43**) shows the same losses as C (**42**) but from the molecular formula, all three of its substituents are hydroxyl groups.[61]

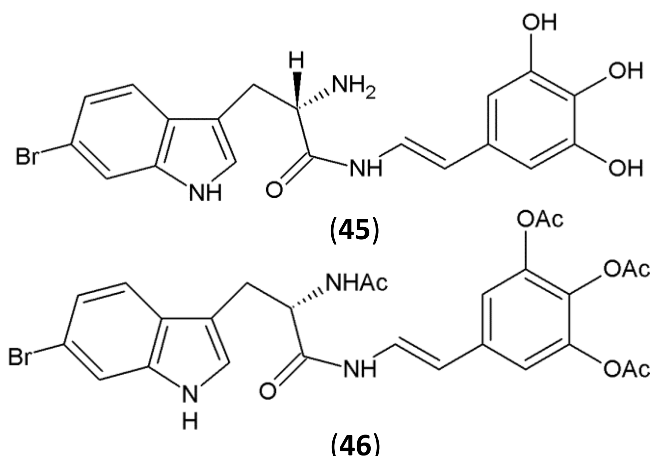
All of the storniamides showed antibiotic activity against *Staphylococcus aureus* and *Micrococcus luteus*. [61] An attempt to synthesise storniamide A resulted in only a partial synthesis, giving a nonamethyl ether analogue (**44**) with no cytotoxic effect.[62] However this compound did have an interesting effect on some human colon cancer multidrug resistant cell lines, reversing their drug resistance and making them vulnerable to vinblastine and doxorubicin treatments.[63]



(**44**)

A sample of the sponge *Cliona celata* was collected from Barkley Sound off Vancouver Island. The sample was extracted in ethanol then the concentrated extract partitioned between ethyl acetate and water. The extract had antibiotic properties when tested against *Staphylococcus aureus.* Subsequent testing that showed the antibiotic activity was in the ethyl acetate partition which contained

also clionamide (**45**). Normal phase, size exclusion and reversed phase HPLC were all used to try to purify the crude extract but they did not result in effective separation. After using ^{1}H-, ^{13}C-NMR, MS and IR spectroscopy, the structure of the derivatised tetracetyl clionamide (**46**) was elucidated.

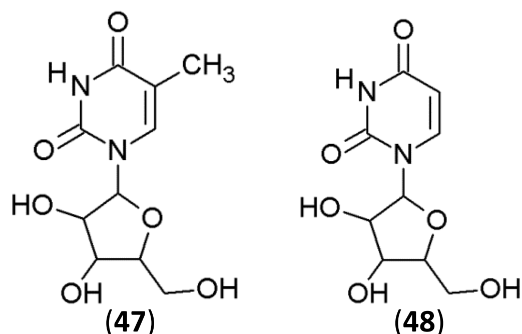


(**45**)

(**46**)

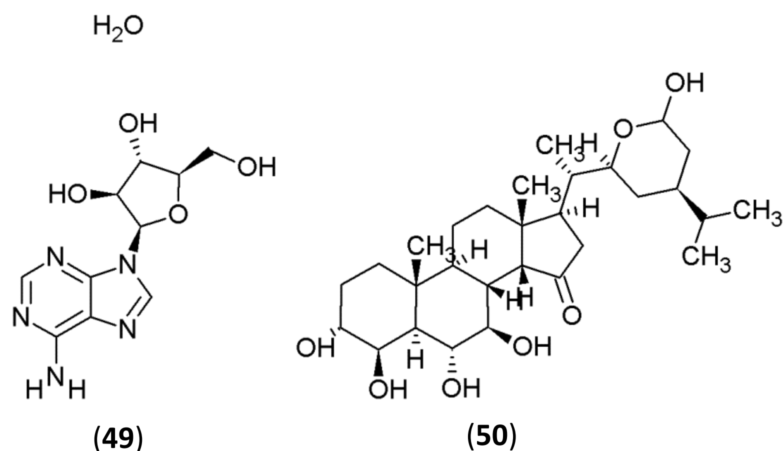
Part of the structure is a brominated indole found in some marine organisms and the N-vinyl amide group was of chemical interest due to the rarity of it at the time of the discovery of Clionamide.[64]

One of the first applications of sponge natural products in modern times was from an extract of *Cryptotethia crypta* in the 1950s. The sponge contained two nucleosides, spongothymidine (**47**) and spongouridine (**48**).

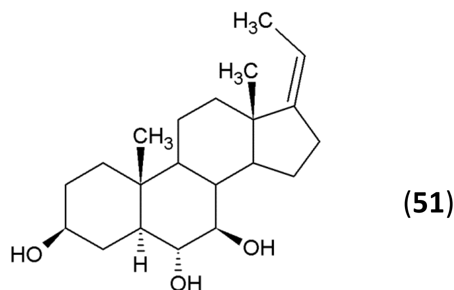


(**47**) (**48**)

These were used as lead compounds to develop the drug Ara-A (**49**) an antiviral compound.[58]



Contignasterol (**50**) is a steroid that was isolated from the sponge *Petrosia contignata* found in Papua New Guinea.[65] When the structure of contignasterol was initially elucidated, the configuration of two of the chiral centres was ambiguous. All four of the possible configurations were synthesised and by comparing their ^{1}H NMR spectroscopic data with that of natural contignasterols the absolute configuration was solved. Contignasterol was found to have cytotoxicity when tested against rat mast cells and was therefore chosen as base structure from which to synthesise variants.[65]



(**51**)

One of these variants IPL576-092 (**51**) showed good antiinflammatory and antiasthma activity. It was then advanced to Phase Two clinical trials.[65, 66]

One of the drawbacks of natural products use from sponge species and marine species in general, is the relative difficulty of obtaining them compared to terrestrial species due to their environment. This means that sourcing large quantities of the compounds of interest can be difficult. In addition to this problem, some species only produce trace amounts of the natural product of interest. This can contribute to the relatively few marine compounds that have been advanced to clinical use.[3] Even if species can be reliably sourced from the

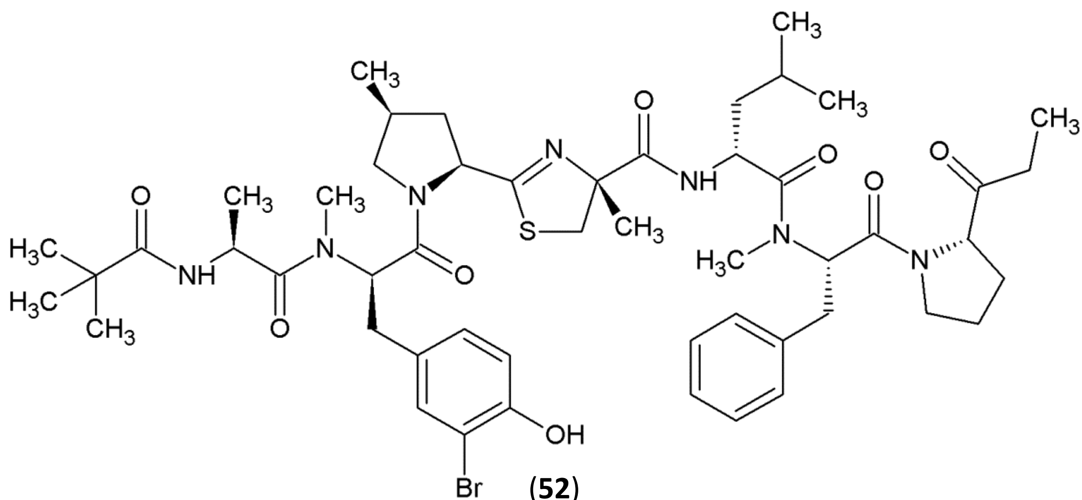
environment ecological impacts might prevent large scale collection. For example populations of the sponge *Xestospongia muta* form a major part of the habitat of reefs off the Florida Keys, so collection would have major ecological impacts.[15] In sponges, bioactive molecules typically appear in concentrations too low to be commercially viable.[67] However, there may be an efficient synthetic pathway to the same product or by cultivating sponges in aqua farms or within aquariums.[67] Alternatively, if a symbiont such as a cyanobacterium (common candidates for symbiosis due to their nitrogen fixing ability) produces the secondary metabolite, it could potentially be isolated and cultivated separately.[67, 68]

1.2.6 Cyanobacteria

Cyanobacteria are a phylum of bacteria and are also called blue-green algae (because they contain chlorophyll).[69] Similar to the sponge they are ancient and relatively simple compared with many other forms of life. [70] They are likely to have been the first oxygen producing photosynthetic organisms on earth and have developed a wide variety of secondary metabolites throughout their ecological history.[71] On account of their adaptability, they have been able to inhabit almost every environment on earth and molecules already found in them have been shown to have antiviral, antibacterial, antifungal and anticancer activity. In addition to having a range of biological activity, cyanobacterial natural products comprise a variety of different structures including indoles, lipopeptides, polyketides, amides and alkaloids.[72] The specific advantages of studying cyanobacteria to look for novel compounds is that different strains can have their own unique sets of secondary metabolites and once a strain is found with a desired molecule, cultivation of that strain may be relatively easy under the correct environmental conditions.[73]

The specific genus of cyanobacteria *Lyngbya* has been found to produce many interesting natural products. In addition to this, they are easily accessible from the tropic and subtropical regions they inhabit and are able to be cultured in the

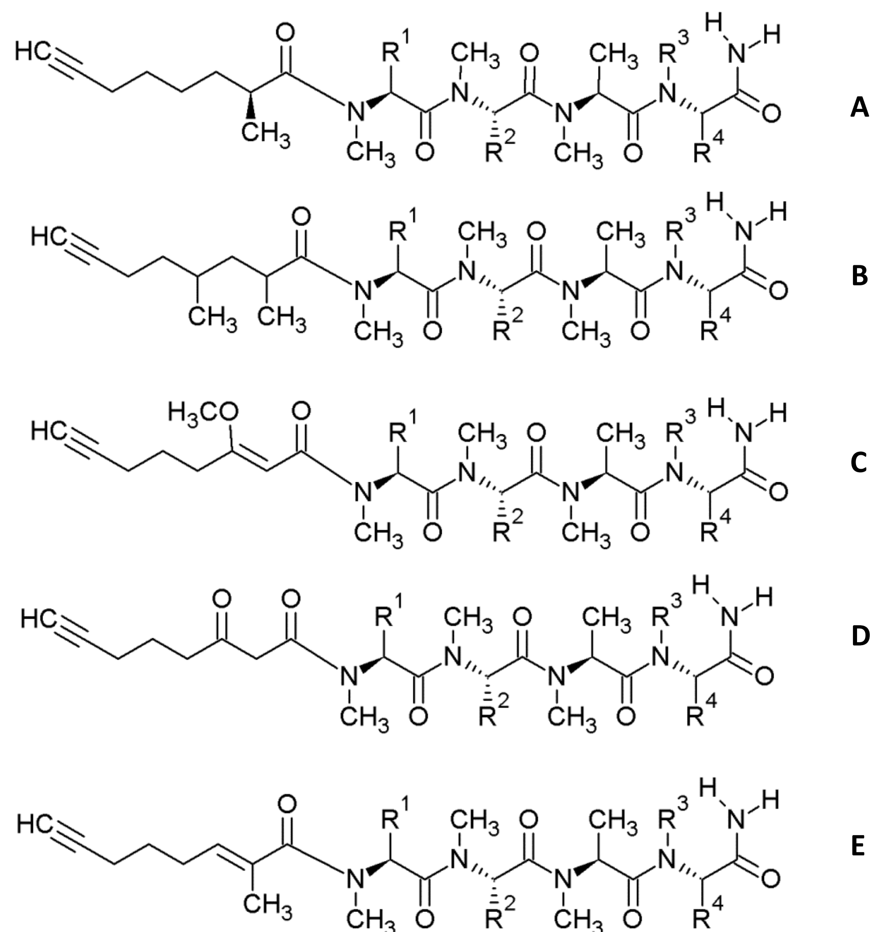
laboratory to produce workable quantities of the natural products of interest.[70] The natural product bisebromoamide (**52**) was isolated from an unknown species of *Lyngbya* collected in Okinawa.[74]



(**52**)

Bioassays were used to direct the fractionation process and HPLC was used to isolate the bioactive bisebromamide. After this, HRESIMS, ^{1}H and ^{13}C NMR spectroscopy were used to deduce the structure. To assign correct configurations to the eight chiral centres, the molecule was broken into chiral subunits and compared to enantiomeric standards by chiral HPLC. It was found that bisebromoamide contains a large proportion of D-amino acid chiral centres.[74] Where D-amino acids have been found in many other marine and terrestrial organisms, their presence has been essential to maximise the compounds bioactivity. These D-amino acids within organisms have shown biologically specific functions to regulate systems L-amino acids do not, such as parts of the nervous and endocrine systems.[74] In marine organisms it has been speculated that they assist in maintaining osmoregularity in response to environmental changes.[75, 76] When tested against HeLa cells bisebromoamide gave an IC_{50} value of 0.04µg/mL. It was also tested against 39 cancer cell lines and the average concentration required to inhibit growth by 50 % was 40 nmol/L.[74]

From the both the species *Lyngbya majuscula* and *Lyngbya polychroa* dragonamide A (**53**) and six new acyclic peptides with analogous structures (**54-59**) were isolated.[70]



(**53**) R_1=R_2=R_3= *i*-Pr, R_4= $PhCH_2$- Structure =**A**

(**54**) R_1= Bn, R_2= R_3= Me, R_4= 4-MeO-$PhCH_2$- Structure =**B**

(**55**) R_1= Bn, R_2= R_3= Me, R_4= 4-MeO-$PhCH_2$- Structure =**A**

(**56**) R_1= R_2= R_3= *i*-Pr, R_4= *i*-Pr Structure =**A**

(**57**) R_1= R_2= R_3= *i*-Pr, R_4= *i*-Pr Structure=**C**

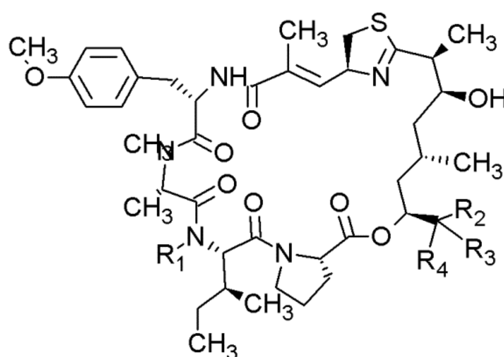
(**58**) R_1= R_2= R_3= *i*-Pr, R_4= *i*-Pr Structure =**D**

(**59**) R_1= R_2= R_3= *i*-Pr, R_4= $PhCH_2$- Structure =**E**

The seven compounds were tested for their antimalarial and antileishmanial activity. Only carbamin A (**54**), dragomabin (**55**) and dragonamide A (**53**) showed significant antimalarial activity, with IC_{50} values of 4.3, 6.0 and 7.7 µmol/L respectively. Dragonamides A (**53**) and E (**59**) showed antileishmanial properties

but the analogue dragonamide B (**56**) did not. This indicates that the aromatic group that is missing in B (**56**) but contained in A (**52**) and E (**59**) is important for the bioactivity of the molecules.[70] Malaria and Leishmaniasis infect millions of people in tropical regions each year and with the diseases gaining resistance to existing therapies finding compounds that can lead to new treatments is important for global health.[77]

Apratoxins are another group of compounds isolated from a *Lyngbya sp.* (possibly *Lyngbya bouillonii*) with apratoxins A-C (**60-63**) sourced from areas around Guam and Palau.[78]



(**60**) R_1= R_2= R_3= R_4= Me

(**61**) R_1=H, R_2= R_3= R_4= Me

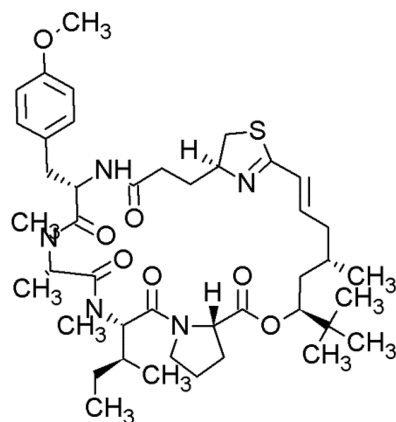
(**62**) R_1= R_3= R_4= Me, R_2= H

(**63**) R_1= R_2= Me, R_3= H, R_4= $(CH_3)_3CCH_2$-

Apratoxin D (**63**) was isolated from samples of *Lyngbya majuscula* and *Lyngbya sordida* found in Papua New Guinea.[70] The apratoxins are a group of depsipeptides defined by a macrocycle containing a mixture of polyketide sections and amino acid residues. The structure of apratoxin A was solved by analysis of the mass spectrum and several types of NMR data with the stereochemistry of the amino acid residues being determined by acid hydrolysis followed by chiral HPLC.[79] Studies of the bioactivity of the apratoxins indicate that they inhibit cancer cells in many ways, including interfering with heat shock protein 90 and the secretory pathway.[73] Apratoxin A has not been seen as a good candidate for further testing because of unsatisfactory *in vivo* test results.[80] It has potent cytotoxicity but lacks the selectivity to make it a viable drug.[79] It could still be useful as a lead compound for analogues developement as there is a current

synthetic pathway to apratoxin A and its mode of action is becoming better understood.[80, 81]

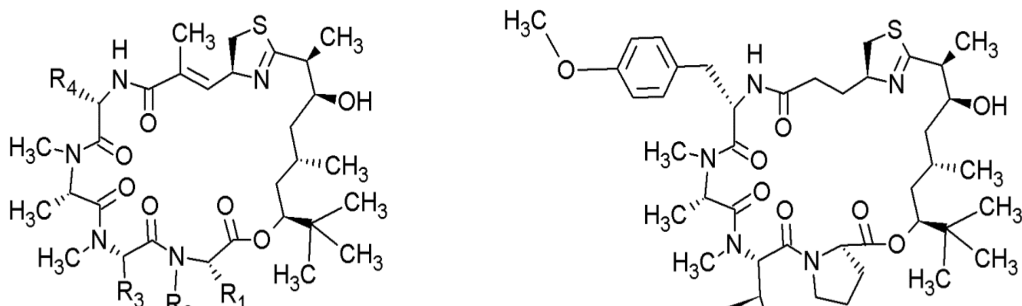
Apratoxin E (**64**) was isolated from a sample of *Lyngbya bouillni* in Guam.[82]



(**64**)

The structure of apratoxin E was solved by mass spectrometry, NMR spectroscopy and chiral HPLC. Apratoxin E was tested against a variety of cell lines along with other compounds including apratoxin A and *E*-dehydroapratoxin A. Apratoxin A has more potency than both its dehydro analogue and apratoxin E. It has been concluded from this that the hydroxyl group on the polyketide unit is important for the cytotoxic effect.[82]

Apratoxins A and E, as well as some of their analogues were analysed to identify their unique mode of action to inhibit cell growth through the secretory pathway. Cancer cells can be characterised by an overexpression in pro-growth signalling by the receptor tyrosine kinase (RTK). Apratoxin A was found to stop the secretion of RTK activating ligands and RTK itself.[81, 83] After the mechanism was understood, other less potent analogues were synthesised to try to find a more viable drug candidate with less toxicity problems that apratoxin A. Four analogues were developed; S1-3 (**65-67**), S4 (**68**) and the related compound apratoxin F (**69**) were also studied.



(**65**) R_1= R_2= Me R_3= (S)-sec-Bu R_4= PMB

(**66**) R_1=R_2= -$(CH_2)_3$- R_3= Me R_4= PMB

(**67**) R_1=R_2= -$(CH_2)_3$- R_3= *i*-Pr R_4= PMB

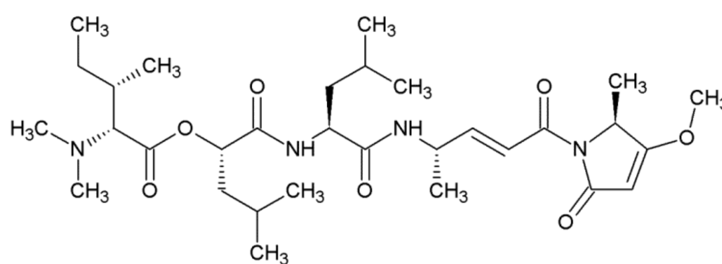
(**69**) R_1=R_2= -$(CH_2)_3$- R_3= (S)-sec-Bu R_4= Me

(**68**)

PMB= p-Mehtoxybenzyl ether

The analogue S4 (**68**) was found to have even more potency than apratoxin A and was developed from combining elements of apratoxins A and E to try to get good tumour selectivity and strong anticancer properties.[83]

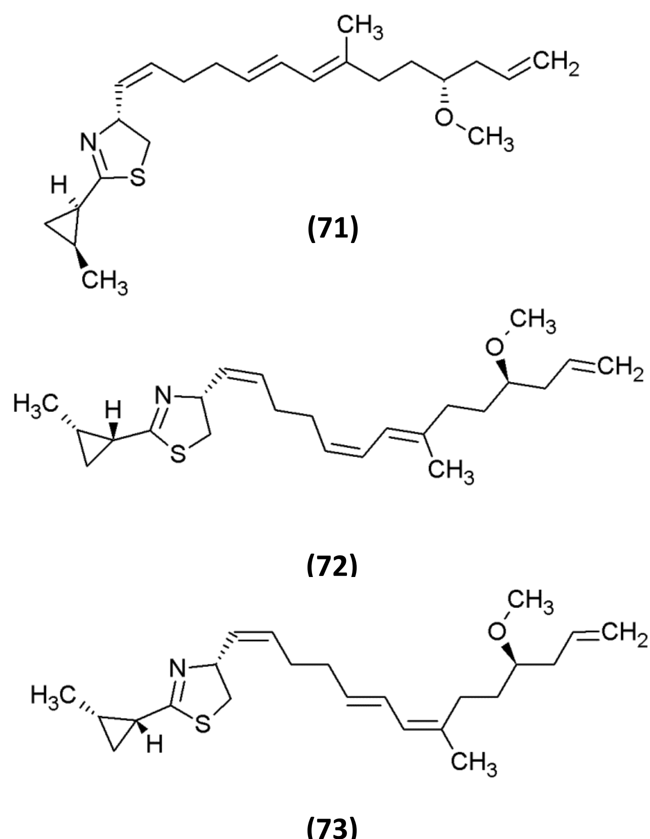
A sample of the cyanobacterium *Schizothrix sp.* obtained by SCUBA divers off the coast of Panama was found to contain gallinamide A (**70**), a potent antimalarial compound.[84]



(70)

After initial fractionation, a pure sample of gallinamide A was isolated by HPLC. This was tested for its potency against Malaria and found to be moderately active with an IC_{50} value of 8.4 µmol/L.[84] Interestingly, the parent fraction was also tested against Malaria and showed an IC_{50} value of 1 µmol/L.[84] This could either suggest another molecule in the fraction with stronger bioactivity or possibly a synergistic effect of gallinamide A with another compound in the fraction.[84]

In 1995 two isomers of curacin A (**71**), Curacins B (**72**) and C (**73**) were isolated from a sample of *Lyngbya majuscula*, a cyanobacterium obtained in Curacao.[85]



(73)

The molecules are geometric isomers of curacin A (**71**) which had shown strong antimitotic activity.[86] The *Lyngbya majuscula* sample was extracted and fractionated by chromatography. Curacins B (**72**) and C (**73**) were found to be related to A because of similar ^{1}H and ^{13}C NMR spectra and through further analysis were determined to be geometric isomers. Two different mixtures of the compounds were tested in a tubulin polymerisation assay using 95:5 and 1:1 C:B

ratios. It was found that ratios of 95:5 and 1:1 gave IC_{50} values of 1.6 and 1.7 µmol/L respectively only slightly higher than curacin A (**71**) (IC_{50} 1.4 µmol/L). The study concluded that for the curacin molecules geometric, isomerism has little effect on the compounds antimitotic potency.[85]

1.3 Bioassays and Cell Lines Used in Natural Product Research

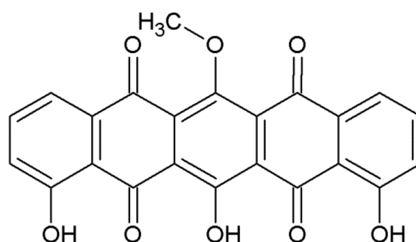
The above review mentioned bioassays and some of the information that they can give about the bioactivity of natural products. This section will detail specific cell lines used in bioassays and the use of the MTT assay to determine cytotoxicity of compounds

1.3.1.1 HeLa Cells

The HeLa cell line was the first human cancer cell line cultivated, as well as one of the most robust.[87] The origin of the line is a sample of cervical carcinoma cells taken for a biopsy from the patient Henrietta Lacks (hence the name HeLa) who was receiving treatment at John Hopkins Hospital in 1951.[88] The cells were placed in culture and found to grow rapidly. They can grow indefinitely in culture and their physiology is very well understood with over 65,000 scientific papers on HeLa cells published since their propagation.[89] In practice the cells can grow indefinitely but like other cell lines, after many generations, mutations begin to build up and the cell biology can have significant differences from earlier passages. If the cell lines continue to be used at very high passages, this can have a detrimental effect on the reliability and reproducibility of results as higher passages can alter cells growth rates and response to stimuli.[90] HeLa cells are useful to work with due to their adaptability to culture conditions, as well as a short doubling time (24 hours). The drawback of these characteristics is that the cell line can easily contaminate others and overgrow them, meaning careful technique and monitoring of cells lines is essential to avoid cross contamination.[87]

1.3.1.2 Using HeLa Cells to find bioactive compounds

As a proliferative cancer cell line, the HeLa cell line has been commonly used for bioassays to indicate anticancer activity of compounds. By dosing the cells with known quantities of the compounds of interest (or a crude extract) and measuring cell activity (or cell death), potentially bioactive molecules can be identified and further assessed.[91] If a crude extract is used, then bioassay can help decide whether fractionation is needed or not and if it is, which of the sub fractions contains the bioactive compound(s). The bioactivity of the pure fractions can be compared to the crude extract and an indication of which components are more or less potent can be obtained.[92] This is what was done to find the compound benzobijuglone.[93] Researchers used the MTT bioassay to guide the fractionation of an extract from the plant *Juglans mandshurica* and eventually found and characterised the quinone compound p-hydroxymethoxybenzobijuglone (**74**) using spectroscopic methods.[93]



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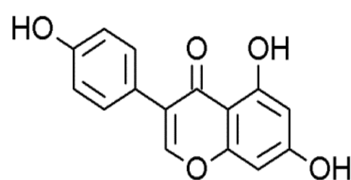
A study looking at natural products contained in soybeans also used HeLa cells and the MTT assay to determine how well they induce apoptosis.



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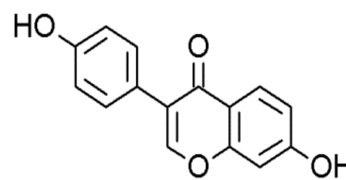

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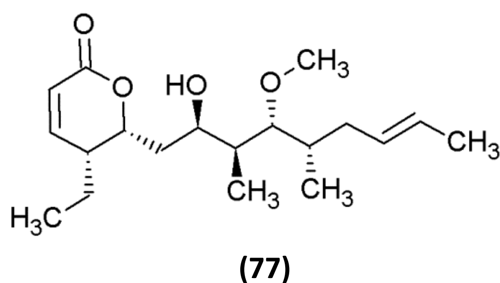
The isoflavones genistein (**75**) and daidzein (**76**) have a synergistic effect, which was indicated by comparing the inhibitory effect of the crude soybean extract on HeLa cells to the pure genistein when concentration is accounted for. The synergistic effect was further confirmed when it was found that the daidzen did not induce apoptosis but genistein with daidzen had more effect than genistein alone.[16]

1.3.2 P388 Cells

P388 are a murine leukaemia cell line commonly used to test for antitumour activity both *in vivo* and *in vitro*.[94, 95] They are very chemosensitive and therefore very useful for detecting bioactive compounds from extracts.[96] One study using both P388 and HL-20 (human leukaemia) cells, illustrates the useful information that can be gained from varied cell line testing. In the study, the compound pironetin (**77**) which previously tested positive for cytotoxic activity, was further examined with the aim of deducing the mechanism by which apoptosis was induced.[97]



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By the use of the two different cell lines, researchers were able to compare the biological effects of pironetin to get a better understanding of its mode of action.[97, 98]

1.3.3 The MTT assay

The 1,3,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) assay works on the basis that the mitochondrial dehydrogenases in healthy cells will reduce soluble yellow MTT to its insoluble purple formazan product.[99]

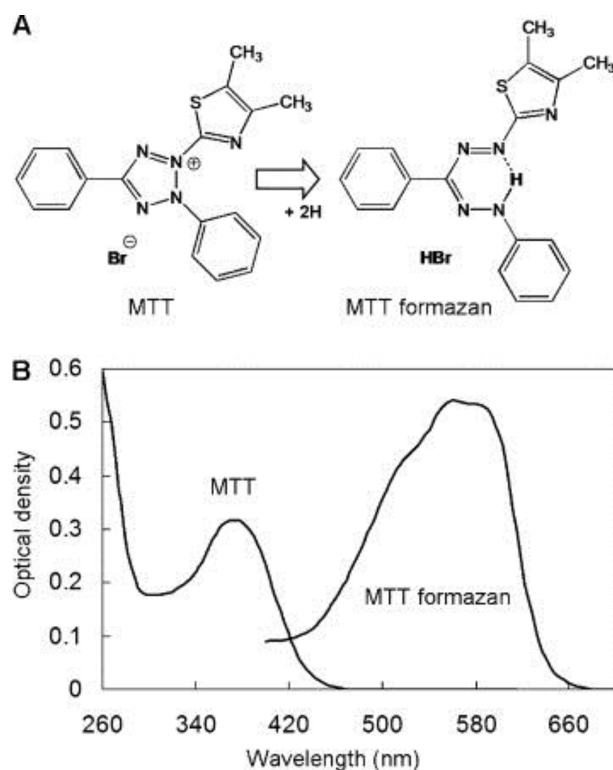


Figure 1: (**A**) Metabolisation of MTT to Formazan and (**B**) absorbance spectra of MTT and MTT Formazan

As shown in Figure 1, the absorbance ranges of the MTT and formazan differ sufficiently with their absorbance spectra and max absorbance peaks about 100nm apart, that the MTT does not interfere with the spectroscopic measurement of the formazan. Therefore the mitochondrial activity of the cells can be measured accurately by using the correct wavelength. The measured absorbances are proportional to incubation time and the concentration of MTT as well as to cell metabolism.[100]

MTT has been used extensively for bioassays to test if samples are killing cells or inhibiting their mitochondrial activity and therefore measuring the samples cytotoxic potency. Despite its wide use, the factors that can lead to the reduction of MTT in cell culture are still not completely understood and many reducing agents in the cells, media or even in the added samples can be involved in reducing MTT to formazan.[100] For example, the flavonoids luteteolin and quercetin have both been shown to reduce MTT in solution with no cells present. They were also shown to have greater reducing ability in RPMI-1640 with 10%

foetal bovine serum more than they did in ethanol.[101] This illustrates why sample controls should be run as a part of all MTT assays.

2 Chemical Analysis of Coastal Marine Samples

2.1 Introduction

Marine organisms provide a rich source of natural products, due to their biodiversity and development of chemical defenses for survival. This results in a number of unique molecules that are of interest both for their chemical structures and their bioactivity. It is useful to know the structures of these molecules for synthesis or further study and any useful bioactivity they may have (anticancer or antibacterial properties etc.).

A summer research project by undergraduate Biological Sciences student Nikki Webb, supervised by Professor Chris Battershill resulted in the collection and cataloguing of 58 marine samples from three sites around Mount Maunganui, New Zealand. Of the samples collected, eighteen were from Leisure Island (LI) (rocky shore habitat), twenty were from Rabbit Island (RI) (rocky reef habitat) and twenty from the Waikareao Estuary (WE). Photographs of each of the samples were taken and preliminary assignment of their taxonomy carried out. These samples were weighed, frozen and are currently stored at the Chemistry Department of the University Of Waikato.

The taxonomic data given for these samples show that they comprise a diverse range of species. Of the 58 samples, fifteen are algae (eight Chlorophyta or green algae, four Rhodophyta or red algae and two Phaeophyta or brown algae with one unidentified to fit any of the groups). Twelve of the samples are sponges (Porifera), all from the class Demospongiae, three are sea snails and three are bivalve molluscs, five are Cnidarians consisting of four anemones and one coral. Other taxonomic groups represented include cyanobacteria and ascidians. A complete list of samples with their taxonomy is provided in Appendix One: Initial Survey of the Marine Samples.

The aim of this research was to screen these samples and determine if they contain any novel compounds with interesting structures or biological properties. The rationale of examining many different species is that high biological diversity will translate to high chemical diversity and hopefully the discovery of novel compounds.

2.2 Initial Survey of the Marine Samples

To analyse this collection of marine species, approximately two grams (wet weight) of subsample was taken from each and the biomass exhaustively extracted in methanol:dichloromethane. These crude extracts were stored as reference samples. A subsample of each was taken for analysis by LCMS, run on a reversed phase C_{18} column. Duplicates of each sample were run to confirm the reproducibility of the traces. For each sample, data was collected over a mass spectral range of 100-1500 Da and UV data with a wavelength range of 200-800 nm.

Analysis of the data identified halogenated compounds from their characteristic isotope patterns and the major peaks each chromatogram contained. On account of a soft ionisation technique being used (electrospray ionisation) most peaks were assumed to be pseudo-molecular ions ($M+H^+$ or $M+Na^+$) with very few if any fragment ions seen. A few common losses were identified in some mass spectra such as water (18 Da), carbonyl (28 Da) and ammonium (17 Da) losses.

General analysis showed that in the majority of crude extract chromatograms (Appendix One: Initial Survey of the Marine Samples), metabolites eluted between 25 and 40 minutes. The common ions that appear in many of the traces were noted. This could indicate that primary metabolites common to many of the marine species are major components of these extracts. Even though these might not be of any chemical interest, as they are unlikely to be novel metabolites due to their commonality. Most of these compounds are likely

primary or common secondary metabolites which assisted the decision on whether to pursue the species for further analysis or not.

2.2.1 Common High Mass Ions

Many of the samples were found to have peaks that eluted at 35-37 minutes with masses between 730 and 750 Da. Six of these peaks commonly appeared as major peaks in the spectrum and were paired two mass units apart. They yielded ions with *m/z* of 731.5, 733.5, 740.5, 742.5, 745.5 and 747.5. Their peaks are very strong in the samples they appear in (Table 1). It is likely that these are either very long chain fatty acids or triglycerides, based on their relatively high molecular mass and their elution off the C_{18} column during the pure acetonitrile solvent phase of the LCMS run. This is also supported by the fact that lipids are common in marine species for energy storage.[102] Whether these are fatty acids, triglycerides or another type of lipid, the two mass unit differences indicates the species are related with only a difference of one double bond group. The small differences that they have in retention time show that this is not an isotope effect but rather a minor functional group change.

Table 1: Common high mass ions observed in Algae Species Surveyed

Taxonomy (sample)	Ions (*m/z*) and their retention times (min)		
	731.5, 733.5 (36.6, 36.9)	740.5, 742.5 (36.4, 36.7)	745.5, 747.5 (35.6)
Ulva sp.(LI 2-5)	✓		✓
Codium sp.(LI 2-6)	✓		
Ulva sp.(WE 1-1)	✓		✓
Green Alga *sp.*(WE 1-5)	✓		
Enteromorpha sp.(WE 2-16)	✓		
Entermorpha sp (WE 2-17)	✓		
Green Alga *sp.*(WE 2-19)	✓		
Brown Alga *sp.*(LI 1-1)		✓	
Sargassum sp.(RI 2-20)			✓
Plocamium sp.(WE 1-2)	✓		
Gigartina sp.(WE 1-4)		✓	

Algae are at the bottom of the food chain and tend to be the species that produce fatty acids.[102] From the fifteen algal species surveyed eleven showed at least one the following six ions with *m/z* values 732.5, 734.5, 741.5, 743.5, 746.5 and 748.5 with paired two mass unit differences eluting at 35-37 minutes. Table 1 shows that the compounds with ions at *m/z* 746.5 and 748.5 have the same retention time, which could lead to the conclusion that it is an isotope pattern but closer analysis of the chromatogram showed that these ions only elute closely off the column and are probably related but not isotopes. The other algal species did not contain these peaks but did have ions that elute around that retention time with two mass unit differences that could potentially be lipids.

2.2.2 Analysis of the Mangrove Samples and More Common Ions

Four of the samples analysed were different parts of a mangrove and one was lichen species that grew on the mangrove. These were the seeds (WE 1-9), leaves (WE 2-14), lichen (WE 2-15) and pneumatophores (WE 2-12). Unlike the other samples where a representative cross section of the species was taken for analysis this gives an insight into the localised chemistry of the species features. The chromatograms of the leaves, lichen and pneumatophores showed the same ions (740.5, 742.5) as those of the algae but the seeds did not (Table 2). This could mean that the compounds are produced later in the life cycle during the growth of the mangrove.

Table 2: Compounds of interest in Mangrove samples

Taxonomy (sample)	Ions (*m/z*) and their retention times (min)	
	740.5, 742.5 (36.4, 36.7 min)	Other prominent ions
Mangrove Leaves (WE 2-14)	✓	117 (8.1 min)
Pneumatophores (WE 2-12)	✓	
Mangrove Lichen(We 2-15)		757.5, 759.5 (36.3, 36.6 min)
Mangrove Seeds (WE 1-9)		117 (8.1 min)
Root by-product (WE 2-13)	✓	

The 117 *m/z* ion is included, as it was seen in the chromatograms of thirteen of the marine samples, indicating that this is a primary or a very common secondary metabolite. It is found in such diverse groups such as algae, cnidarians, tunicates, molluscs (which make up five of the thirteen), in addition to the mangrove. This could also explain two other common ions found in a number of marine species, 219 *m/z* (8.6 min) identified in twenty two of the species chromatograms and 138 *m/z* (8.2 min) found in fourteen species.

The last common ions identified in the chromatograms were potential sterols that had masses of 328 and 560 *m/z* with retention times of 27.2 and 27.9

minutes respectively. They are both quite small peaks and often appear in the same sample. Their retention times showed that they elute from the column just after the mobile phase becomes pure acetonitrile. The 328 *m/z* ion could be a fragment of cholesterol. The difference between them is 57.5 Da which is the same molecular mass as an isobutyl group part of the side chain of cholesterol. If this was a cholesterol fragment the number of large number of species it appears in would not be surprising due to the commonality of cholesterol. Neither of the ions are seen in the chromatograms of three of the twelve sponges where sterols are common which indicate they may not be sterols. Alternatively, the peaks could have been too small to be identified in those three chromatograms.

2.3 Analysis of the Halogenated Compounds

Halogenated molecules can be identified by the presence of characteristic isotope patterns and even if they are in low concentrations, peaks can often still be seen, as halogenated compounds ionise well. The most common halogens seen are chlorine and bromine whose isotopes appear two mass units apart in ratios 3:1 and 1:1 respectively. On the mass chromatogram, this is seen as two peaks with identical retention times differing by two mass units. Depending on the peak ratios, the molecule can be determined to either contain chlorine or bromine. This can be extended to doubly halogenated molecules. In this case, three peaks will be seen in the ratio 9:6:1 for chlorine and 1:2:1 for bromine.

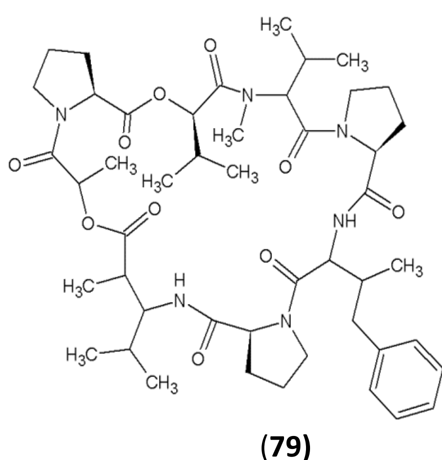
From analysis of the chromatograms of the marine samples analysed in this research, six chlorinated and twelve brominated metabolites were detected. In addition, thirty two other ions identified appeared to be halogenated but the peak ratios were not distinctive enough to determine which halogens the molecules contain. Many of them seem to contain multiple halogen atoms and if they contained different halogens, would give an isotope ratio that may be difficult to identify. It is not surprising that more bromine than chlorine containing compounds were found. Despite the fact that the chlorine concentration in the marine environment is much greater than that of bromine it

more brominated compounds were found as bromine is more easily oxidised and therefore incorporated into a biosynthetic pathway.[103, 104]

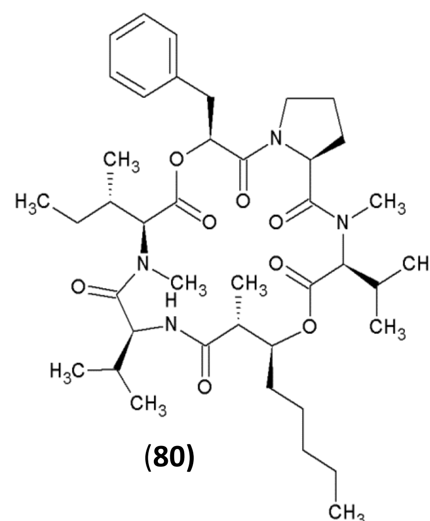
Based on the information gained in the chemical survey two of the marine species the cyanobacterium *Lyngbya sp.* (WE 2-20) and the sponge *Cliona celata* (RI 1-11) were selected for bulk extraction and further analysis. Three more samples selected for less detailed analysis were the tunicate *Aplidium sp.* (RI 2-13), the cnidarian coral *Alcyonaria sp.* (RI 2-14) and *Ircinia sp.* (RI 2-17) a sponge. These underwent micro-extractions, LCMS and tandem mass spectrometric analysis of some potentially novel compounds.

3 Analysis of *Lyngbya sp.* (WE 2-20)

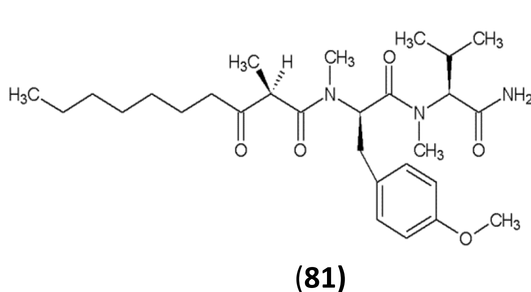
Lyngbya is a genus of cyanobacterium that has been a source of many new natural products due to high secondary metabolite production.[105] From just one study of the species *Lyngbya majuscula* twelve new polyketide-polypeptide secondary metabolites were discovered. The compounds dolastatin 16 A (**78**), hantupeptin C (**79**), majusculamide A (**80**) and isomalyngamide (**81**) were found to have antifouling properties shown to reduce settlement of larvae.[106]



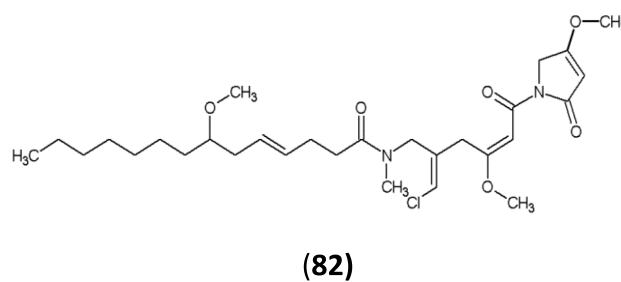
(**79**)



(**80**)

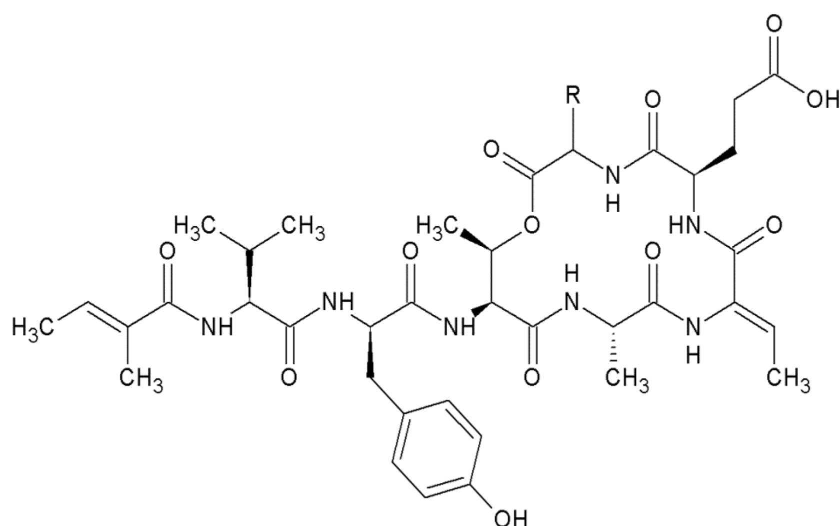


(**81**)



(**82**)

Another study of the species *Lyngbya confervoides,* resulted in the isolation of tiglicamides A-C (**82-84**), three new cyclodesipeptides. These compounds were tested against porcine pancreastic elastase and found to have IC_{50} values of 2.14-7.28 µM.[107] It has also been demonstrated with *Lyngbya birgei* that growth within the laboratory is possible under the correct conditions, which allows for more biomass to be obtained for extraction without external sourcing.[108]



(**82**) R = $-CH_2\,CH_2(C_6H_4)OH$

(**83**) R = $-CH_2(C_6H_4)$

(**84**) R = $-CH_2\,CH_2S(O)CH_3$

Lyngbya sp. was chosen on the basis that historically this genus has been an excellent source of novel metabolites. An LCMS analysis of the extract yielded a relatively simple chromatogram containing several peaks with half mass units including a major 656.6 m/z peak with several other minor peaks (710.5, 718.5, 741.5 m/z). It was thought that this could be a novel peptide due to the half mass units of the peaks and no compounds found in the MarinLit database with a 656.5 Da mass in the *Lyngbya* genus. There was also a large amount of biomass available for this species. The decision was based on the chemical and taxonomic data alone, as no bioassay data was available at the time.

For further analysis, a bulk extraction of the sample was carried out with the resulting lyophilised powder being fractionated on a C_{18} reversed phase column. Twelve fractions were collected and analysed by LCMS. Three of the fractions

contained major amounts of the 656.5 *m/z* and also 741.5 *m/z* ions. One of these fractions contained approximately equal amounts of both compounds and was further fractionated by gel permeation chromatography on an LH-20 column to try to separate the two compounds. These fractions were then analysed by LCMS to determine the effectiveness of the separation. Despite their difference in mass, the two compounds were not well separated by the size exclusion column. It is unknown why but could possibly be due to structural features causing similar retention times.

The fractions collected before and after those previously discussed were then fractionated separately by size exclusion chromatography, as they each contained one of the compounds of interest as major components along with minor metabolites. The fraction collected directly before had 656.5 *m/z* as a major component. This was then fractioned again to try to isolate the 656.6 *m/z* and analysis of the sub-fractions revealed that two of the fractions contained 656.5 *m/z* as a major ion but still contained minor amounts of a 710.5 *m/z*. The fraction collected after contained 741.5 *m/z* as the major ion and was fractionated again to further isolate the component that gave rise to this ion. Analysis of the sub-fractions showed that one of the fractions contained 741.5 *m/z* as the major ion of interest.

^{1}H NMR spectra in $CDCl_3$ were acquired for fractions containing 656.5 *m/z* and 741.5 *m/z* to gain structural information. Both spectra looked similar, containing six major signals each with identical chemical shifts. The spectrum showed a small triplet at 0.8 ppm and a large singlet at 1.25 ppm. These are a methyl triplet and a large number of methylene groups in the same chemical environment. There is also a signal at 5.5 ppm indicating double bond(s) in the molecule and three signals at 1.6, 2.0 and 2.3 ppm that could be representative of methylene groups in three different chemical environments. Judging by the first two signals and information from the mass spectrum, these three signals could be explained by methylene adjacent to an ester group, another two bonds from an ester group and a methylene adjacent to a double bond.

The 656.5 *m/z* ion was subjected to tandem mass spectrometry (MS^2 and MS^3) for more structural information. The major fragments are given in Table 3. The table also shows subsequent fragmentations of 428.3 *m/z* and 236.1 *m/z* ions.

Table 3: 656.5 *m/z* tandem mass spectrometry.

Parent Ion (*m/z*)	MS^2 fragment (*m/z*)	MS^3 fragment (*m/z*)
656.5	446.3	
	428.3	144.1, 200.1, 341.5, 384.4
	236.1	218, 131, 105.1

The eighteen Da difference between the 236.1 *m/z* and 218 *m/z* ions indicates either water or an ammonia loss. Also the 236 *m/z* ion appears to be cleaved into 131 and 105.1 *m/z* ions. So the compound probably contained a hydroxyl or amine group. The 428.3 *m/z* ions 44 Da loss to the 384.4 *m/z* ion could be an aldehyde group (CH_2CHO). Also the 428.3 fragment has an 87 Da loss forming the 341.5 *m/z* ion. One possibility is this is an alkyl chain terminating in an alcohol $(CH_2)_4CH_2OH$). All of this analysis is speculative and would require other methods to corroborate the information.

4 Analysis of *Cliona celata* (RI 1-11)

Cliona celata is a sponge species and similar to *Lyngbya sp.,* the biology and history of natural products discovery in sponges is well documented. However there has been little chemical study on *Cliona celata* with only a few compounds reported on the MarinLit database. The biology of *Cliona celata* is regarded as very able to adapt to changing environmental conditions.[109] The species is known to produce clionamide as a major metabolite, which was isolated in a derivatised form in 1978.[64] In 2012 a crude extract was tested on smooth muscle cells and was found to inhibit enzyme activity as well as two other pathways.[110] *N*-acetylhomoagmatine has also been isolated from *Cliona celata* but was found to have only low toxicity when tested against corals.[111]

Cliona celata was chosen for further analysis to examine a dibrominated (487.9/489.9/491.9 *m/z*) and monobrominated (408/410 *m/z*) pseudo-molecular ions seen in its chromatogram. In the LCMS trace and it which were not found in the MarinLit database. The sample was extracted in bulk and fractionated on a C_{18} reversed phase column. Fourteen fractions were collected and analysed by LCMS. The fractions RI 1-11.6 showed the strongest peaks of the samples of interest from the LCMS trace. Size exclusion chromatography was used to try to separate the dibrominated from the monobrominated compounds, however this was unsuccessful. This indicates that the compounds are related as they elute off the column at the same time. This is supported by their LCMS data as the 408, 410 *m/z* ions can be explained by the loss of a bromine atom from the 487.9/489.9/491.9 *m/z* ions. This also explains why the dibrominated ion is so much more intense as the soft ionisation used would not result in many bromine losses.

NMR spectra was run on fractions RI 1-11.7 and RI 1-11.6 but the compounds of interest in the samples concentrations were to low give clear signals of what they were. There were also peaks produced by other compounds not separated during fractionation. Similarly a molecular formula was attempted to be

generated by isotope analysis from high resolution mass spectrometry. It was thought that the compound because of its halogenation would give a strong enough signal. However the brominated ions of interest were not abundant enough to produce a signal that a molecular formula could be derived from.

Both the dibrominated and monobrominated ions were subjected to tandem mass spectrometry to obtain more structural information about the ions. The fragment ion are given in Table 4. For the MS^3 fragmentation the strongest ions from the MS^2 were targeted as well as the ones retaining bromines to confirm the presence of bromine from their losses.

Table 4: Dibrominated and monobrominated tandem mass spectrometry.

Parent Ion (*m/z*)	MS^2 fragment (*m/z*)	MS^3 fragment (*m/z*)
488/490/492	470/472/474(dibrominated), 306/308	
	428/430/432 (dibrominated)	349/351, 278/280, 227
408/410	393/395	306/308, 228, 172/174,
	350/352	195, 228, 271
	271	

The appearance of the 306/308 monobrominated ion indicated the loss of a bromine in addition to an unknown a 102 Da fragment. The 428/430/432 ion fragmented into 350/352 fragment gives differences of 81 Da and 79 Da indicating a bromine loss, supporting the observed isotope pattern. Analogous to this is the monobrominated 350/352 ion losing a Br^{79}/Br^{81} to form the 271 fragment ion. This also occurs from the 306/308 monobrominated ion fragmenting to the 228 Da. None of the data is sufficient to give any more definitive structural data but it does confirm the presence of the bromines in the compound.

In addition to the brominated compounds, the sterols of *Cliona celata* were also investigated. Three of the fractions of *Cliona celata* were separated by reversed

phase chromatography and analysed by gas chromatography mass spectrometry (GCMS) with two of the fractions showing several sterols. Based on the data the samples were given probable matches from the NIST database. The three fractions analysed were RI 1-11.9, RI 1-11.10 and RI 1-11.14. From two of these fractions (RI 1-11.9, RI 1-11.10) data was obtained indicating both contained stigmasterol and sitosterol in minor amounts. The major sterol in both fractions was cholesterol at 41.47% in RI 1-11.9 and 34.81% in RI 1-11.10. In total eight sterols, were identified in RI 1-11.9 and thirteen in RI 1-11.10.

5 Analysis of Three Additional Extracts

In addition to the bulk extractions three of the samples underwent another small scale extraction. The samples were *Aplidium sp.* (RI 2-13), *Alcyonaria sp.* (RI 2-14) and *Ircinia sp.* (RI 2-17). These extracts were then separated into five fractions each using a small scale reversed phase chromatography column and analysed by LCMS. The samples were chosen from ions seen in their chromatograms from the chemical survey and the crude extracts re-analysed by tandem mass spectrometry to generate fragements of these ions.

Aplidium is a genus within the Tunicata subphylum. Generally the species from this subphylum lack the structural defenses like spicules and therefore likely rely on chemical defenses.[15] *Aplidium sp.* (RI 2-13) had two ions analysed 452 *m/z* and 524 *m/z* by tandem mass spectrometry. The results are given in Table 5.

Table 5: RI 2-13 452 and 524 ions tandem mass spectrometry.

Parent Ion (*m/z*)	MS^2 fragment(s) (*m/z*)	MS^3 fragments (*m/z*)
452	382, 354, 329, 128	
	340	281, 253, 217, 160, 146, 142, 128
524	479, 399, 208, 278	
	415	345, 339, 299, 265, 228, 231, 175, 356, 370, 329

The fragments generated by 452 and the subsequent 340 *m/z* fractionation still give reasonably large ions making only speculative analysis possible. The fragment lost when 340 fragments to give 281 is 59 Da. This could be the loss of a $CH_2CH_2CH_2OH$ alcohol chain but this is only speculative. The lowest difference in the fragmentation of the 524 ion is 45 Da when it fragments to the 479 ion. There are several possibilities of what this could be including alcohol, carboxylic acid or amine groups (CH_3CHOH, $COOH$, $CH_3CH_2NH_2$).

Alcyonaria (Octocorallia) is a subclass from the phylum Cnidaria that occupy anywhere from the intertidal zone to the deep ocean. They can live either burrowed into hard substrates or dug into soft substrates.[112] The sample of *Alcyonaria sp.* (RI 2-14) was found to contain a monobrominated compound (324/326 *m/z*). The tandem mass spectrometry of it is summarised in Table 6. On the initial chromatogram double and triple charged versions of this ion were seen.

Table 6: RI 2-14 324/326 ions tandem mass spectrometry.

Parent Ion (m/z)	MS^2 fragments (*m/z*)	MS^3 fragments (*m/z*)
324-326	264.9/266.9	142, 186, 222.9/224.9
	222.9/224.9	

The 264.9 *m/z* to 186 *m/z* ion gives a 79 Da loss showing the bromine is being lost. The 44 Da difference between the 142 and 182 *m/z* ions could mean that CO_2 as well as a bromine atom is lost. If this is the case, the compound may contain a carboxylic acid group. From the rest of the data, nothing else can be determined due to the low number of fragments and their difference giving too many potential sub-structures.

The sponge (Porifera) from the genus *Ircinia* (RI 2-17) was analysed and one compound was identified as potentially interesting. Sponges produce many potentially interesting metabolites either directly or from the symbiotic microorganisms that tend to inhabit them and make up as much as 35% of their biomass.[113] The identified compound yielding the 606 *m/z* ion, had no matches found on MarinLit within its genus. The table below summarises the tandem mass spectrometry data.

Table 7: RI 2-17 606 *m/z* ion tandem mass spectrometry.

Parent Ion (*m/z*)	MS^2 fragments (*m/z*)	MS^3 fragments (*m/z*)
606	208, 249, 415, 546, 589	
	502	440, 485, 381, 216, 191, 149, 468

From 606 *m/z* to 589 the loss of a 17 Da OH or NH_3 group can be seen. This is repeated in the 502 *m/z* to 485 *m/z* ion difference. Another 17 Da difference between the 485 and 468 fragments indicating there could be another OH or NH_3 group lost. Like the other two samples, there are more potential structures for fragments but with no corroborative evidence and the large number of combinations that can arise with this data, they are speculative.

While this analysis has not revealed much substantial information about the compounds that these three species contain, it does provide some information if further research on these samples was to be pursued.

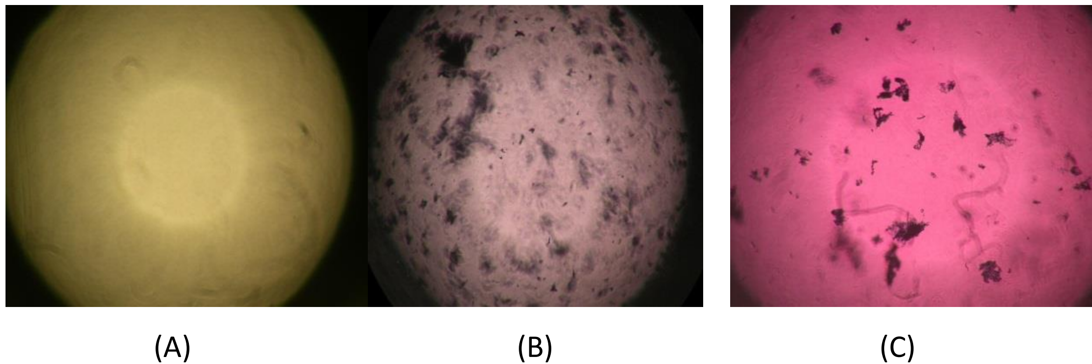
6 Bioassay Screening and Method Development

A common way to determine if a crude extract contains any bioactive natural products is to screen it through a bioassay. Initially, a pre-screening of all 58 marine samples utilising HeLa and P388 cell lines was to be undertaken to determine which samples would be good candidates for further study. However, because of the time taken for method development and to screen all the samples, the chemical analysis continued with the bioassay results to be added later.

The purpose of the *in vitro* MTT assay used in this research was to assess the cytotoxicity of the crude extracts to generate IC_{50} values. The assay is based on mitochondria of cells reducing yellow MTT to its purple formazan product and the spectroscopic absorbance is then measured (see Figure 1: (A) Metabolisation of MTT to Formazan and (B) absorbance spectra. (Section 1.3.3 page 31)) .[99] If cell growth is inhibited or apoptosis induced by cytotoxic agents less MTT will be reduced and the measured absorbances will be lowered. The cells lines used for biological screening were the immortal human cancer-derived HeLa cells and murine P388 D1 lymphocytic leukaemia cells. Different cell lines were used because they may have different responses to bioactive metabolites. HeLa cells are largely used to test for anti-cancer properties. P388 cells have been used for testing antitumour potency.[96]

The initial method used for the MTT assay was based on protocols from the University of Canterbury.[114] Following this protocol, several experiments gave inconclusive results of cell growth, including no inhibition seen from the known cytotoxin pterocellin A with a reported IC_{50} value of 477 ng/mL against the P388 cell line.[115] The method of the assay was examined to determine the reasons for this and a review of the literature found that acidified isopropanol which was being used as a solubiliser, did not effectively dissolve formazan crystals, even with agitation. It also stated that DMSO dissolved formazan crystals rapidly.[23]

An experiment was conducted to compare solubilising agents. HeLa cells were seeded on a 96 well plate at the same seeding density and for the same length of time as indicated in the provided protocol.[114] MTT was added and after four hours of incubation two different solubilisers were each added to half the wells. These were acidified isopropanol 0.1M and acidified isopropanol 0.1M with 10% triton X.



(A) (B) (C)

Figure 2: (A) Media (B) acidified isopropanol added (C) acidified isopropanol+10% triton X

Figure 2 shows the results of the experiments with acidified isopropanol (Figures 2B+2C) leaving much of the formazan (black spots) undissolved and the acidified isopropanol with triton X more effective at dissolving the crystals. Because neither solubiliser completely dissolved the formazan, a small amount of DMSO was added to some of the wells for comparison. The media control well was also photographed (Figure 2A) showing no reduction of the yellow MTT as expected.

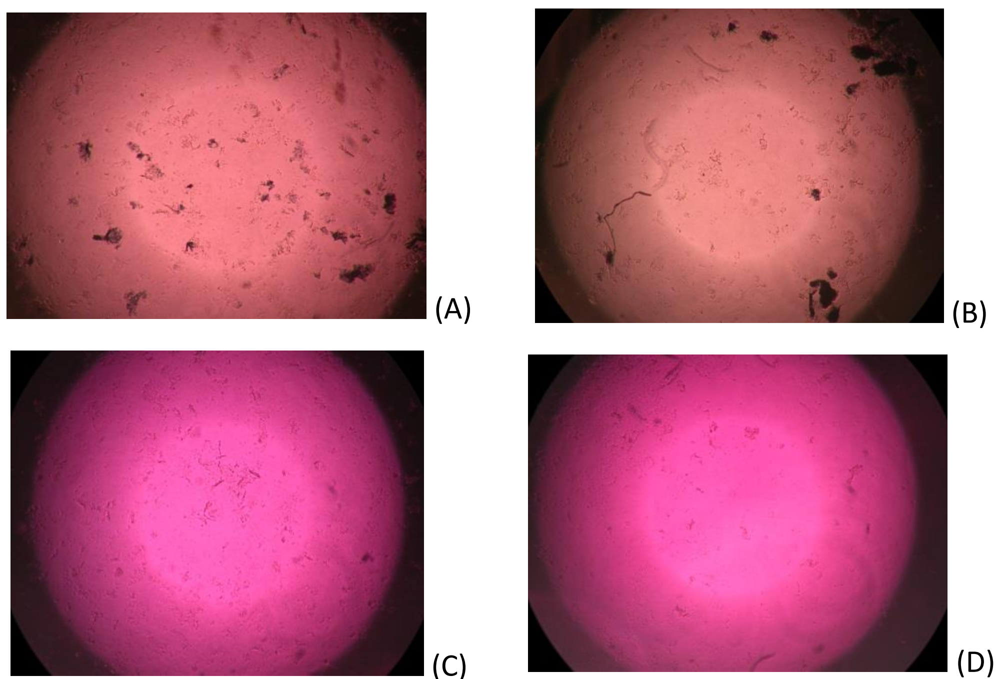


Figure 3: (A) Acidified isopropanol+DMSO, (B) acidified isopropanol+1hr incubation, (C) acidified isopropanol+triton X+DMSO and (D) acidified isopropanol+triton X+1 hr incubation.

Figure 3Figure 1 shows the effectiveness of DMSO in dissolving the formazan when added to the acidified isopropanol (Figure 3A) and the complete solubilisation of formazan when DMSO is added to acidified isopropanol with triton X (Figure 3C). By incubating the plate at 37°C for one hour, the isopropanol+triton X (Figure 3 (**D**)) also completely dissolves the formazan. The observed results agree with the measured absorbance values in Table 8. Values are calculated from a 570 nm test measurement minus a 655 nm reference measurement.

Table 8: Absorbance values for different solubilisation conditions (5000 HeLa cells/well)

Solubiliser	**Additional Conditions**		
	None	DMSO 20µL	1hr incubation
Acidified isopropanol	0.380	0.582	0.478

Acidified isopropanol+triton X	0.620	1.22	1.14

Based on these results, the solubiliser used in the protocol was modified to 100 µL of acidified isopropanol (0.1M)+10% triton X with 20µL of DMSO added to each well and a one hour incubation time, to ensure that all of the formazan was dissolved.

In addition to testing the solubiliser, the HeLa cells were tested for growth response over six days against sodium azide (Figure 4).

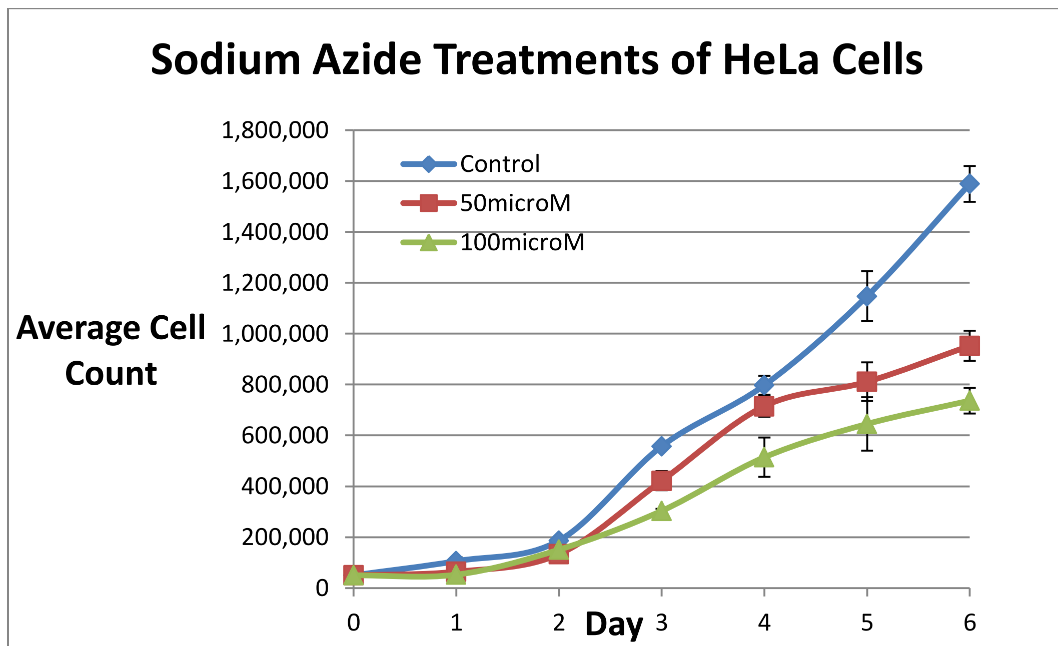


Figure 4: Sodium azide treatments of cells

It was found that the HeLa cells were being inhibited by the sodium azide and were therefore a response from the cells could be obtained with addition of a cytotoxin. The P388 cells showed inhibition measuring 61% of the cell control when grown in wells with 50 mM of sodium azide. For subsequent testing sodium azide was used a positive control for both P388 and HeLa cells with 50 mM concentrations being added to some wells.

Using the altered method, the samples were tested against the HeLa and P388 cell lines. However, there was still inconsistent data observed. Many of the samples showed activity either much lower or much higher than the cell control.

For the lower activity, this could be explained by an effect of the extract except all the concentrations produced approximately the same response rather than a dose response curve that would be expected from a cytotoxin. The crude extract from the brown alga *Xiphophora* (LI 2-3) did exhibit an expected trend for both the P388 and HeLa cell lines.

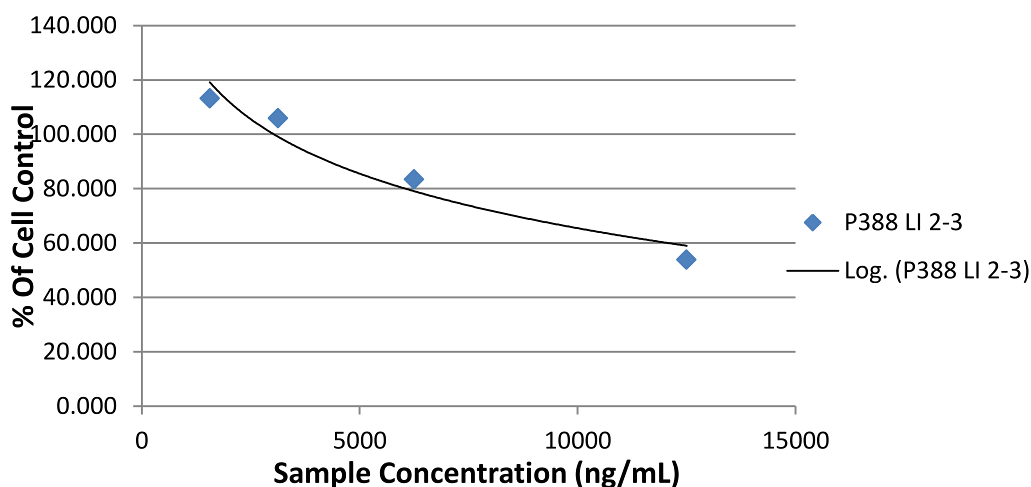


Figure 5: P388 LI 2-3 MTT Response curve

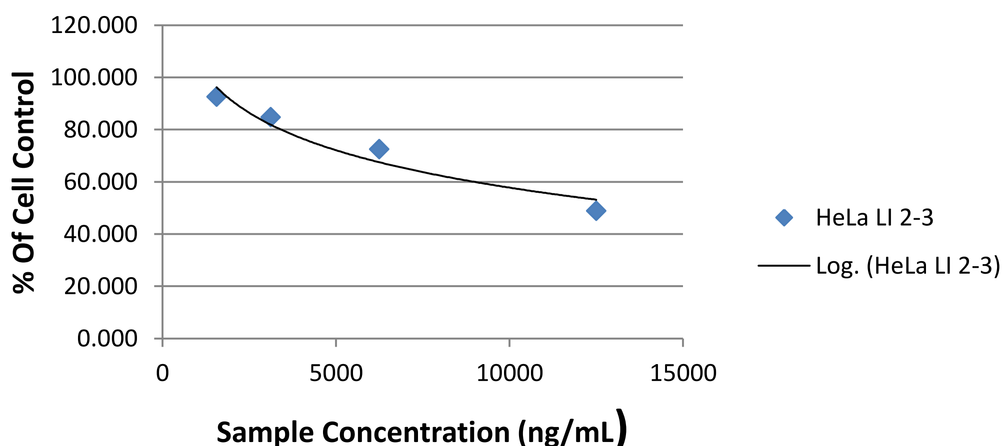


Figure 6: HeLa LI 2-3 MTT Response curve

Using the trend lines from the graph, IC_{50} values of 17079 ng/mL for P388 and 14547 ng/mL for HeLa were calculated. Two of the P388 data points gave responses about 7% higher than the cell control. This could be random error masking any effect at the low concentrations. Because of the inconsistencies

with the cell controls and the small amount of data used to calculate each point this does not confirm if the sample actually contains a cytotoxin. From the bioassay survey it shows the clearest dose response effect on the cells. More reliable results and replication of the experiment is needed to confirm if there is a reproducible effect.

7 Experimental Methods

7.1 General Experimental Methods

All of the marine samples were stored at -20°C and removed only to take a subsample for extraction.

Crude extraction was carried out using drum grade distilled methanol:dichloromethane (3:1) for the solvent and a Janke and Kunkel IKA Labortechnik Ultra Turrex T25 blender stick for homogenising the samples. Solvent was removed using a rotary evaporator (BÜCHI Rotovapor R110 with BÜCHI Vacuum Pump V-700) with samples warmed in a water bath (BÜCHI 461) at 40°C. Lyophilisation was carried out in a Labconco freeze dry system.

The LCMS data was generated on a Dionex Ultimate 3000 HPLC system with a C_{18} column connected to a Bruker amaZon X electrospray ionisation mass spectrometer in positive ion mode. All LCMS analysis was carried out using the same method with a two solvent gradient profile (Figure 7).The solvents used were water (type 1) with 0.05% trifluroacetic acid (solvent A) and acetonitrile (solvent B).

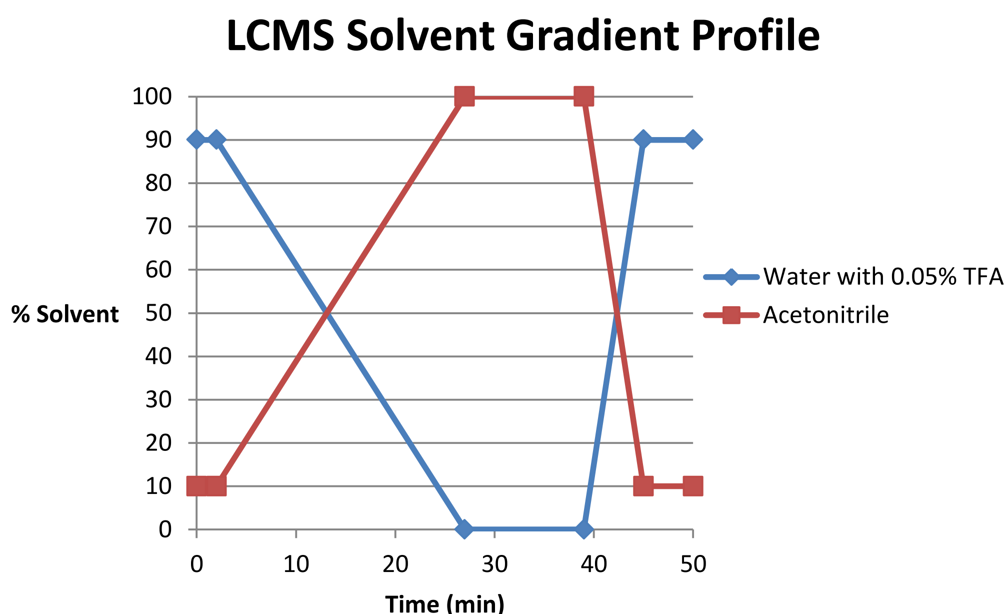


Figure 7: LCMS C_{18} reversed phase chromatography solvent gradient profile

Methanol (250µL) was added to each crude extract vial and the solution was pipetted through a syringe with a 0.2 µm filter into a LCMS vial followed by two 500µL HPLC grade methanol washes. The syringe plunger was used to provide pressure to force the sample and wash methanol through the filter.

Both the C_{18} and LH-20 column fractions were dried down under vacuum by rotary evaporation while being samples heated in a water bath at 40°C to remove the majority of the solvent. All fractions were then transferred into weighed scintillation vials and the remaining solvent completely in a heating block (Lab-Line Multi-block) at 36°C under nitrogen gas before being re-weighed and the weights calculated.

GCMS analysis was carried out on a Hewlett Packard 6890 Gas Chromatogram System connected to a Hewlett Packard 5973 Mass Selective Detector. Underivatised samples were dissolved in distilled dichloromethane and inserted into the machine by direct injection (RI 1-11.9 1µL, RI 1-11.10 5µL). The initial temperature was 80°C and increased at 30°C per minute until it reached 310°C. The temperature increase then changed to 6°C per minute for 5 minutes when the temperature was held at 340°C until the end of the run. The total run times were: RI 1-11.9 18.17 min and RI 1-11.10 33.33 min.

The small scale columns were Pasteur pipettes plugged with cotton wool were packed with C_{18} YMC gel 120Å (500mg). The column was packed dry and methanol then water:methanol 1:1(2mL) and water (2mL) run through the column to equilibrate it. Pressure was applied using a rubber pipette sucker to force the liquids through the column.

Both HeLa and P388 cells used in this research were grown in a humidified Heraeus Cell 240 incubator at 37°C and 5% CO_2. Cell media was changed every 3 days and cells were split once per week. A stock of new cells was grown once the current cells passage number approached 30. All experimental work used cells passaged less than 35 times. Handling of cells was carried out in a Hera Safe

laminar flow hood. All cells were counted using a Nikon Eclipse TS100 Microscope and photos taken on a Coolpix 4500 Nikon camera. The HeLa cells were acquired from the Biology Department of the University of Waikato and the P388 cells provided by AgResearch.

7.1.1 Solution Preparations

7.1.1.1 Phosphate buffered saline (PBS)

8g NaCl (137 nM)

0.2 KCl (2.7 nM)

1.44 g Na_2HPO_4 (4.0 mM)

0.24 g KH_2PO_4

1L of sterile H_2O

7.1.1.2 Acidified Isopropanol

20 mL isopropanol

1.6 mL 1M HCl

7.1.1.3 MTT in PBS

38 mg MTT

10 mL PBS

7.1.1.4 Sodium Azide Solution 100mM

0.065 g NaN_3

10 mL sterile H_2O

Diluted to 1 mM for treatments.

7.2 Chemical Methods

7.2.1 Crude Extraction of Marine Samples

Each sample (~2g) was weighed out and methanol: dichloromethane 3:1 (30mL) added. The sample was then blended and vacuum filtered. This process was repeated for exhaustive extraction of the biomass. Distilled water (100 mL) was

added to the resulting filtrate and the solution was frozen by dropwise addition to liquid nitrogen. The samples were then freeze-dried for 48 hours to remove the solvent. The crude extract was transferred into pre-weighed vials and the weight calculated. The extracts are stored in the Chemistry Department of the University of Waikato as reference extracts.

7.2.2 Bulk Extraction Method

Bulk extractions were carried out on *Lyngbya sp.* (WE 2-20)and *Cliona celata* (RI 1-11). Two hundred grams of sample was weighed out and blended with 75mL of solvent (methanol: dichloromethane ratio 3:1). This was then vacuum filtered and the filtrate collected. The residue was recovered then 50mL of solvent (methanol: dichloromethane ratio 3:1) added and the sample was blended, filtered and collected. This process was repeated five times to exhaustively extract the biomass with the residue being recovered each time. The solvent was then removed using a rotary evaporator, followed by lyophilisation to yield a crude extract.

7.2.3 Reversed Phase Chromatography of *Cliona celata* (RI 1-11) and *Lyngbya* sp. (WE 2-20)

Solid samples were loaded onto a reversed phased C_{18} YMC gel (120Å, 150g) packed glass column and eluted using the solvent gradient outlined (Tables 9/10) with applied nitrogen gas pressure (20 psi).

Table 9: Reversed phase chromatography solvent gradient of RI 1-11

Solvent	Volume (mL)
H_2O	100
H_2O:MeOH(7:3)	100
H_2O:MeOH(1:1)	100
H_2O:MeOH(3:7)	100
H_2O:MeOH(1:9)	100
MeOH	100
MeOH	100
MeOH:CH_2Cl_2(9:1)	100
MeOH:CH_2Cl_2(1:1)	100
CH_2Cl_2	100
CH_2Cl_2	100
MeOH	100
MeOH: H_2O(1:1)	100
H_2O	150

Table 10: Reversed phase chromatography solvent gradient of WE 2-20

Solvent	Volume(mL)
H_2O	100
H_2O:MeOH(1:1)	100
H_2O:MeOH(3:7)	100
H_2O:MeOH(1:9)	100
MeOH	100
MeOH	100
MeOH:CH_2Cl_2(9:1)	100
MeOH:CH_2Cl_2(1:1)	100
CH_2Cl_2	100
MeOH	100
MeOH: H_2O(1:1)	100
H_2O	150

7.2.4 Small Scale Reversed Phase Chromatography

Samples were loaded onto the column as solids and eluted using a five step solvent gradient with 5mL fractions from each solvent collected and the column run dry between fractions. The solvent gradient was; H_2O, H_2O:MeOH (1:1), MeOH, MeOH:CH_2Cl_2 (1:1), CH_2Cl_2. Pressure was using a rubber dropper bulb applied to force all the solvent through the column.

7.2.5 Size Exclusion Chromatography

Fractions of *Cliona celata* and *Lyngbya* sp. extracts were dissolved in minimal methanol and loaded onto a Sephadex LH-20 (150g) packed glass column. The samples were eluted off the column using methanol and sub-fractions were collected at regular intervals.

7.3 Cell Culturing and Bioassay Methods

7.3.1 Splitting of HeLa Cells

The media was removed and trypsin/EDTA (2 mL) was added. The cells were incubated for 10 minutes. The flask was removed from the incubator and tapped to dislodge the cells. After the cells were dislodged media (Gibco Life Technologies) was added (8mL). The cell solution was transferred into a Falcon tube and centrifuged for five minutes (400 rpm). The supernatant liquid was poured off and the cell pellet re-suspended in additional media (4 mL). An aliquot of this solution(1 mL) was pipetted into a new culture flask (60 mL Nunclon) containing fresh media (9 mL). The flask was then labelled with the date and the passage number then re-incubated.

7.3.2 Splitting of P388 Cells

The adherent cells were scraped from the bottom of the flask. The cell solution was transferred to a Falcon tube and centrifuged at 400 rpm for five minutes. The media was poured off and the remaining pellet of cells was re-suspended in fresh media (4 mL). One quarter of the suspension was seeded to a new culture flask (60mL Nunclon) containing fresh media (9mL), the date and passage number were recorded on the flask and the cells re-incubated.

7.3.3 Preparation of the Cell Media

In a laminar flow hood (Heraeus) sodium bicarbonate (15 g/L, 2.5 mL), of 10mM nonessential amino acids (1 mL) and penicillin-streptomycin (2mL) was pipetted into an autoclaved 100 mL measuring cylinder. Dulbeccos Modified Eagle Medium (Gibco Life Technologies) was added until the total volume was solution up to 100 mL, 10 mL was discarded and fetal bovine serum (10 mL) added. The solution was then filtered using a sterile 60 mL syringe with a 0.2 µm filter.

7.3.4 MTT Assay Methods

7.3.4.1 Counting the Cells

The cells were split and an aliquot from the suspension (50 µL) added to 0.4% trypan blue dye in phosphate buffered saline (50 µL). Half of this solution was transferred onto a hemocytometer, the cells were counted and the cells per millilitre in the suspension calculated. An aliquot determined by the cells/mL of the suspension was then transferred to a Falcon tube and cell media added, to give a cell solution with the appropriate seeding density. Seeding density for HeLa cells was 5000 cells/well and for P388 cells 11,300 cells/well.

7.3.4.2 Preparing the sample dilutions

Serial dilutions of the samples were prepared on a 96 well plate (Greiner Bio-one CellStar) (Figure 8). Media (280 µL) was added to well 5A (see Figure 8) then 150µL of media was added to each of the 6-12A wells. An aliquot (40 µL) of the test samples (1 mg/mL of crude extract in methanol) was added to the 5A (1/8 dilution). Then 150 µL of the resulting solution was transferred to 6A and triturated. This process continued down the whole row to give a series of half dilutions. Figure 8 gives the resulting serial dilution concentrations in row A (5-12) in ng/mL. The process was repeated to prepare additional samples to be tested.

	1	2	3	4	5	6	7	8	9	10	11	12
A					125000	62500	31250	15620	7810	3900	1950	975

Figure 8: Serial dilution concentration layout of one row of a 96 well plate.

7.3.4.3 Preparing the test plate

The cell solution (135 µL) was pipetted into all of the wells of columns 3-12 of in a 96 well test plate with columns 1-4 used as control wells. The media control (column 1) consisted of the cell media (150 µL) added to each well in the column. The sample control (column 2) had an aliquot of media (135 µL) and an aliquot (15 µL) crude extract in methanol (concentration 1mg/mL). Media (15 µL) was added to column three (cell control). For the solvent control, a 1:80 dilution of

methanol in cell media (15 µL) was added to each well in column four. The methanol 1:80 dilution was used as for the solvent control to give the same methanol concentration as column 5, which has the highest concentration of methanol from the test samples.

	Control Wells				**Sample Test Dilutions**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	**Media**	**Sample**	**Cell**	**Solvent**								
B												
C												
D												
E												
F												
G												
H												

Figure 9: Diagram of a 96 well plate

7.3.4.4 Adding the samples into the test plate

Columns 5-12 had an aliquot (15 µL) of the sample dilution from the test plate added (1/10 dilution from the concentrations in Figure 8). The position of the serial dilutions on the sample plate corresponded to the position they were added to the test plate. So for the 12A well from the sample dilution plate, 15 µL of solution was transferred to the 12A well on the test plate. The samples were run in duplicate so the same sample dilutions were used for every two rows of the test plate. Therefore an aliquot of 12A (sample plate) was also added to the well 12B on the test plate.

7.3.4.5 Measuring the Formazan Product

The cells were incubated for three days at 37°C, then MTT (3.8 mg/mL, 20 µL) was added to each well and the cells incubated for four hours. After incubation, a solution of acidified isopropanol (0.1N HCl) with 10% triton X (100 µL) and DMSO (20 µL) was added to each well on the test plate. The plate was then incubated for one hour after which it was shaken on an IKA MS 1 Minishaker for 5 minutes

to assist in solubilising the formazan. The absorbances were measured at 655 nm (reference) and 570 nm (test) on a Bio-Rad 680 Microplate Reader. The reference numbers were subtracted from the test numbers to give the corrected values.

7.3.5 Sodium Azide (NaN_3) Cell Treatments

Cells were seeded at 50,000 cells/mL in each well of three 24 well plates. Sodium azide solution (1 mM) was added to each well of two plates at 50 µL and 100 µL respectively. Every 24 hours each plate had the media removed from three of its wells and 200 µL of trypsin/EDTA added. After ten minutes of incubation 800 µL of cell media was added to each of the three wells. For each well, an aliquot of the suspension (50 µL) was added to 0.4% trypan blue dye in phosphate buffered saline (50 µL) and counted on a hemocytometer.

8 Appendices

8.1 Appendix One: Initial Survey of the Marine Samples

Table A1.1: Leisure Island Sample Taxonomy

Leisure Island Samples (subsite-sample number)	Genus Species/Description
LI 1-1	Brown Alga
LI 1-2	Green Anemone
LI 2-3	*Xiphophora sp.*
LI 2-4	*Spiach sp.* (Sausage Alga)
LI 2-5	*Ulva sp.* (Sea lettuce)
LI 2-6	*Codium sp.*
LI 2-7	Filamentous Cyanobacterium
LI 2-8	*Pterocladia sp.*
LI 2-9	*Glossophoria sp.*
LI 2-10	*Panncea sp.*
LI 2-11	*Tethya sp.*
LI 2-12	*Haplosclerida* (Order)
LI 3-13	*Isactinia sp.*
LI 3-14	*Actinia sp.*
LI 3-15	*Actinia sp.*
LI 3-16	*Patiriella regularis*
LI 3-17	*Cliona celota*
LI 4-18	*Xenostrobus pulex*

Table A1.2: Rabbit Island Sample Taxonomy

Rabbit Island Samples (subsite-sample number)	Genus Species/Description
RI 1-1	*Callyspongia regularis*
RI 1-2	*Cnemidocarpa + Aplidium*
RI 1-3	*Chondropsis sp.*
RI 1-4	*Aplidium scabellum*
RI 1-5	*Cnemidocarpa bicornuta*
RI 1-6	*Ancorina alata*
RI 1-7	*Tethya aurautium*
RI 1-8	*Polymastia croceus*
RI 1-9	*Lophon minia*
RI 1-10	*Biemna sp.*
RI 1-11	*Cliona celata*
RI 2-12	*Scutus breviculus*
RI 2-13	*Aplidium* (sea squirt)
RI 2-14	*Alcyonaria sp.*
RI 2-15	*Tethya ingalli*
RI 2-16	*Aptos sp.*
RI 2-17	*Ircinia sp.* (horny sponge)
RI 2-18	*Chrondria sp.* (red alga)
RI 2-19	*Terraclaydia sp.*
RI 2-20	*Sargassum sp.*

Table A1.311: Waikareao Estuary Sample Taxonomy

Waikareao Estuary Samples (subsite-sample number)	Genus Species/Description
WE 1-1	*Ulva sp.* (sea lettuce)
WE 1-2	*Plocamium*
WE 1-3	*Bryozoan*
WE 1-4	*Gigartina*
WE 1-5	Spiky green alga
WE 1-6	Sea grass
WE 1-7	*Macomona liliana*
WE 1-8	*Austrovenus stutchburyi*
WE 1-9	Mangrove seeds
WE 2-10	*Amphibola sp.*
WE 2-11	*Zeacumantus* (screw shell)
WE 2-12	Pneumatophores (aerial roots)
WE 2-13	Aerial root by-product
WE 2-14	Mangrove leaves
WE 2-15	Mangrove lichen
WE 2-16	*Enteromorpha sp.*
WE 2-17	*Enteromorpha/Ulva sp.*
WE 2-18	Bacterial flock
WE 2-19	Green alga
WE 2-20	*Lyngbya sp.*

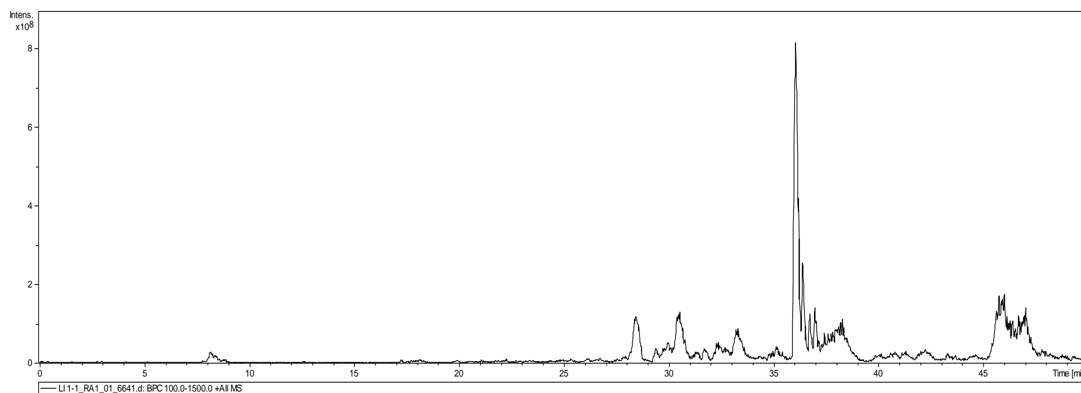


Figure 10: LCMS Chromatogram of crude extract LI 1-1

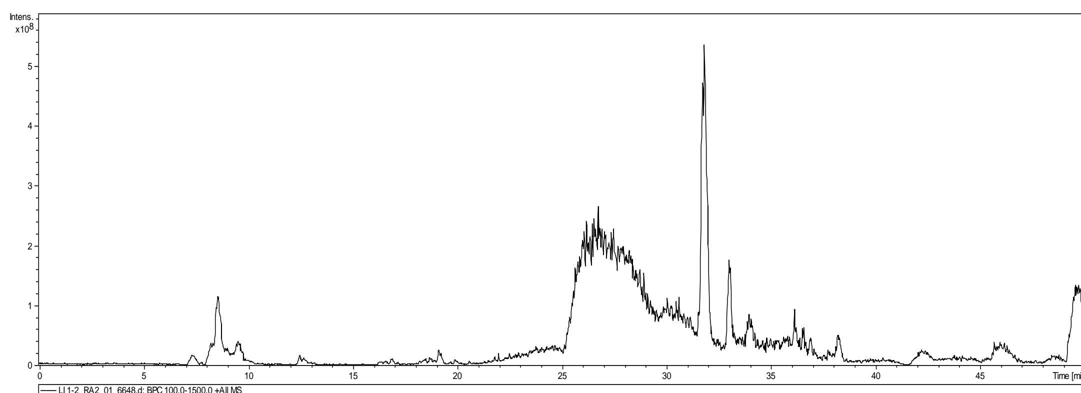


Figure 11: LCMS Chromatogram of crude extract LI 1-2

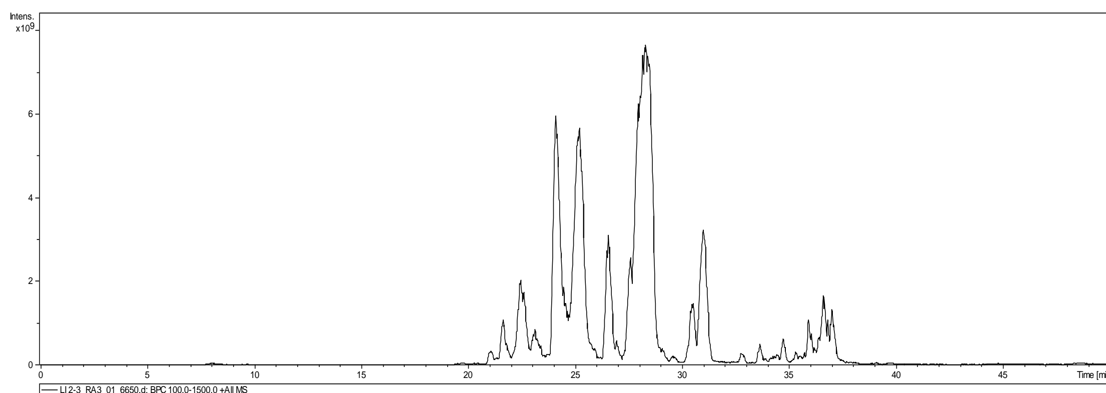


Figure 12: LCMS Chromatogram of crude extract LI 2-3

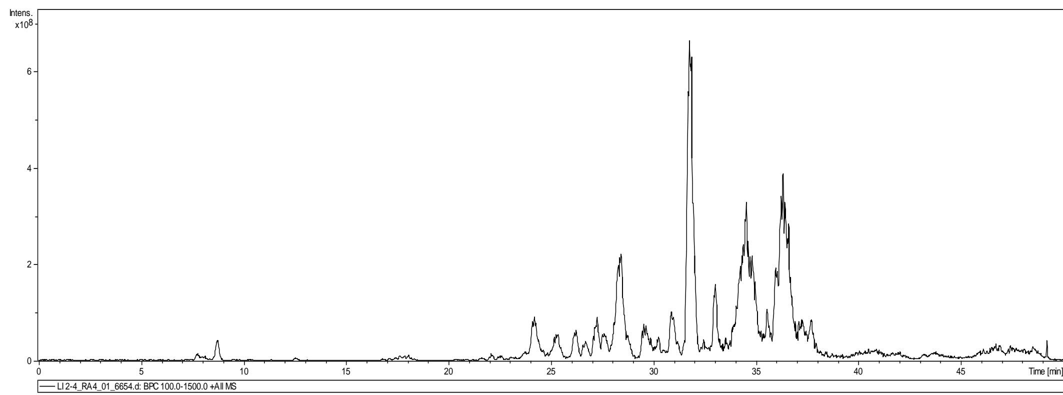


Figure 13: LCMS Chromatogram of crude extract LI 2-4

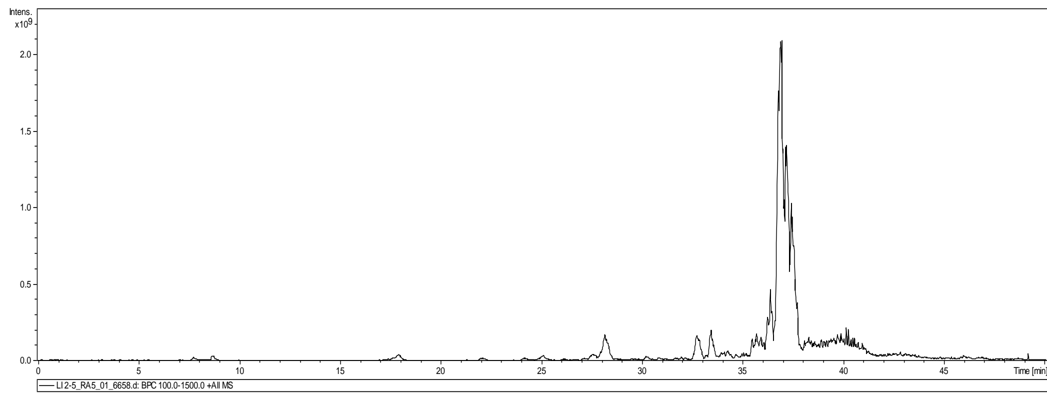


Figure 14: LCMS Chromatogram of crude extract LI 2-5

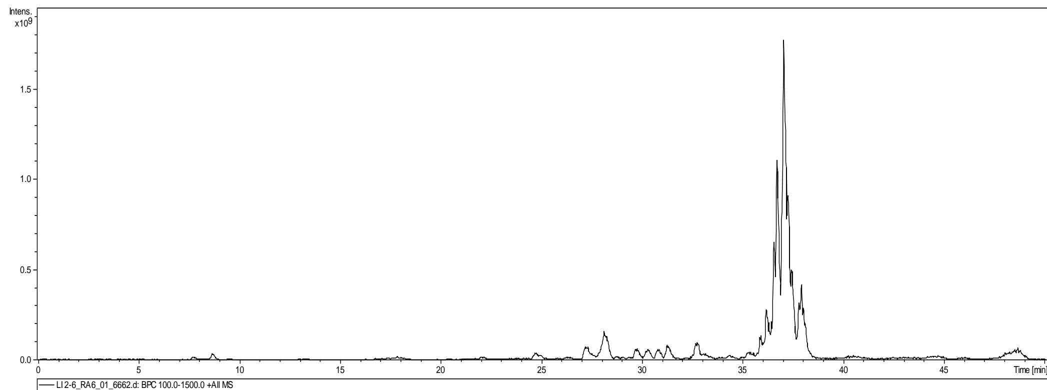


Figure 15: LCMS Chromatogram of crude extract LI 2-6

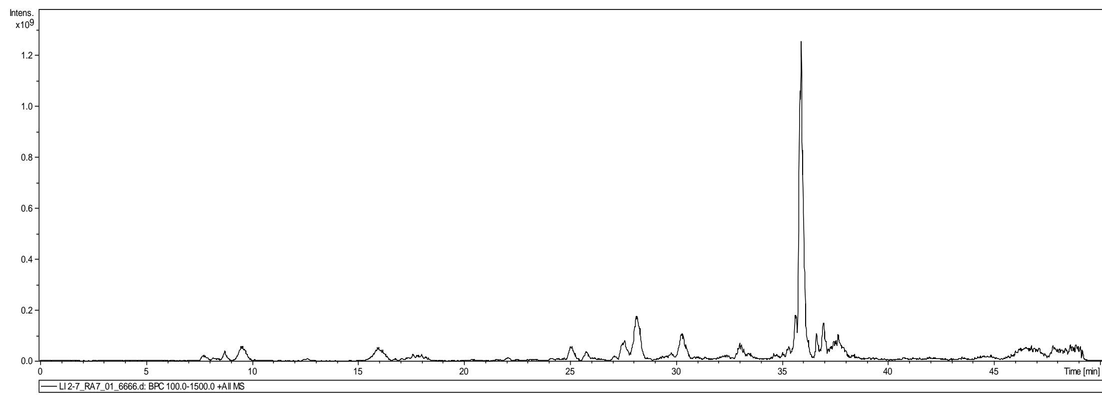


Figure 16: LCMS Chromatogram of crude extract LI 2-7

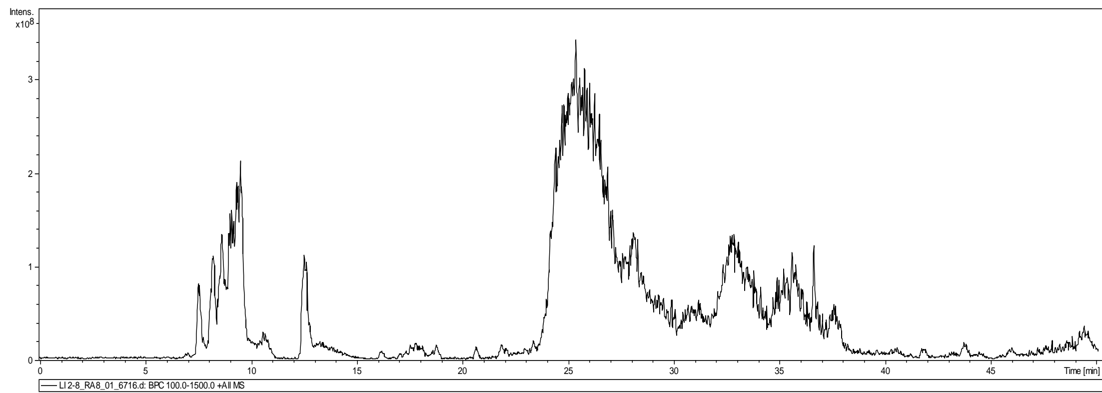


Figure 17: LCMS Chromatogram of crude extract LI 2-8

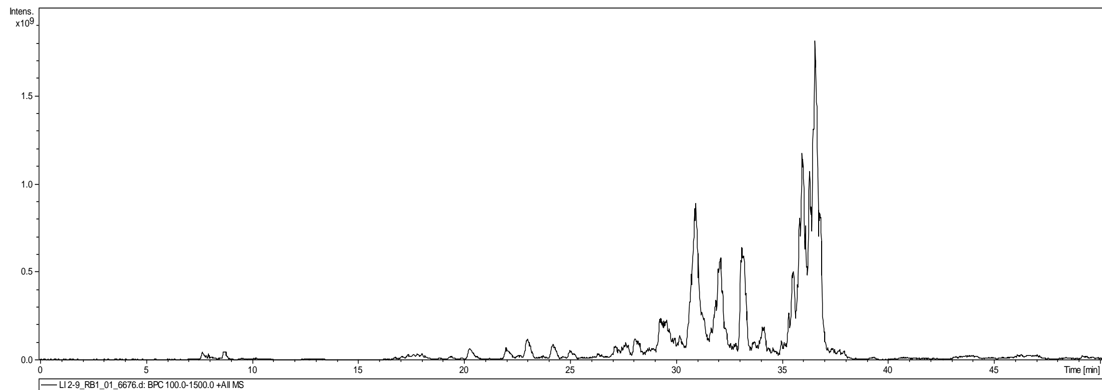


Figure 18: LCMS Chromatogram of crude extract LI 2-9

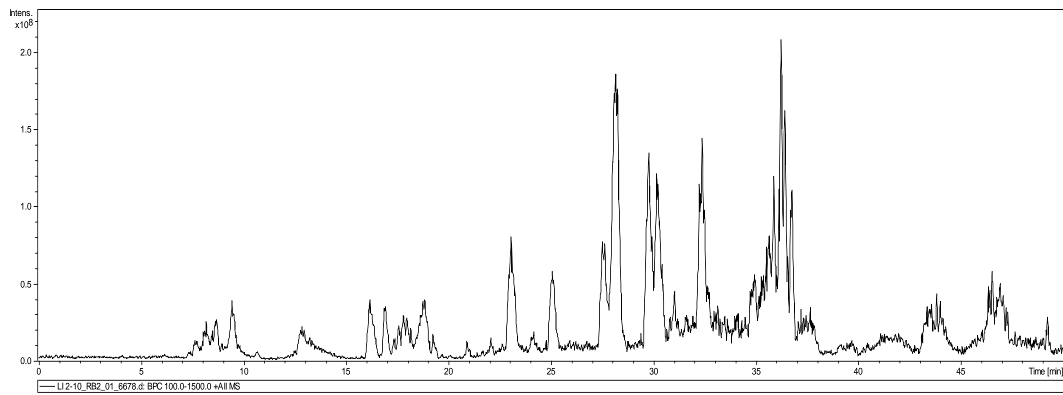


Figure 19: LCMS Chromatogram of crude extract LI 2-10

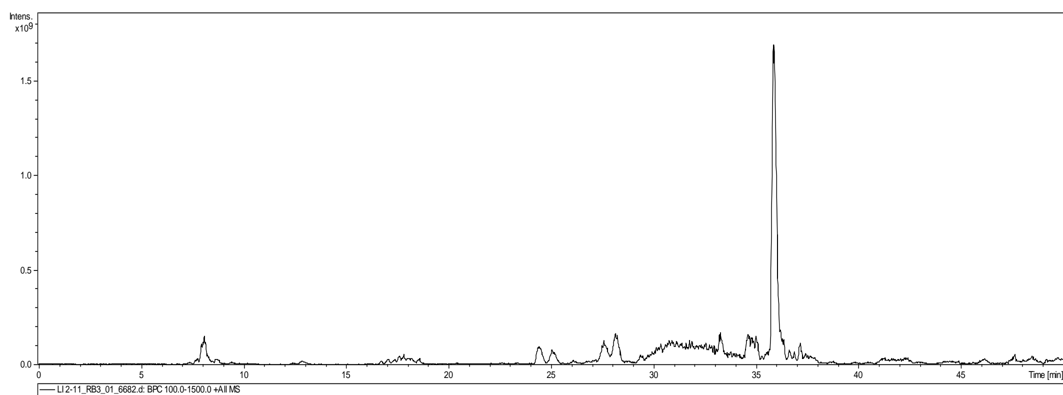


Figure 20: LCMS Chromatogram of crude extract LI 2-11

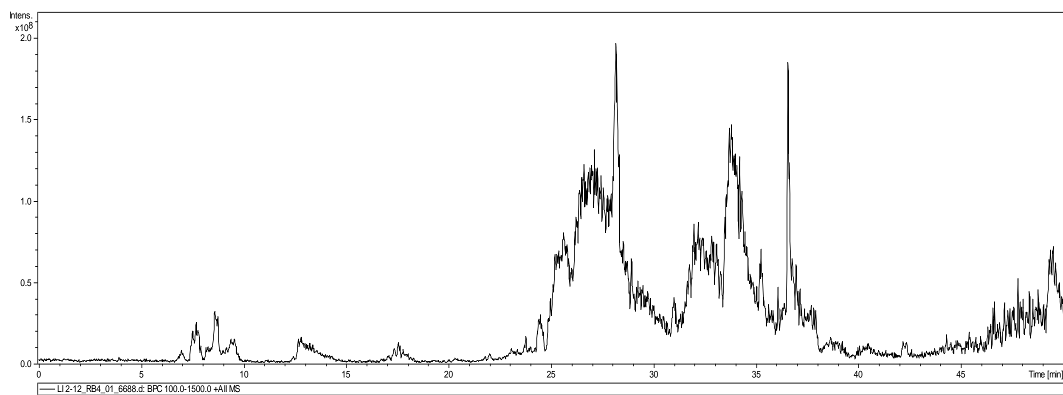


Figure 21: LCMS Chromatogram of crude extract LI 2-12

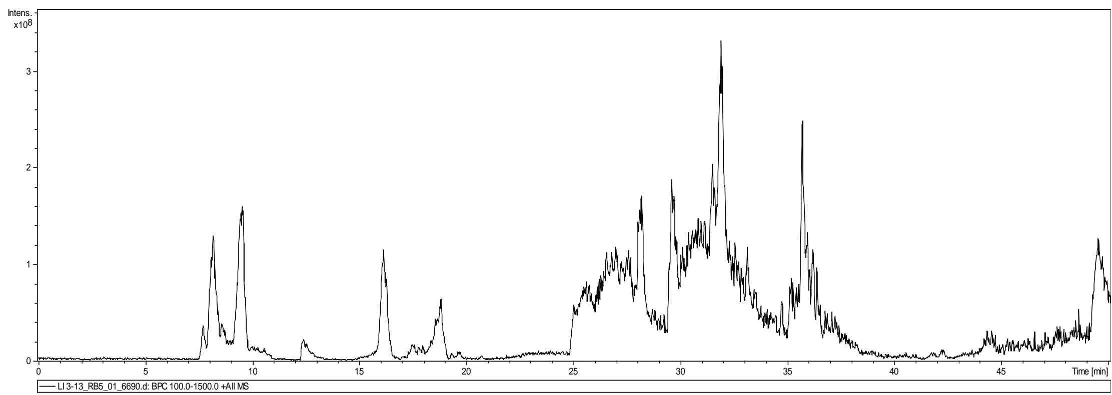


Figure 22: LCMS Chromatogram of crude extract LI 3-13

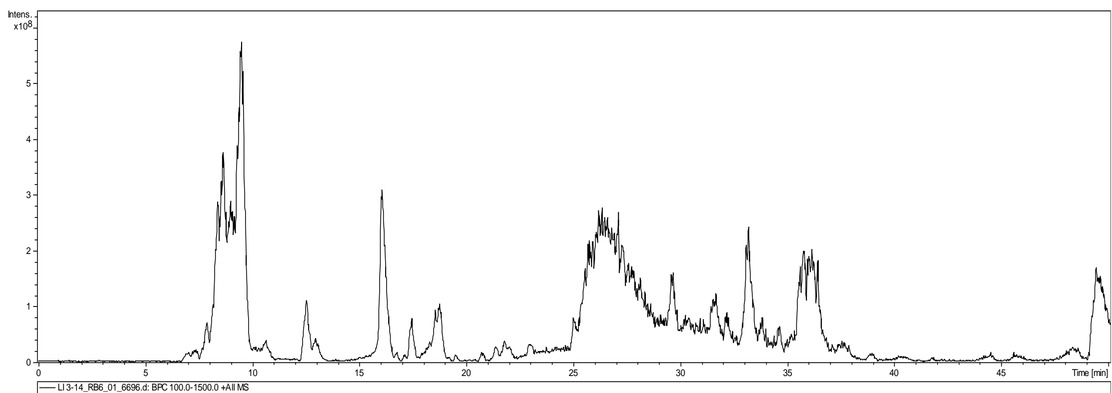


Figure 23: LCMS Chromatogram of crude extract LI 3-14

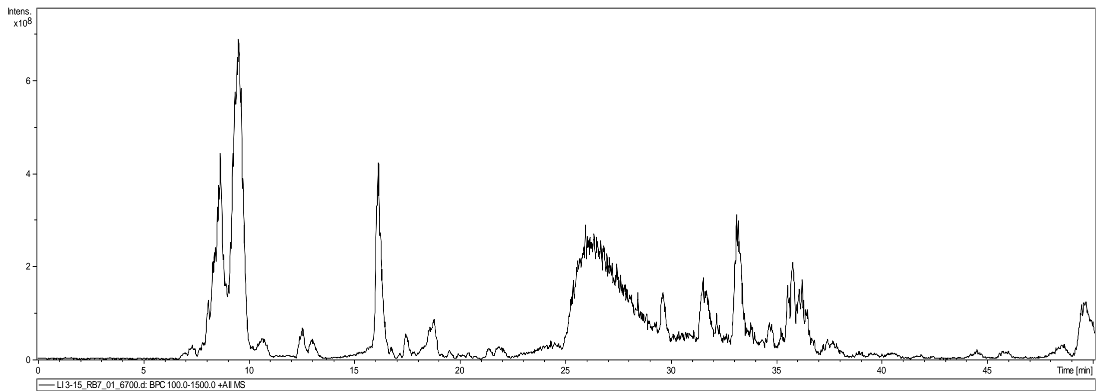


Figure 24: LCMS Chromatogram of crude extract LI 3-15

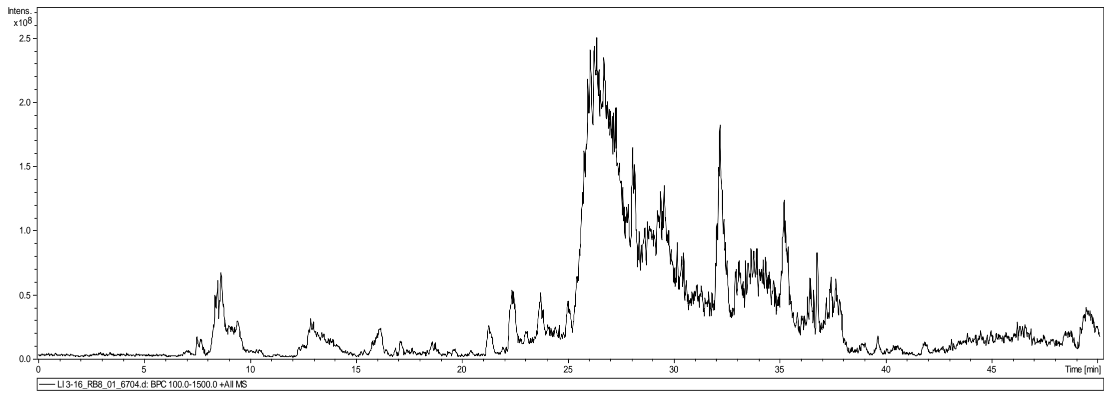


Figure 25: LCMS Chromatogram of crude extract LI 3-16

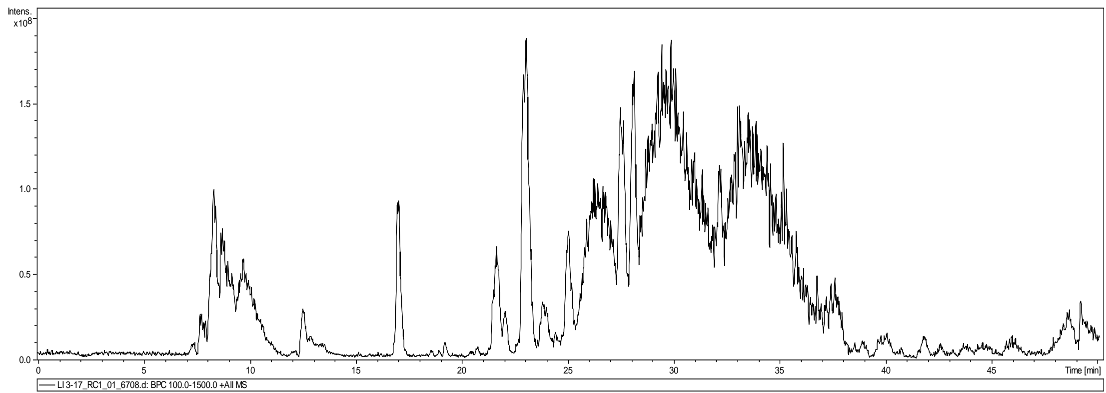


Figure 26: LCMS Chromatogram of crude extract LI 3-17

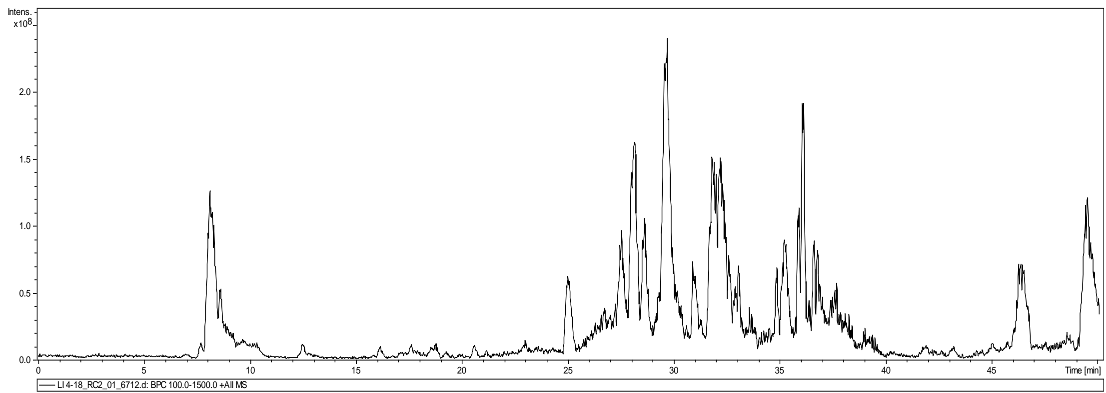


Figure 27: LCMS Chromatogram of crude extract LI 4-18

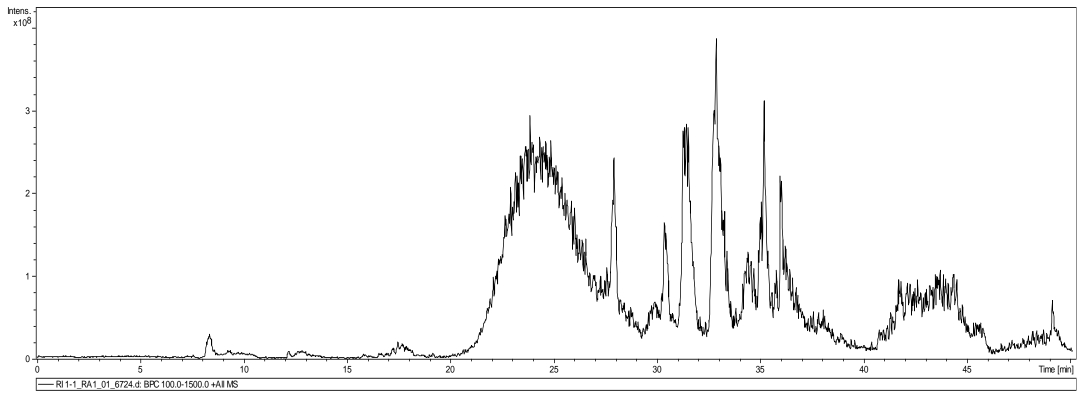


Figure 28: LCMS Chromatogram of crude extract RI 1-1

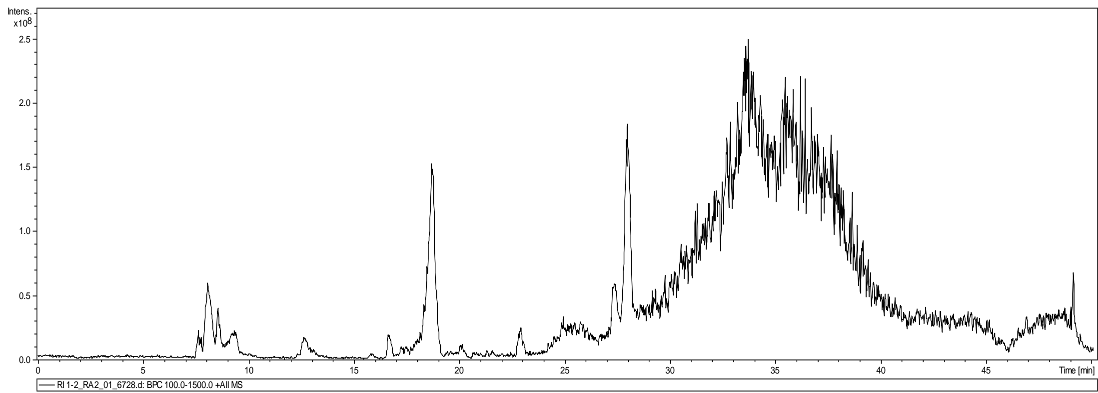


Figure 29: LCMS Chromatogram of crude extract RI 1-2

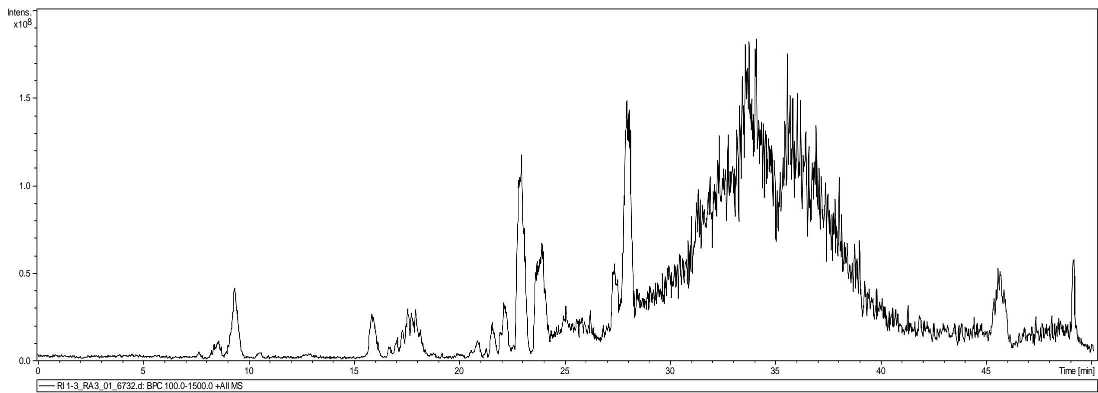


Figure 30: LCMS Chromatogram of crude extract RI 1-3

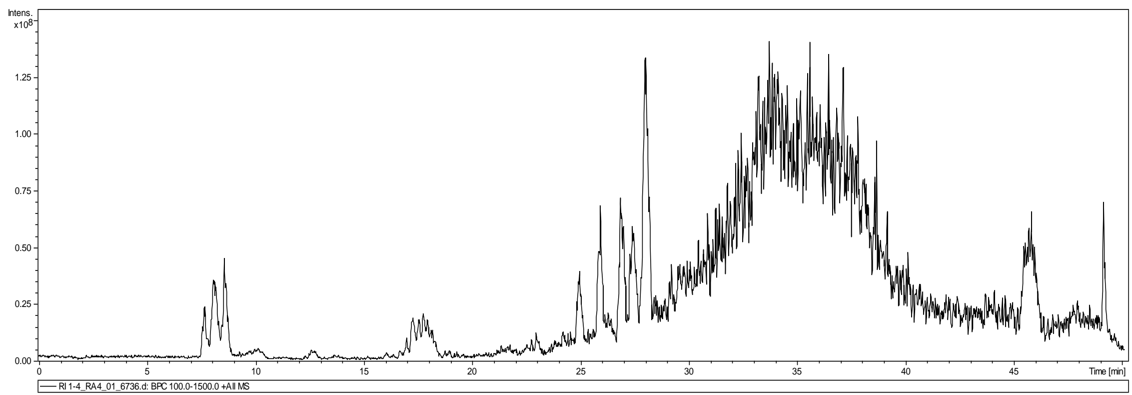


Figure 31: LCMS Chromatogram of crude extract RI 1-4

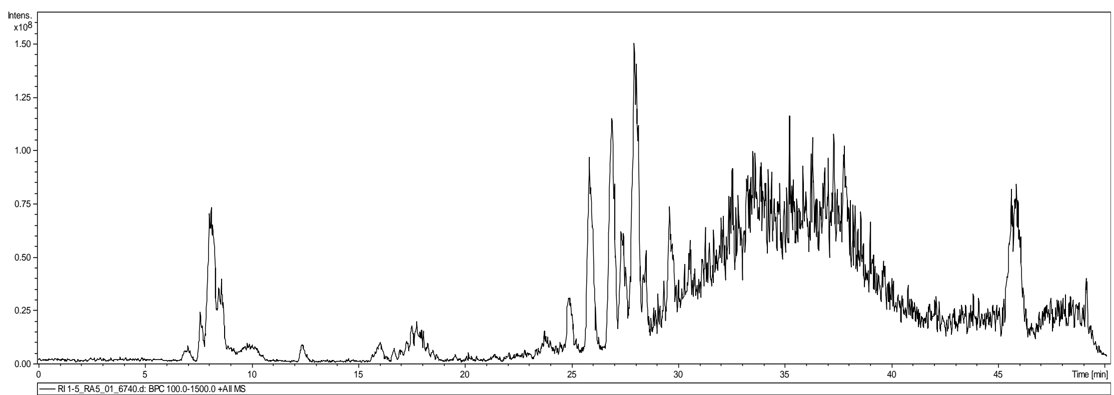


Figure 32: LCMS Chromatogram of crude extract RI 1-5

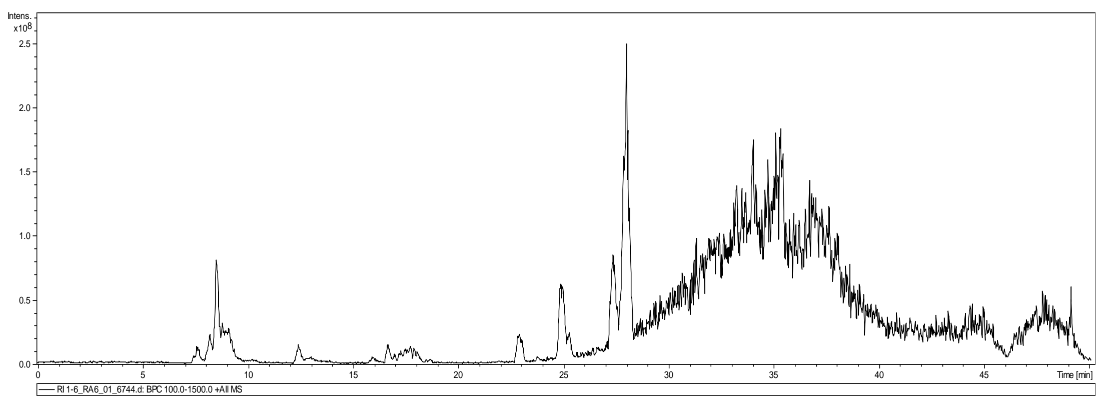


Figure 33: LCMS Chromatogram of crude extract RI 1-6

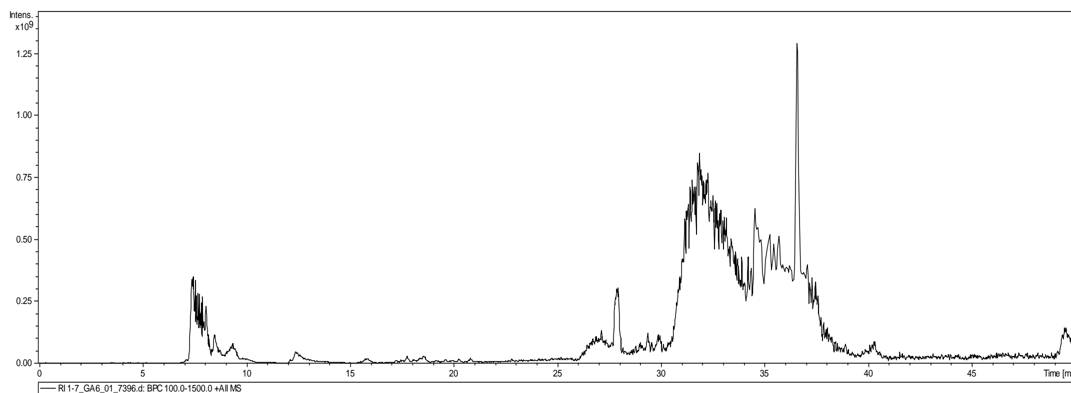


Figure 34: LCMS Chromatogram of crude extract RI 1-7

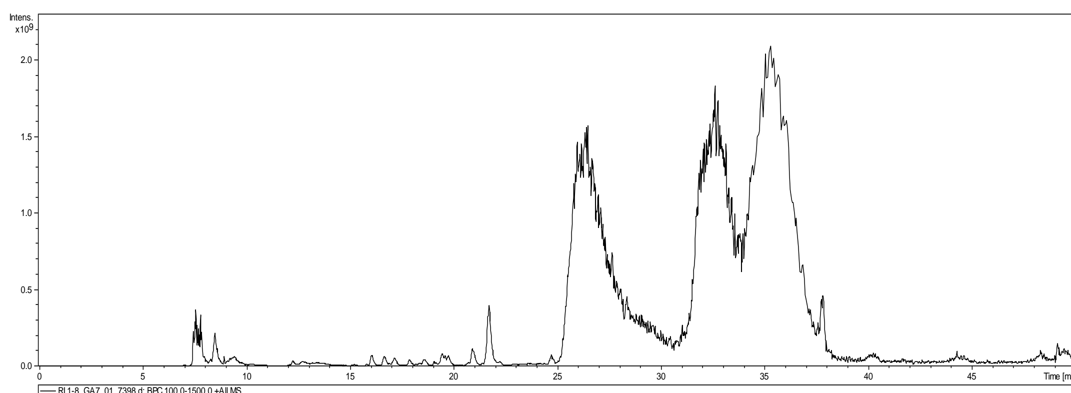


Figure 35: LCMS Chromatogram of crude extract RI 1-8

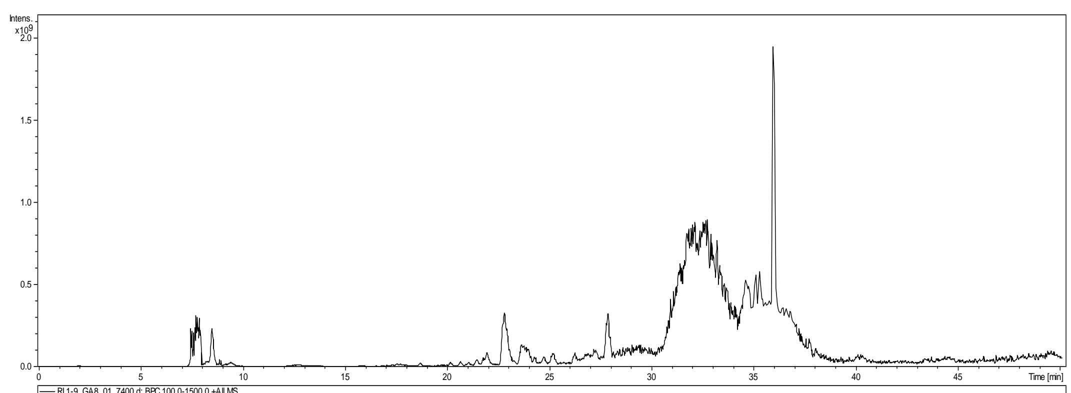


Figure 36: LCMS Chromatogram of crude extract RI 1-9

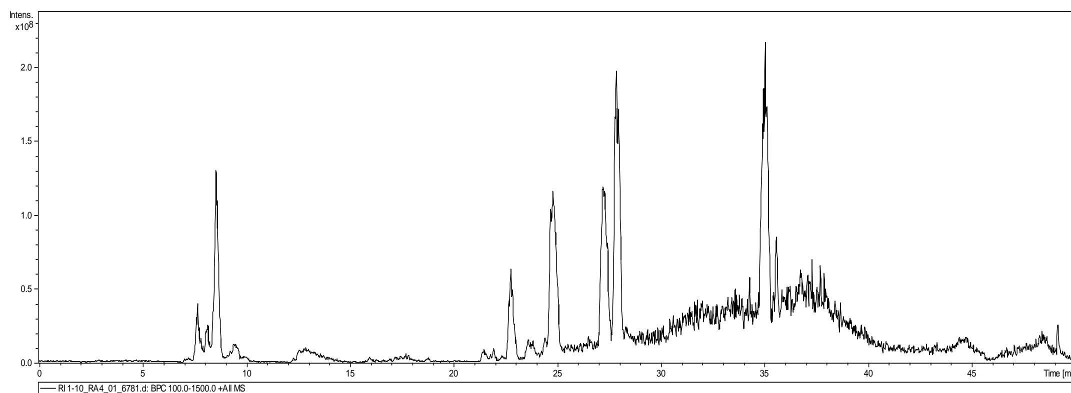


Figure 37: LCMS Chromatogram of crude extract RI 1-10

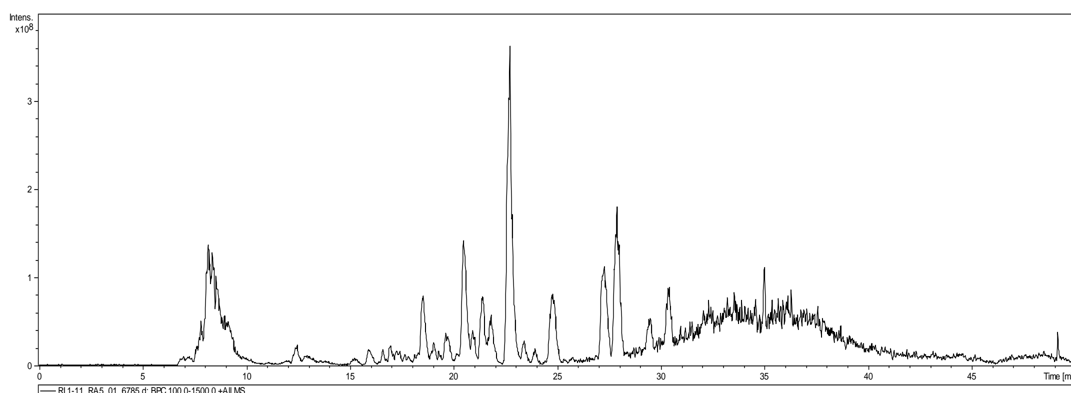


Figure 38: LCMS Chromatogram of crude extract RI 1-11

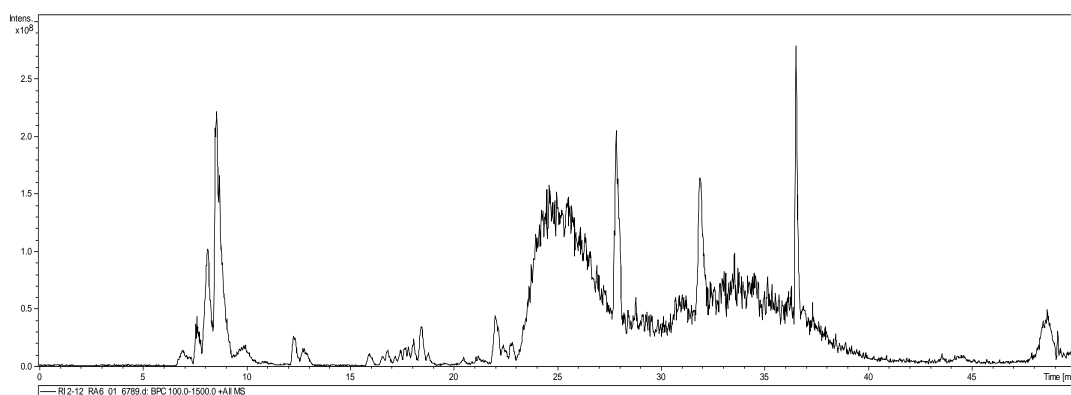


Figure 39: LCMS Chromatogram of crude extract RI 2-12

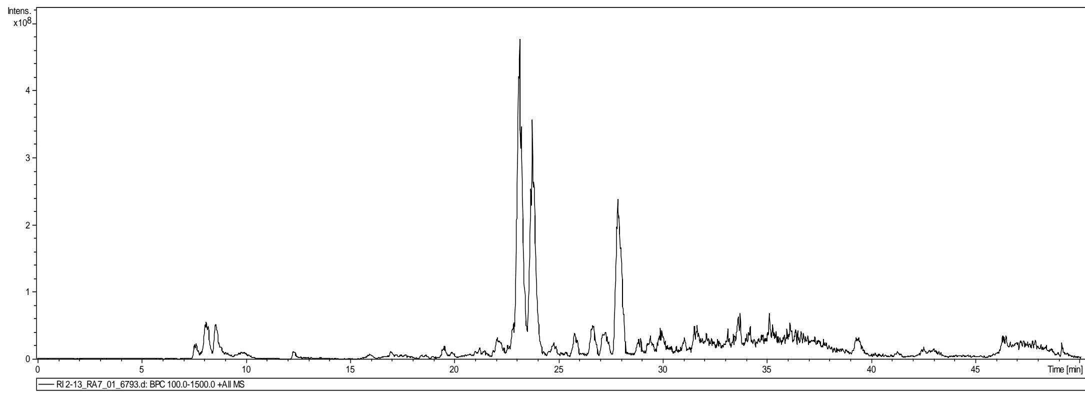


Figure 40: LCMS Chromatogram of crude extract RI 2-13

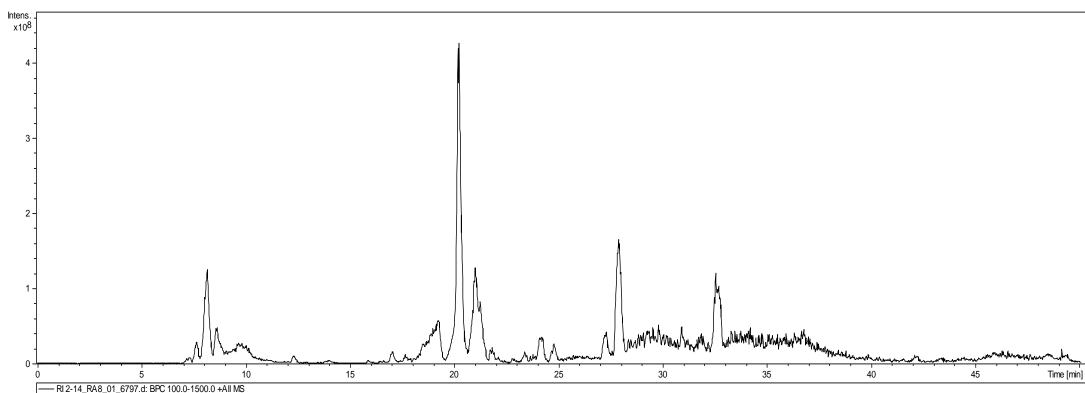


Figure 41: LCMS Chromatogram of crude extract RI 2-14

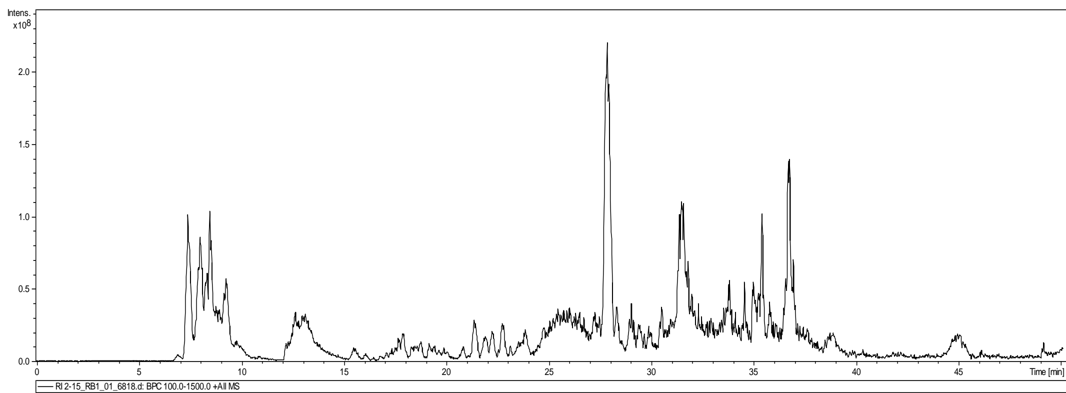


Figure 42: LCMS Chromatogram of crude extract RI 2-15

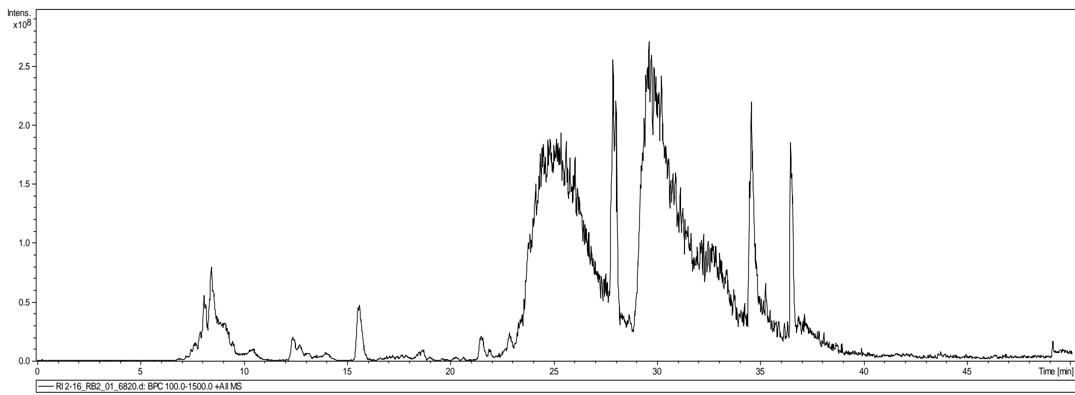


Figure 43: LCMS Chromatogram of crude extract RI 2-16

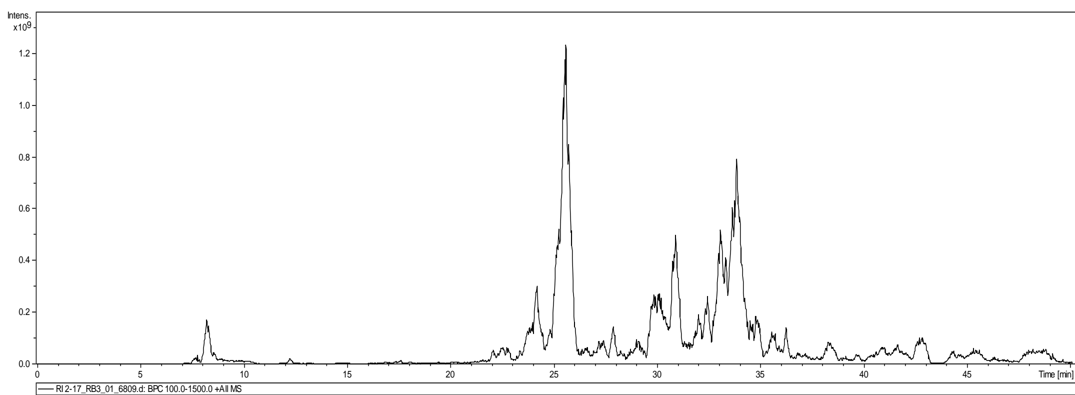


Figure 44: LCMS Chromatogram of crude extract RI 2-17

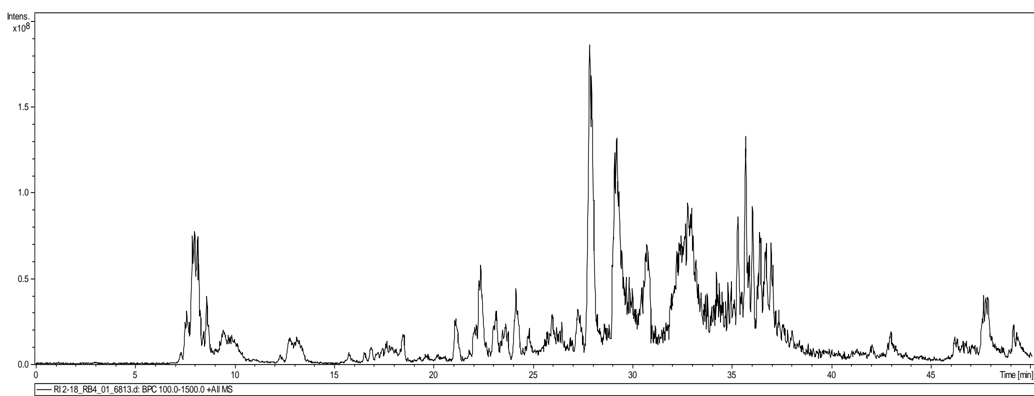


Figure 45: LCMS Chromatogram of crude extract RI 2-18

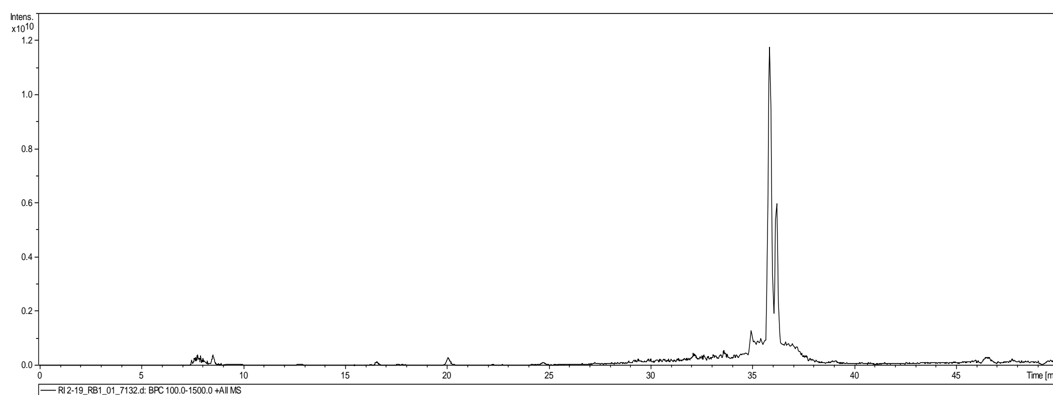


Figure 46: LCMS Chromatogram of crude extract RI 2-19

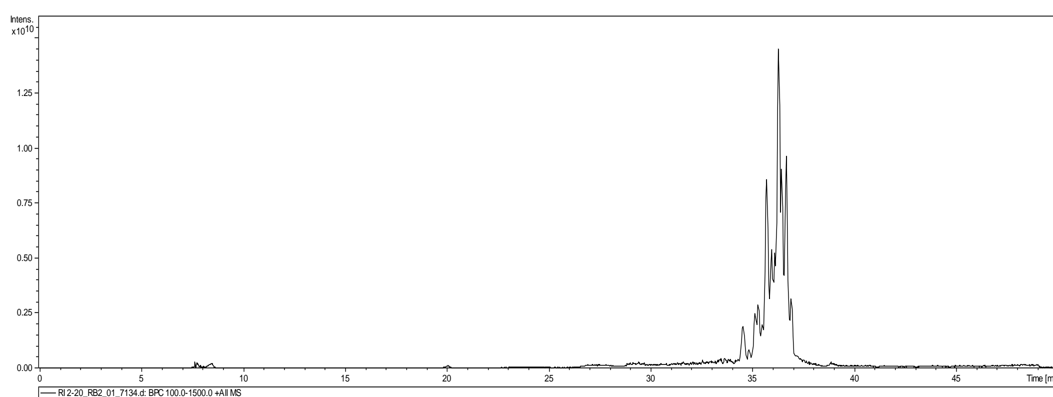


Figure 47: LCMS Chromatogram of crude extract RI 2-20

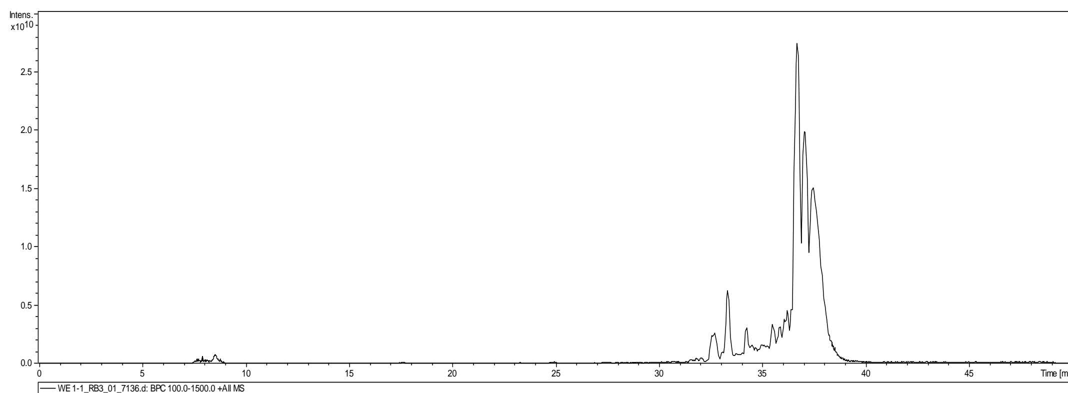


Figure 48: LCMS Chromatogram of crude extract WE 1-1

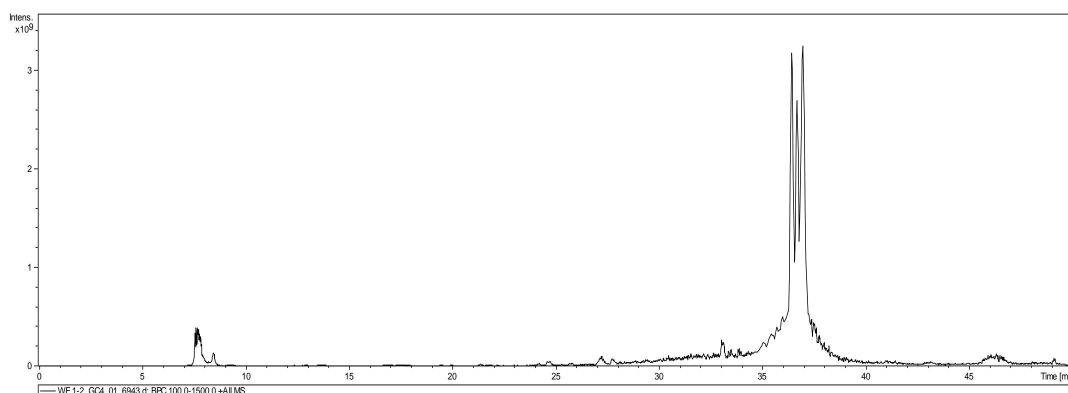


Figure 49: LCMS Chromatogram of crude extract WE 1-2

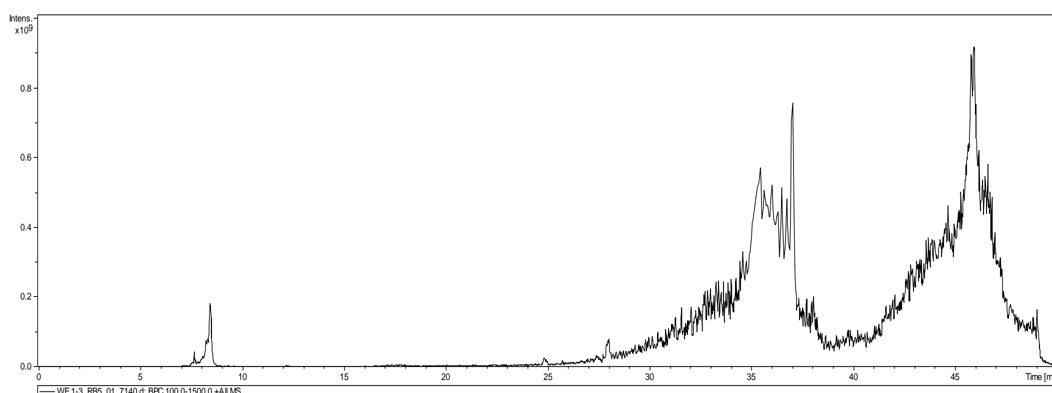


Figure 50: LCMS Chromatogram of crude extract WE 1-3

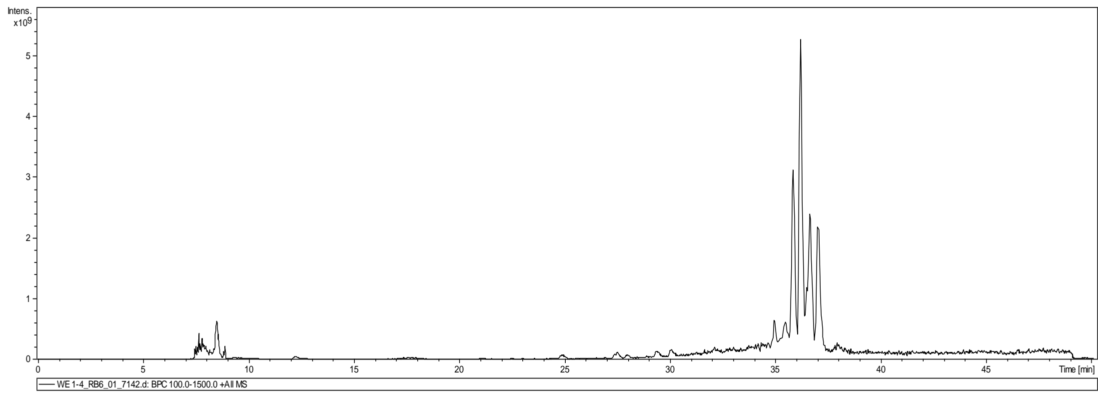


Figure 51: LCMS Chromatogram of crude extract WE 1-4

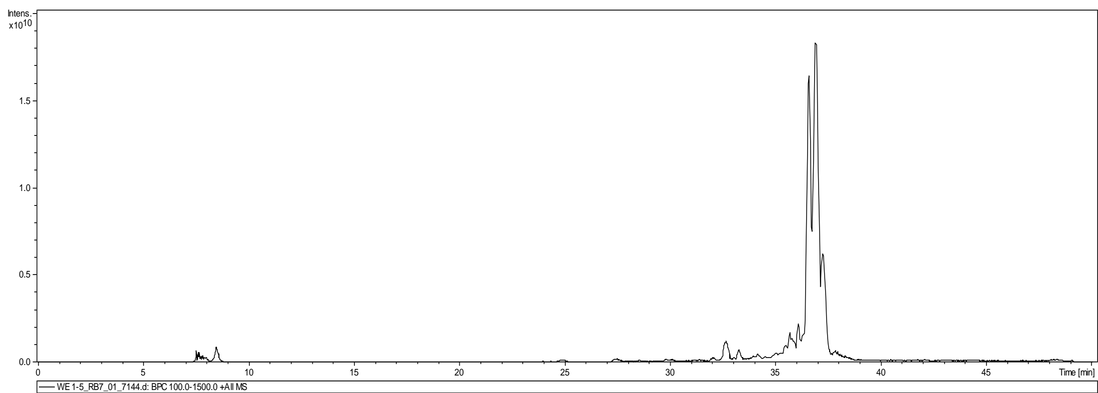


Figure 52: LCMS Chromatogram of crude extract WE 1-5

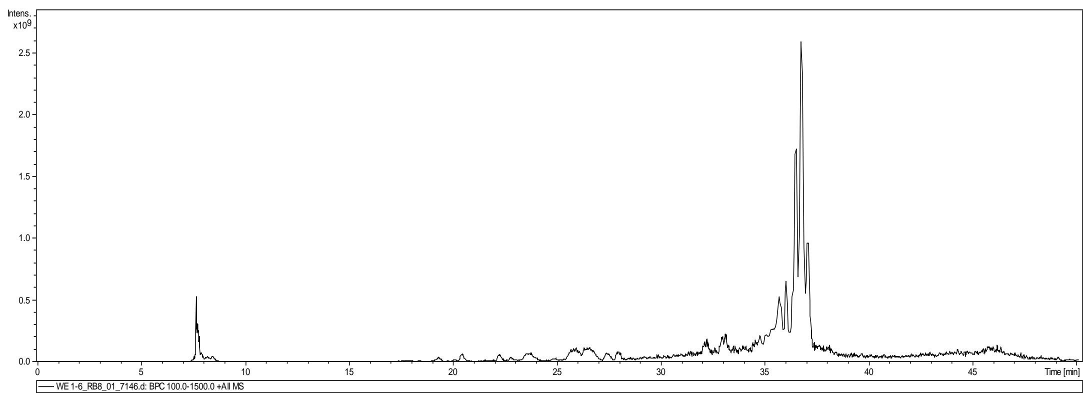


Figure 53: LCMS Chromatogram of crude extract WE 1-6

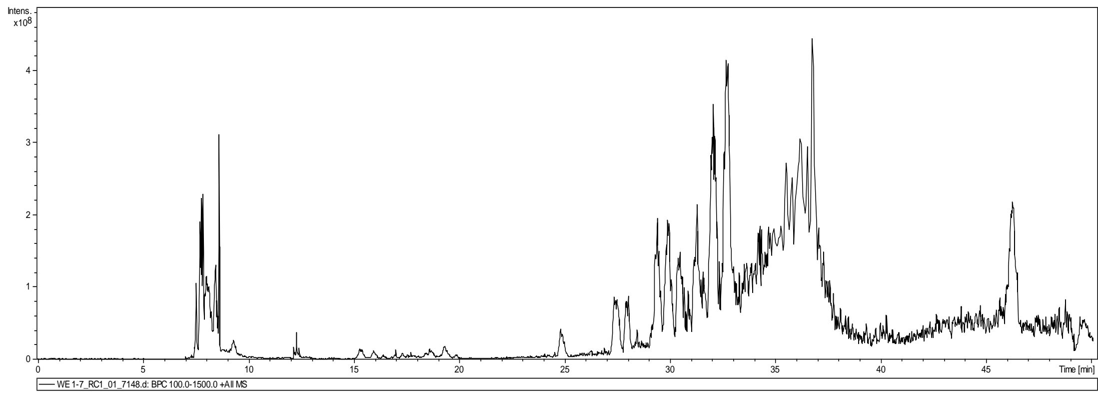


Figure 54: LCMS Chromatogram of crude extract WE 1-7

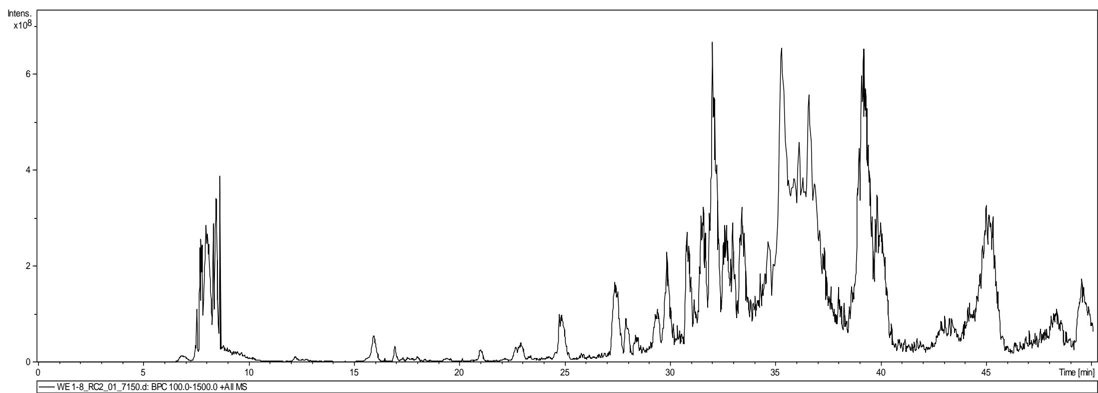


Figure 55: LCMS Chromatogram of crude extract WE 1-8

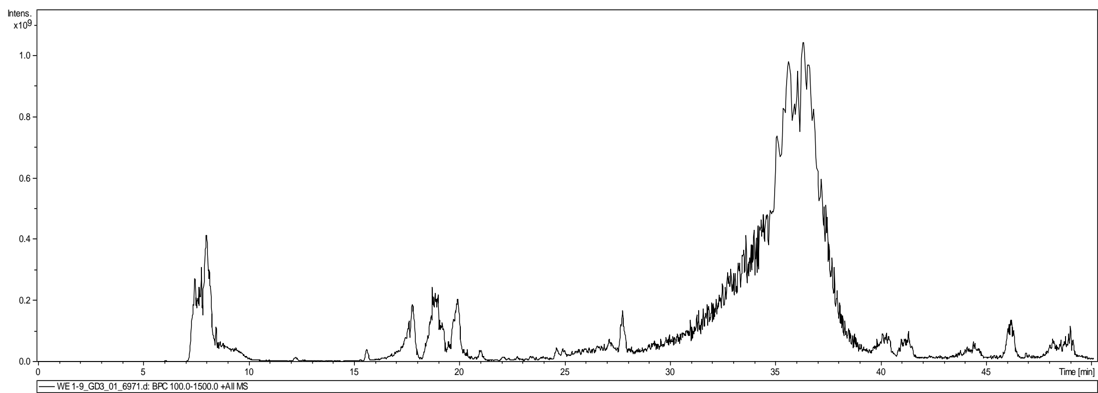


Figure 56: LCMS Chromatogram of crude extract WE 1-9

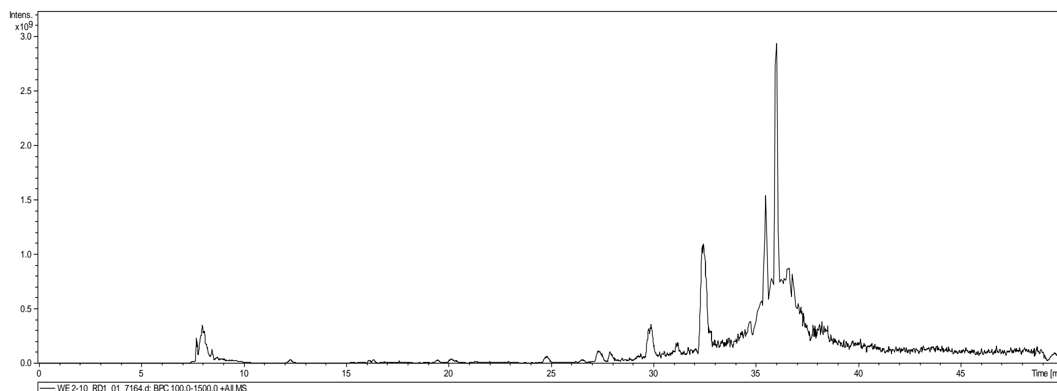


Figure 57: LCMS Chromatogram of crude extract WE 2-10

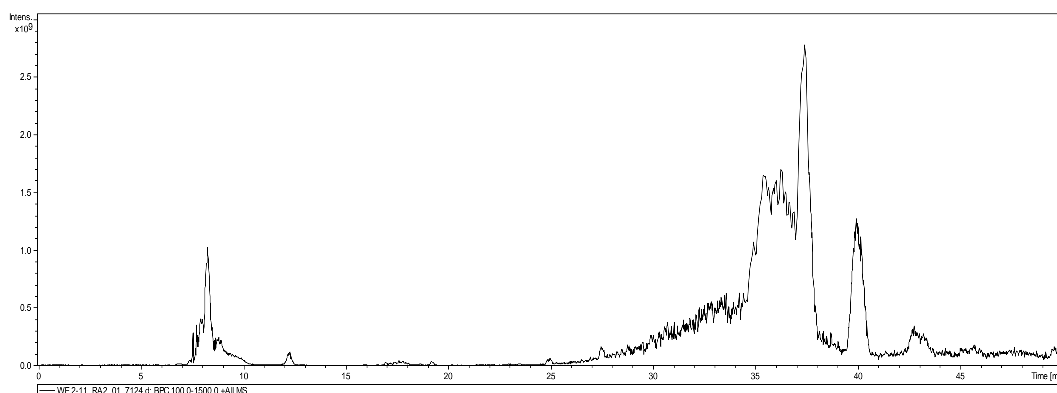


Figure 58: LCMS Chromatogram of crude extract WE 2-11

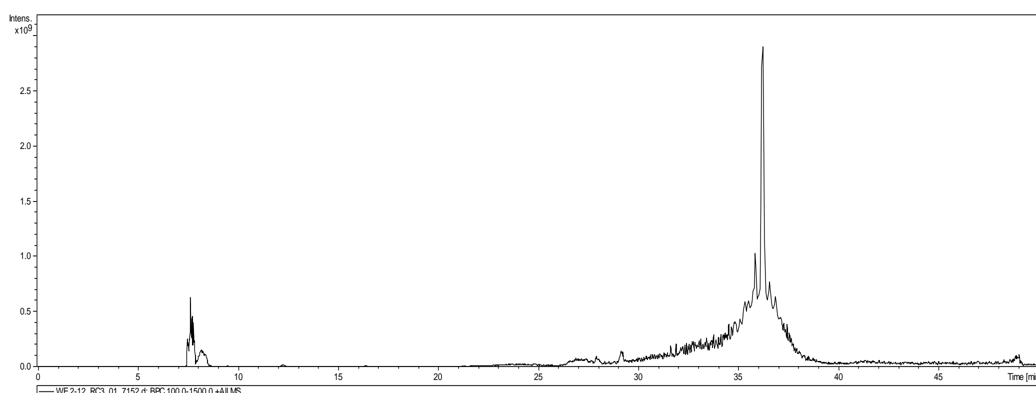


Figure 59: LCMS Chromatogram of crude extract WE 2-12

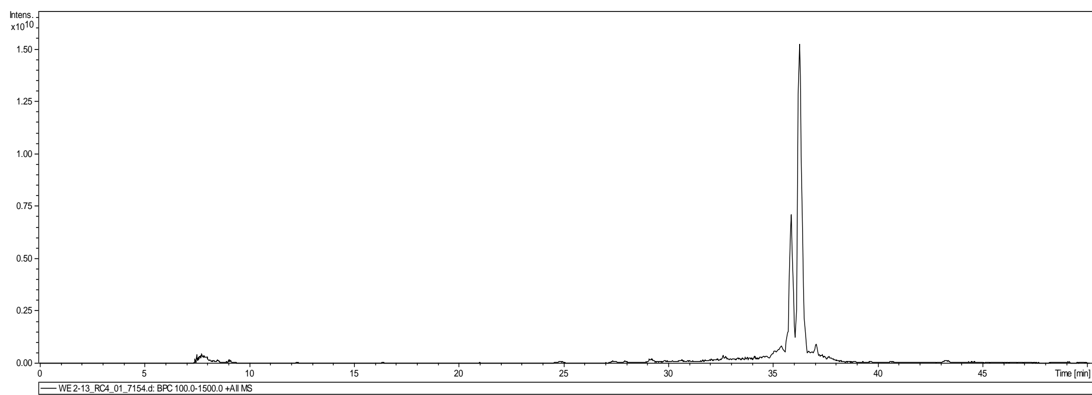


Figure 60: LCMS Chromatogram of crude extract WE 2-13

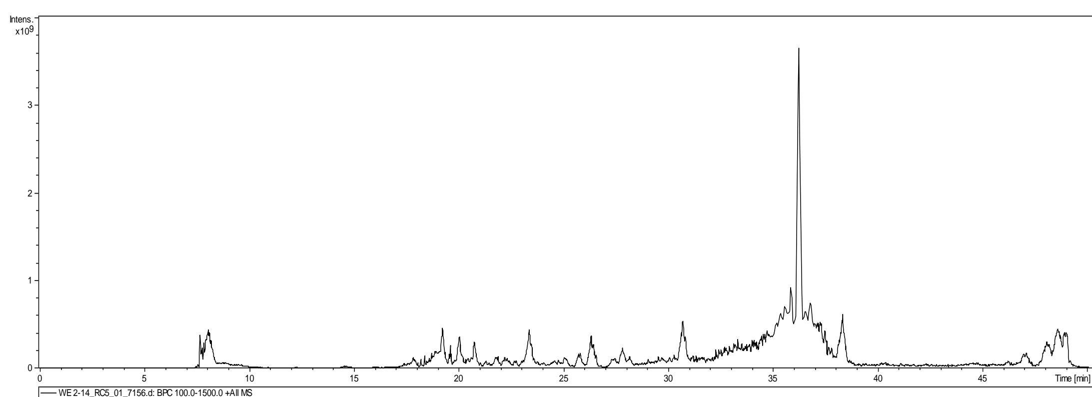


Figure 61: LCMS Chromatogram of crude extract WE 2-14

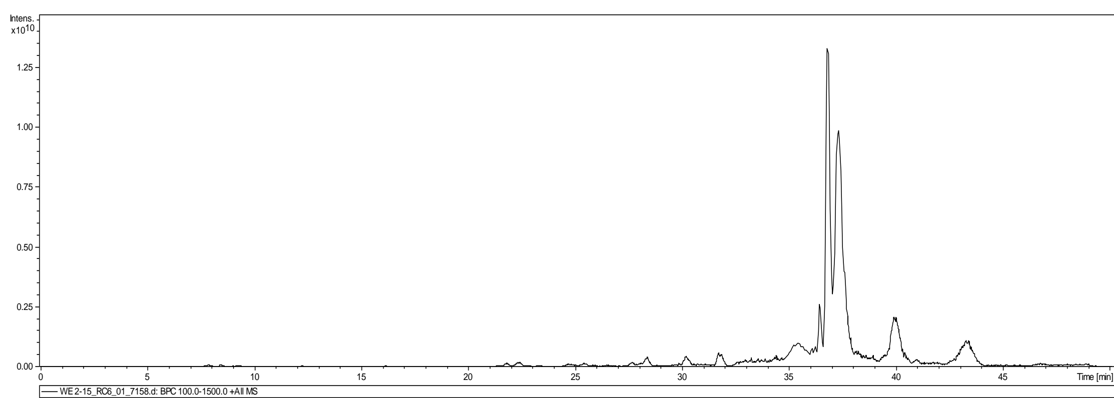


Figure 62: LCMS Chromatogram of crude extract WE 2-15

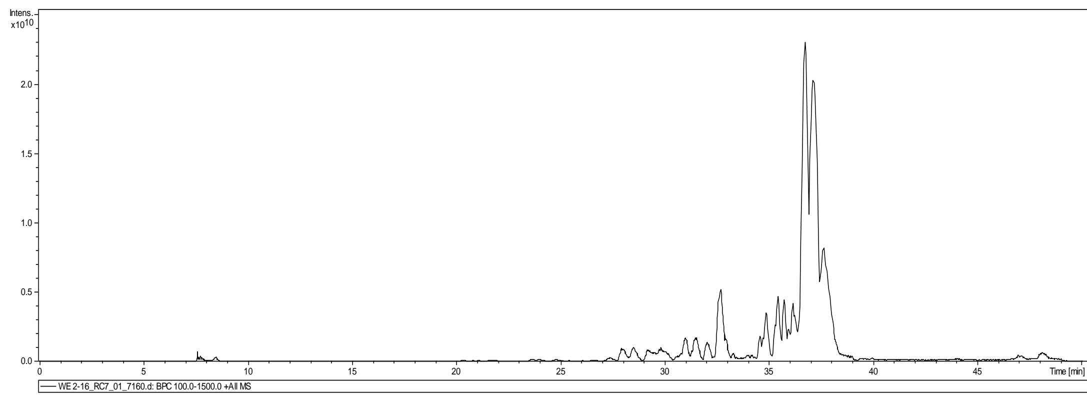


Figure 63: LCMS Chromatogram of crude extract WE 2-16

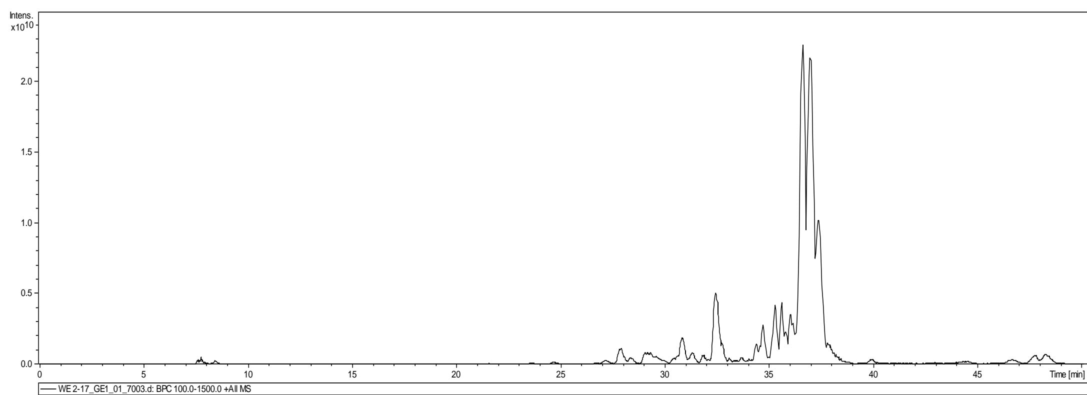


Figure 64: LCMS Chromatogram of crude extract WE 2-17

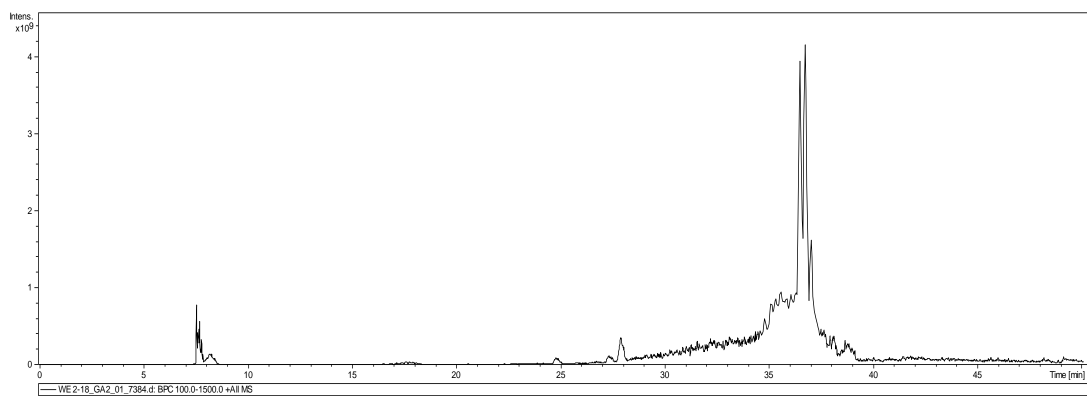


Figure 65: LCMS Chromatogram of crude extract WE 2-18

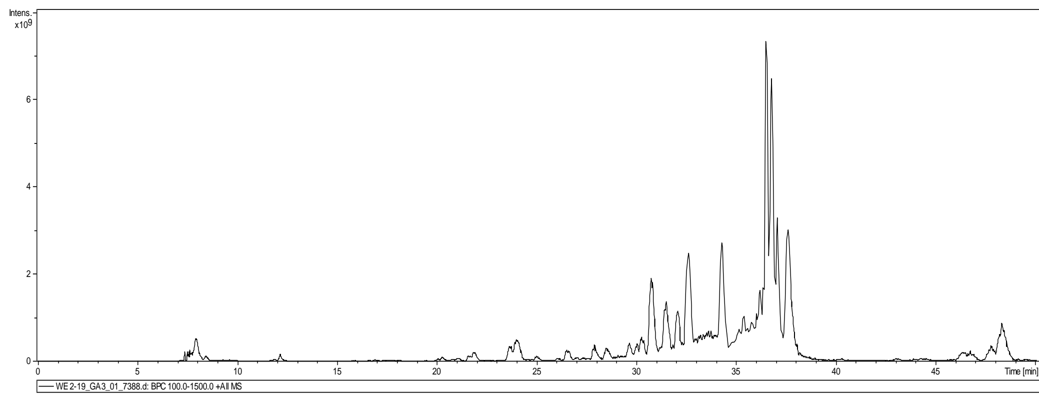


Figure 66: LCMS Chromatogram of crude extract WE 2-19

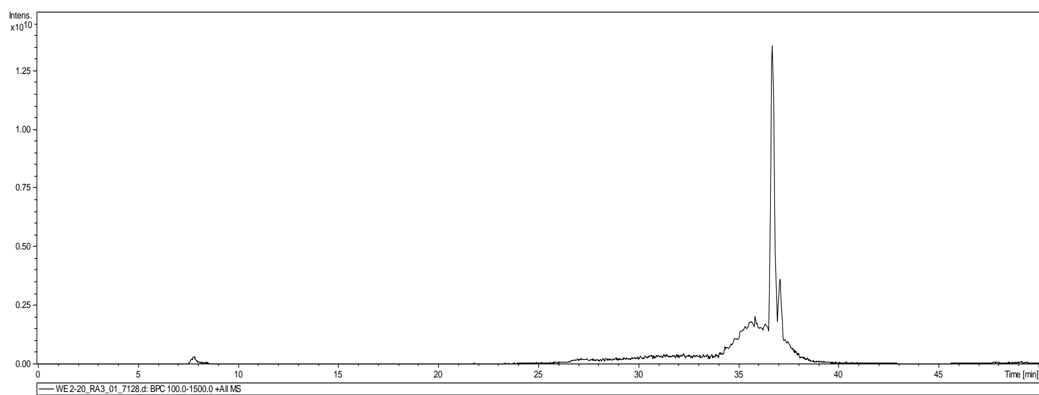


Figure 67: LCMS Chromatogram of crude extract WE 2-20

8.2 Appendix Two: Separation Trees and Diagrams of Marine Species Analysed in Chapters 3-5

Figure 68: Separation tree of *Cliona celata* (RI 1-11)

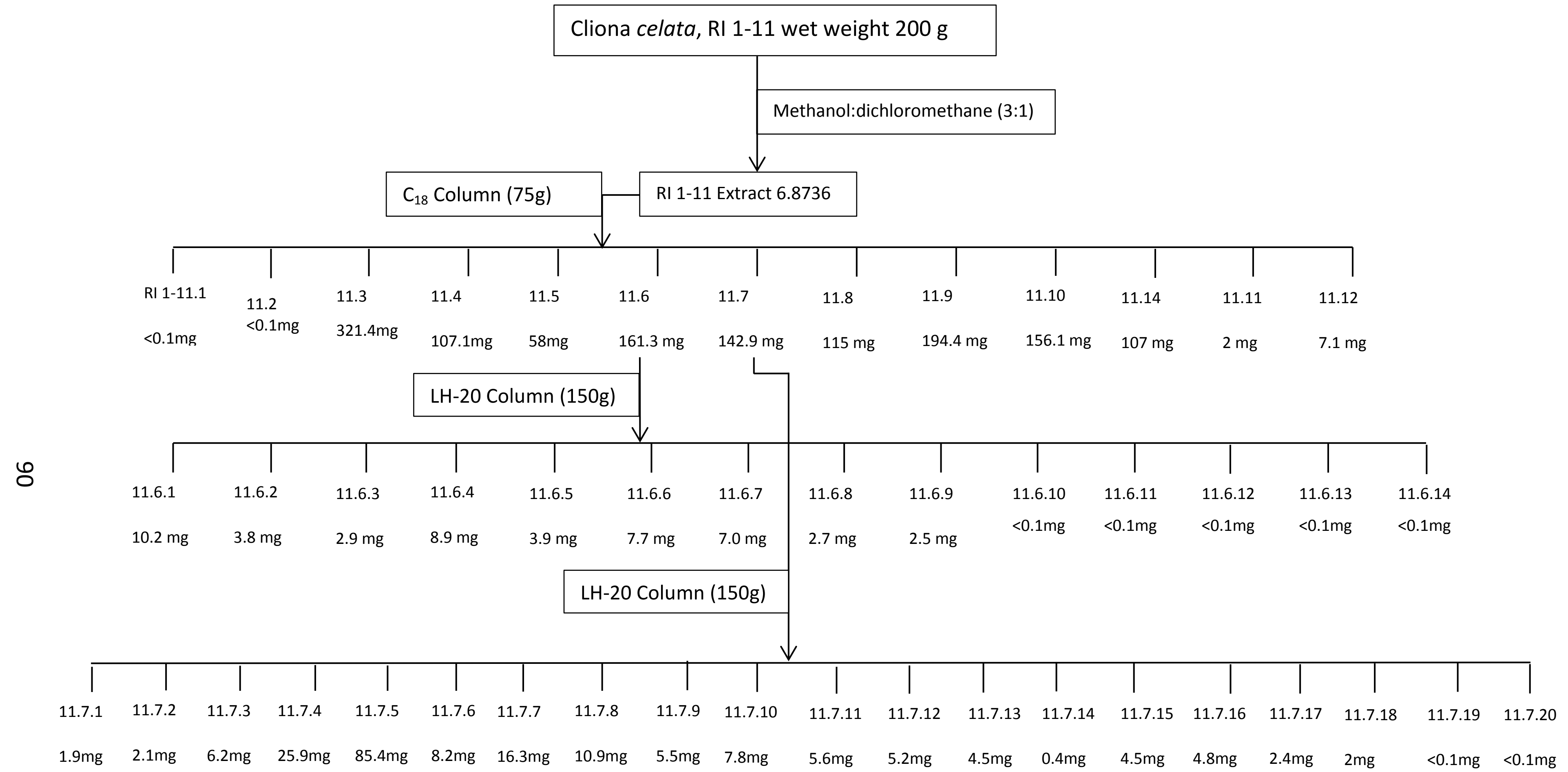
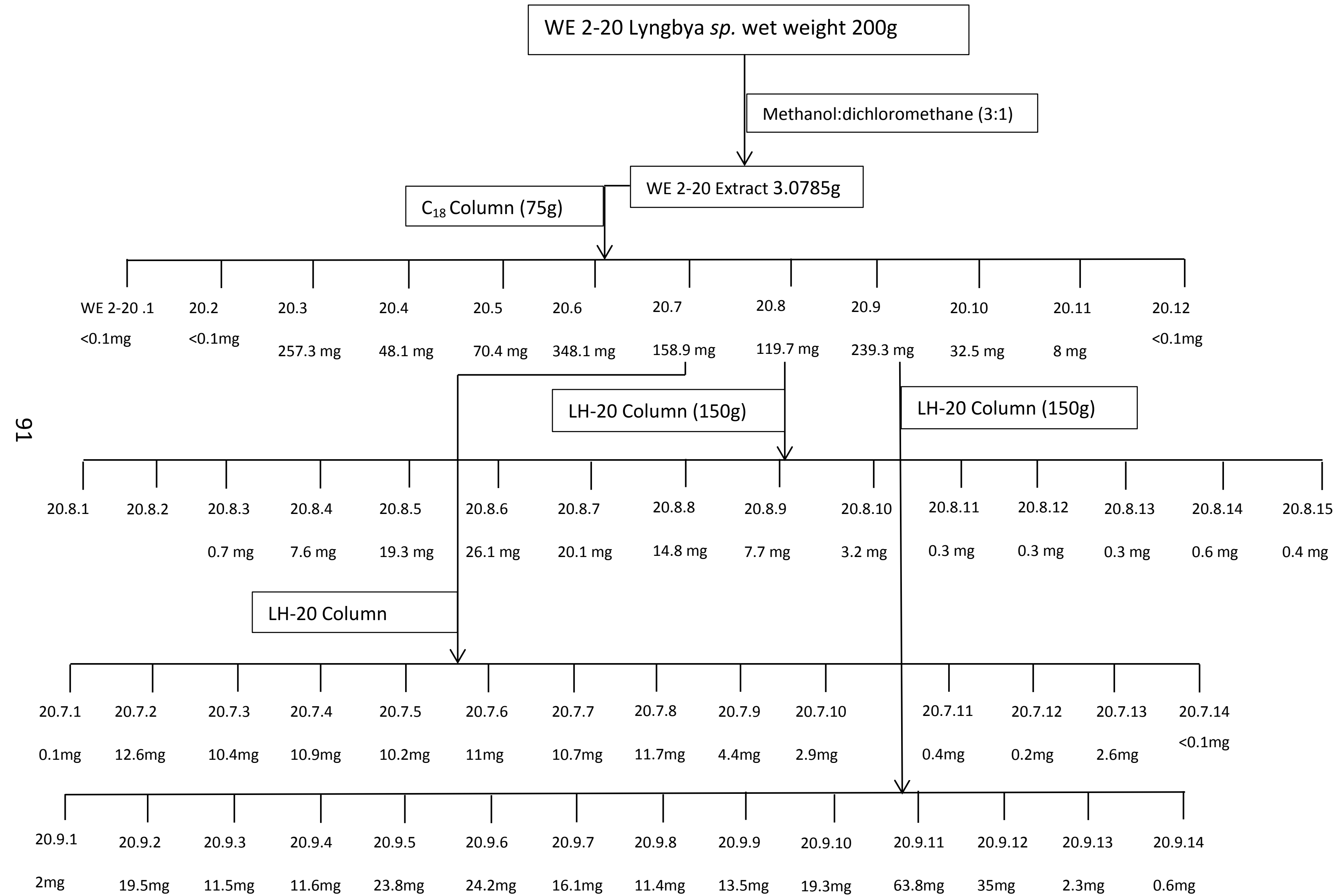


Figure 69: Separation tree of *Lyngbya sp.*(WE 2-20)



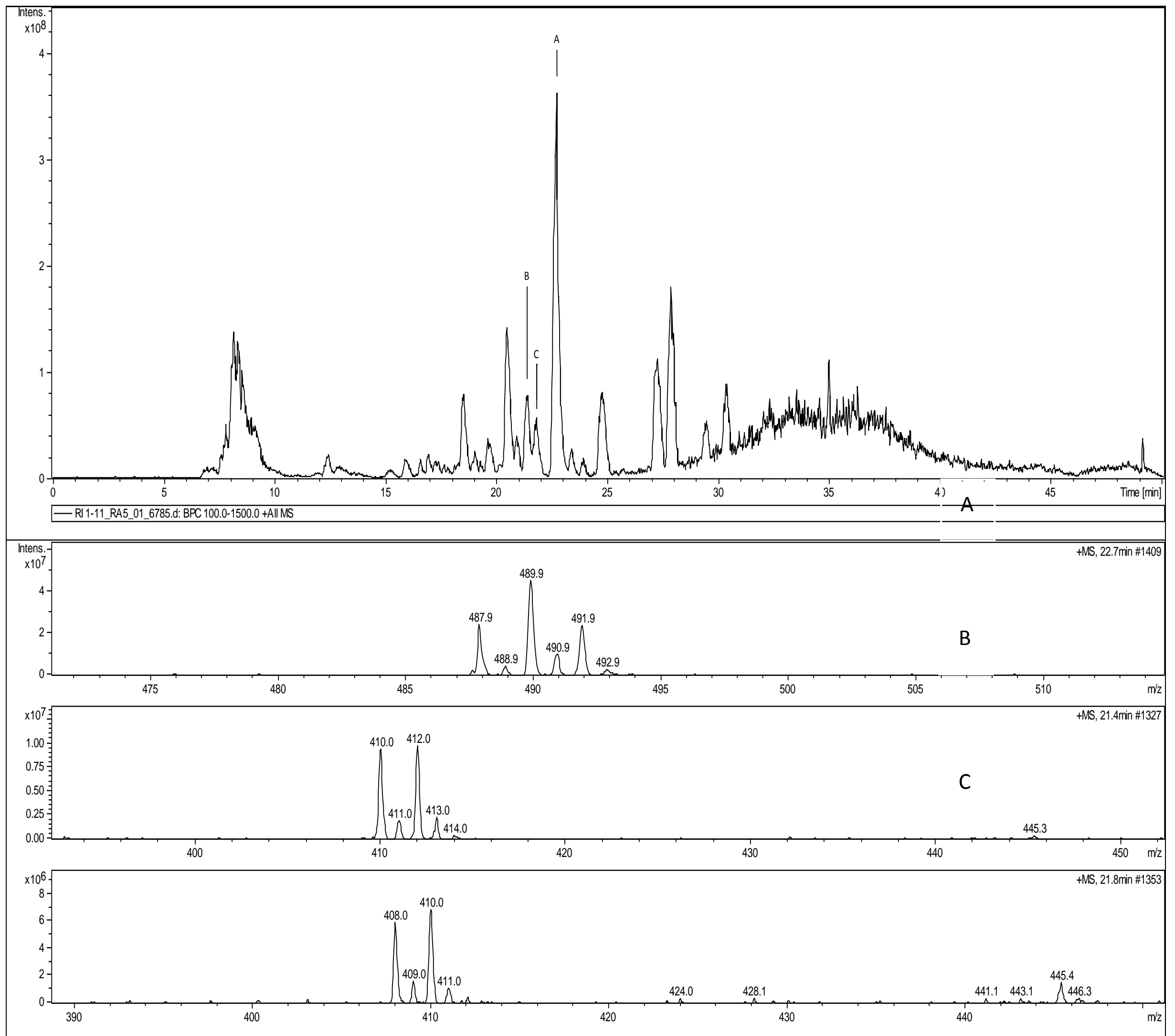


Figure 70: Base peak chromatogram of RI 1-11 *Cliona celata* with mass spectra of metabolites of interest

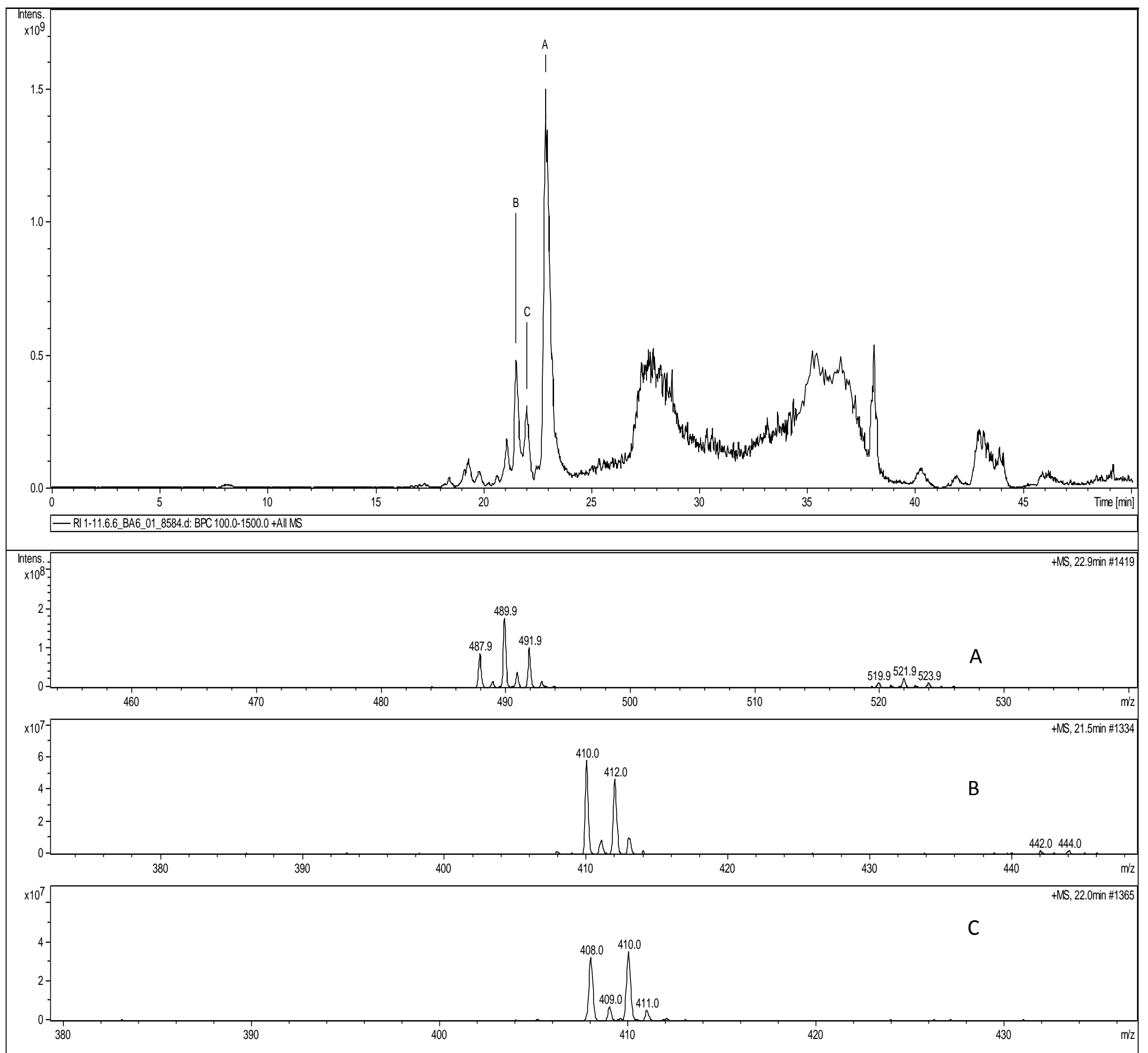


Figure 71: Base peak chromatogram of RI 1-11.6.6 with mass spectra of metabolites of interest

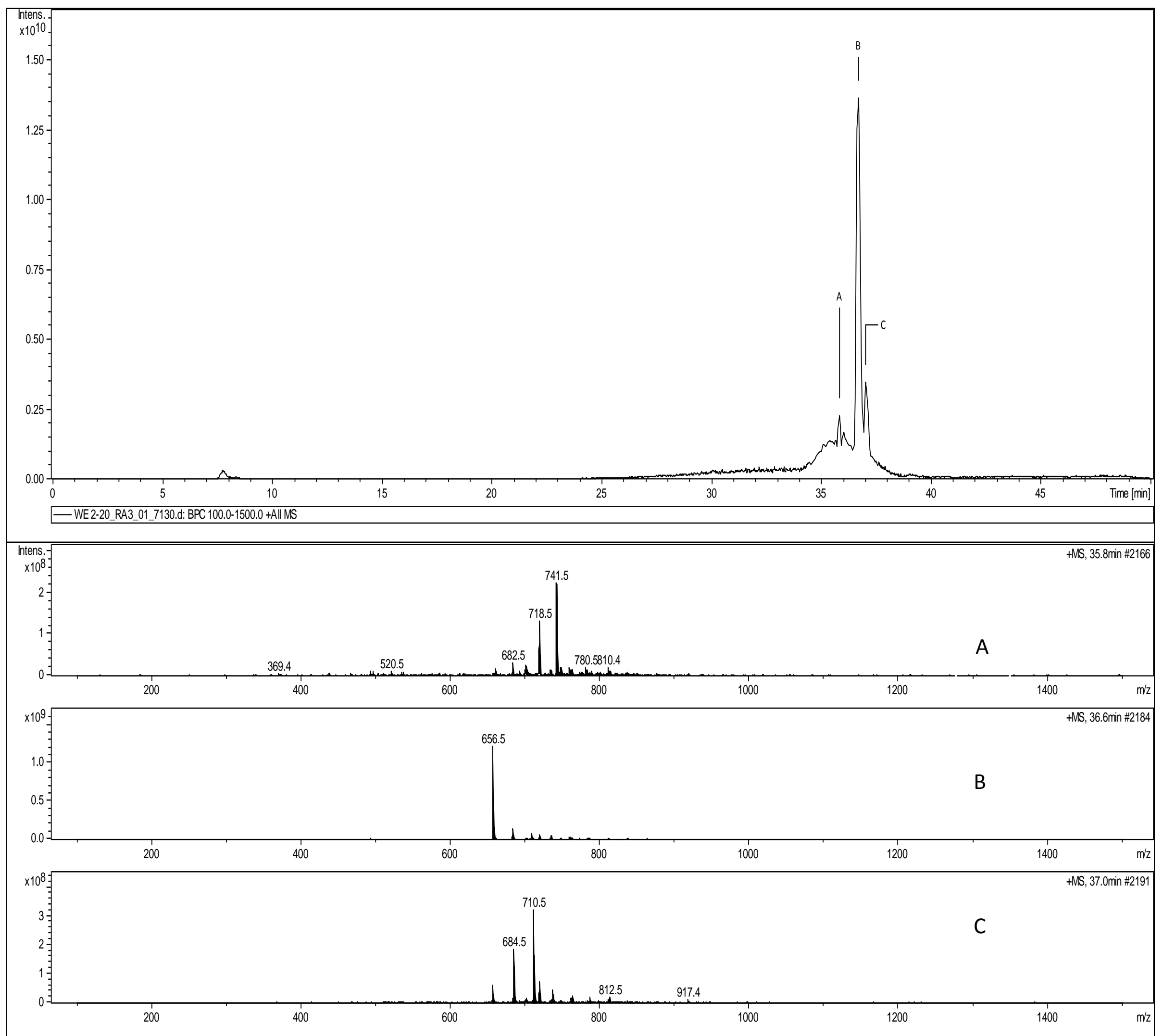


Figure 72: Base peak chromatogram of WE 2-20 *Lyngbya sp.* with mass spectra of metabolites of interest

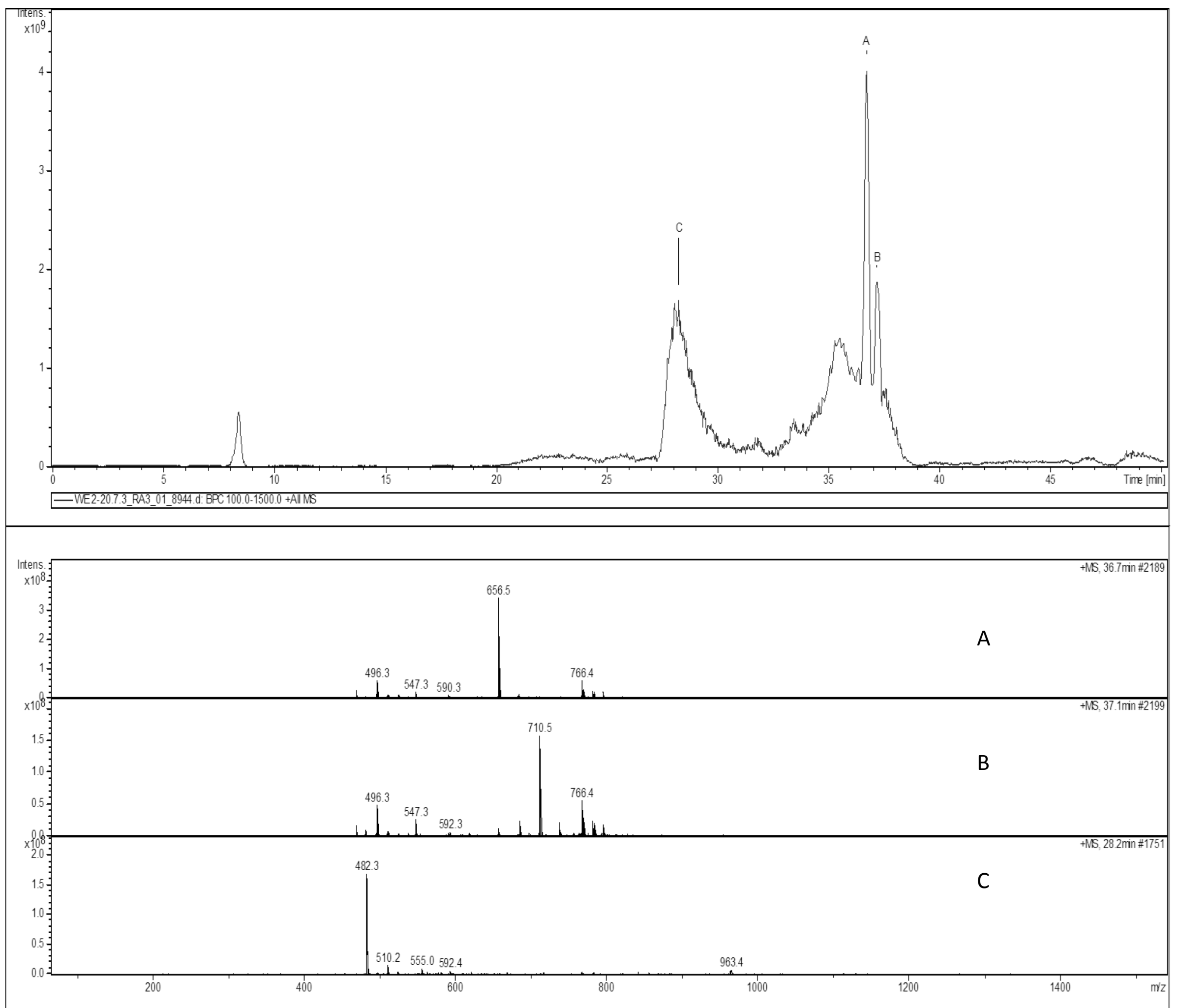


Figure 73: Base peak chromatogram of WE 2-20.7.3 with mass spectra of metabolites of interest

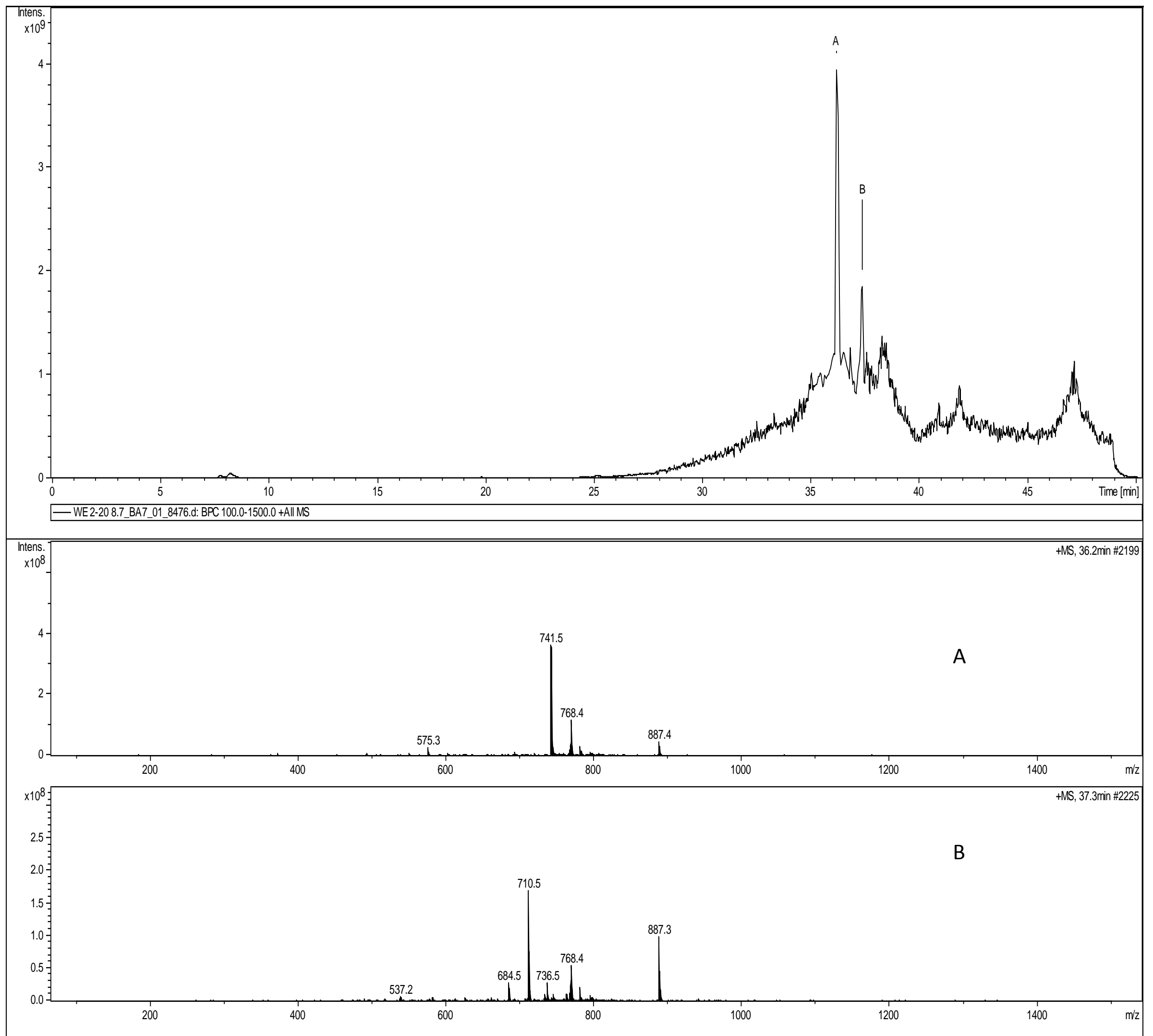


Figure 74: Base peak chromatogram of WE 2-20.8.7 with mass spectra of metabolites of interest

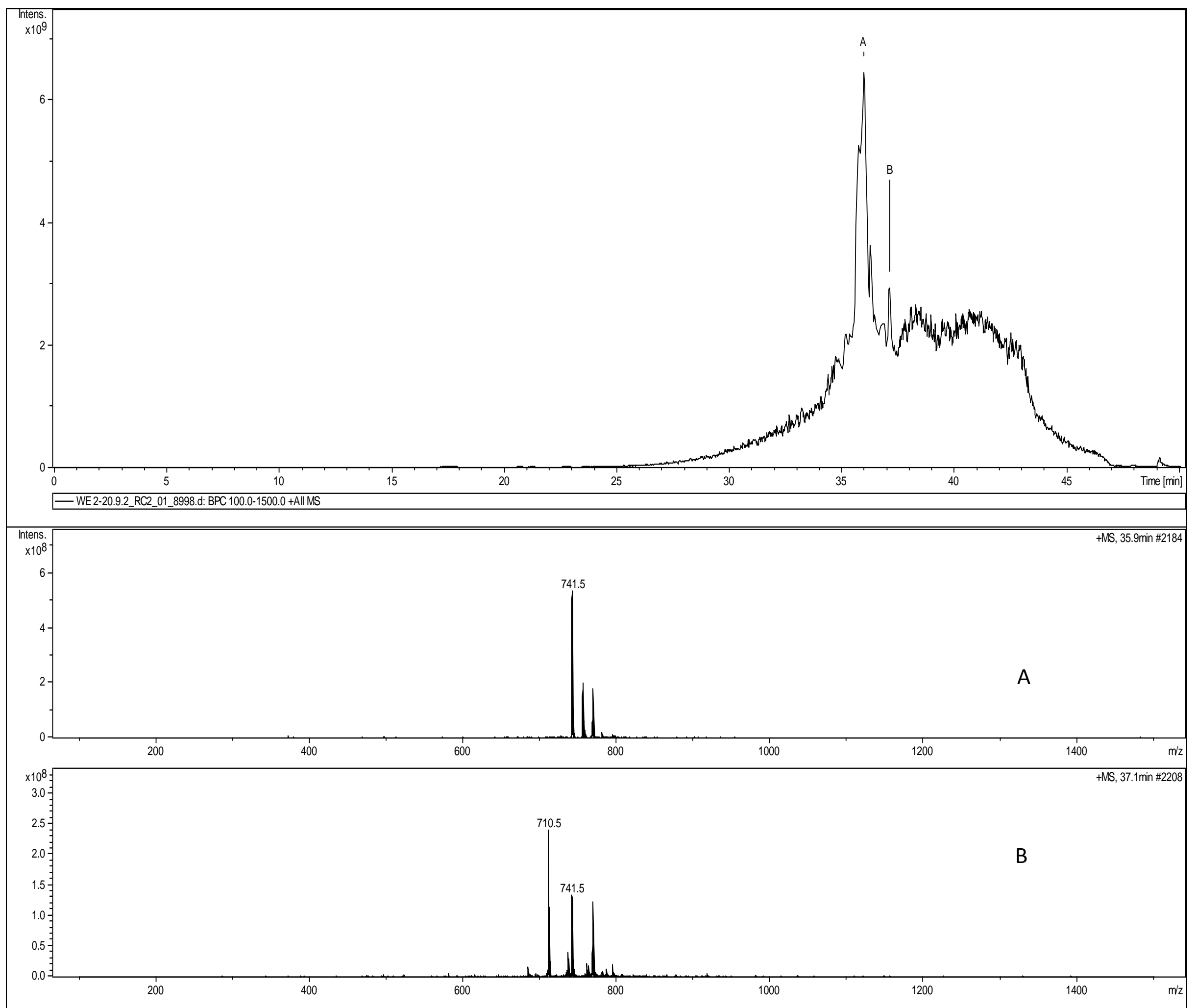


Figure 75: Base peak chromatogram of WE 2-20.9.3 with mass spectra of metabolites of interest

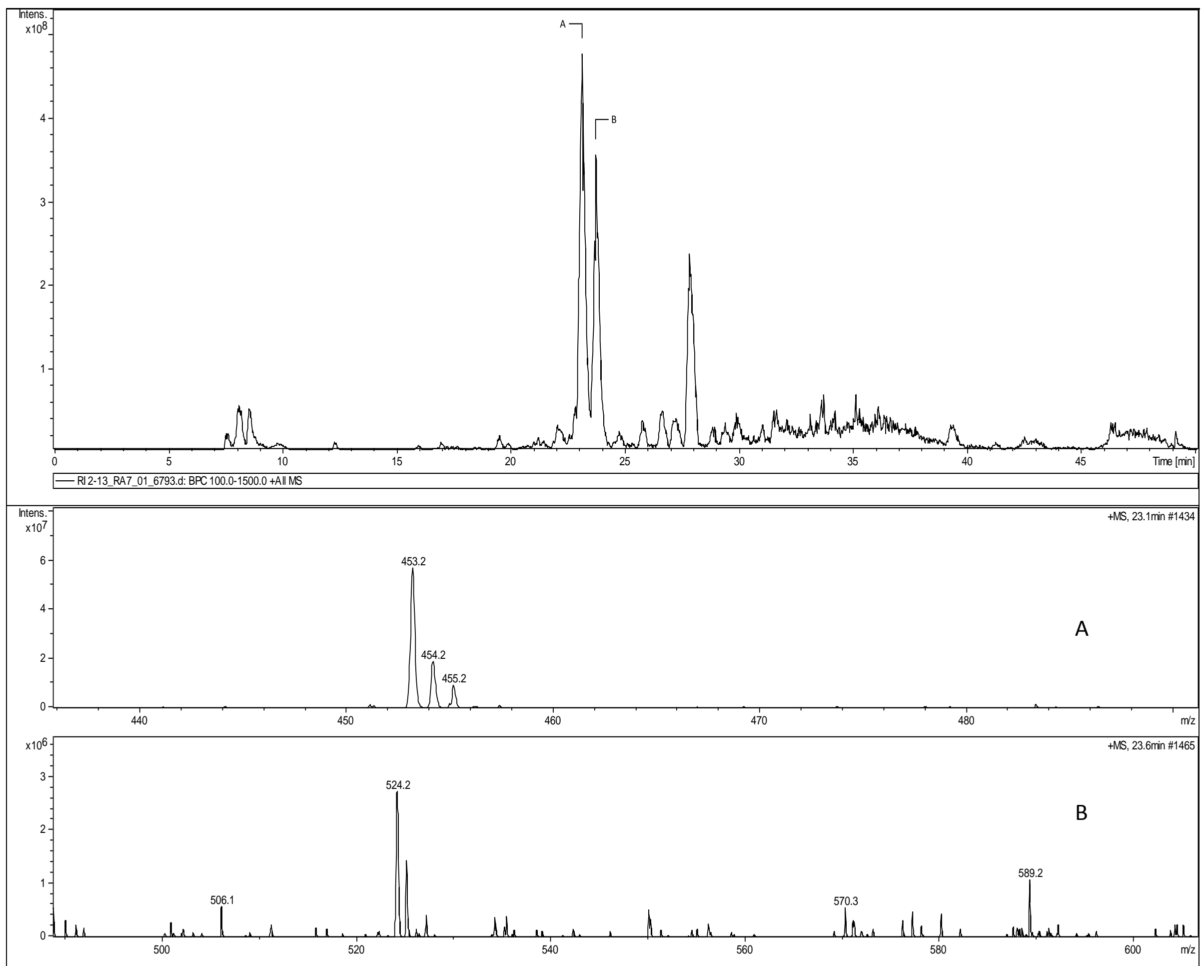


Figure 76: Base peak chromatogram of RI 2-13 *Aplidium sp.* with mass spectra of metabolites of interest

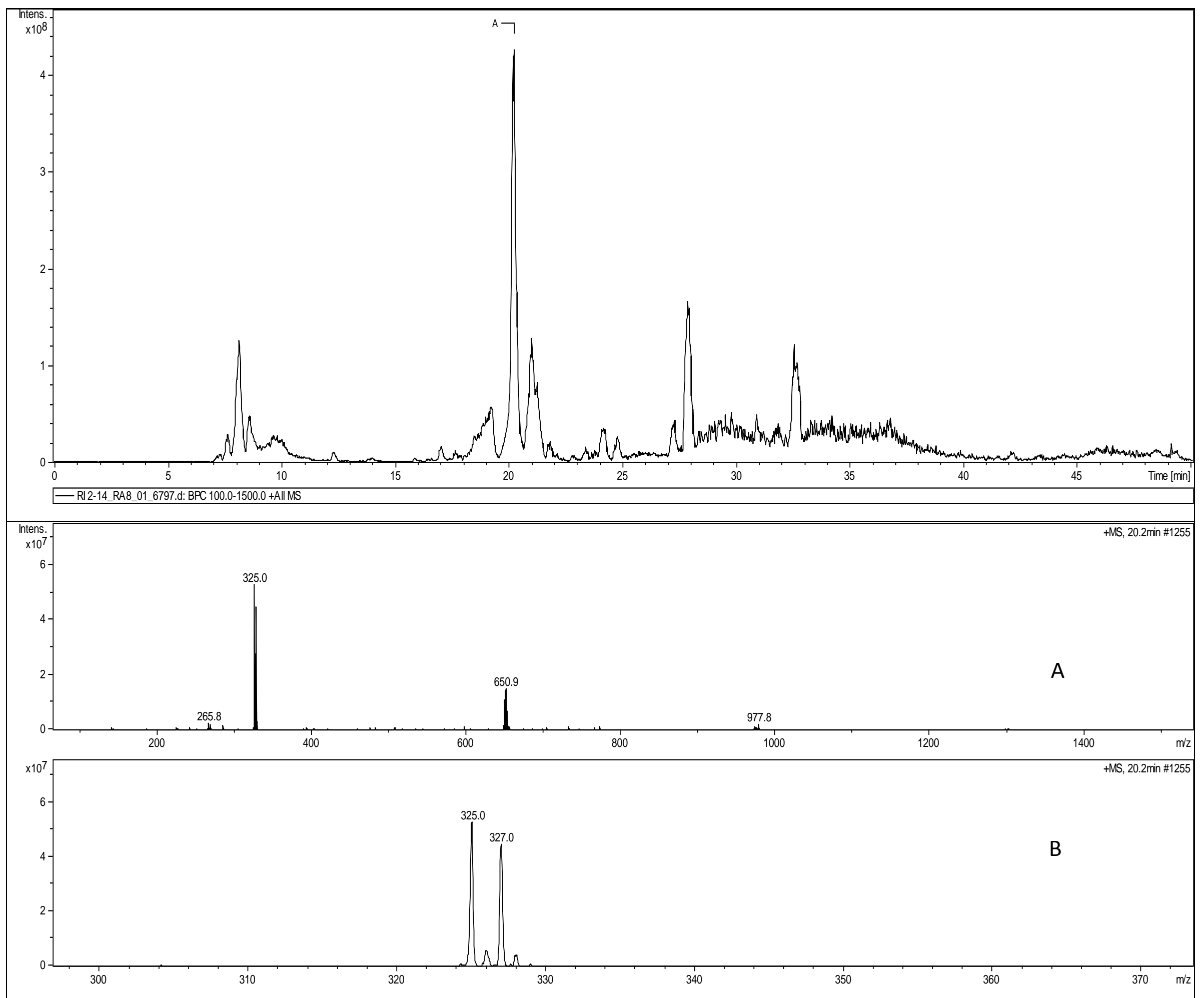


Figure 77: Base peak chromatogram of RI 2-14 *Alcyonaria sp.* with mass spectra of metabolites of interest

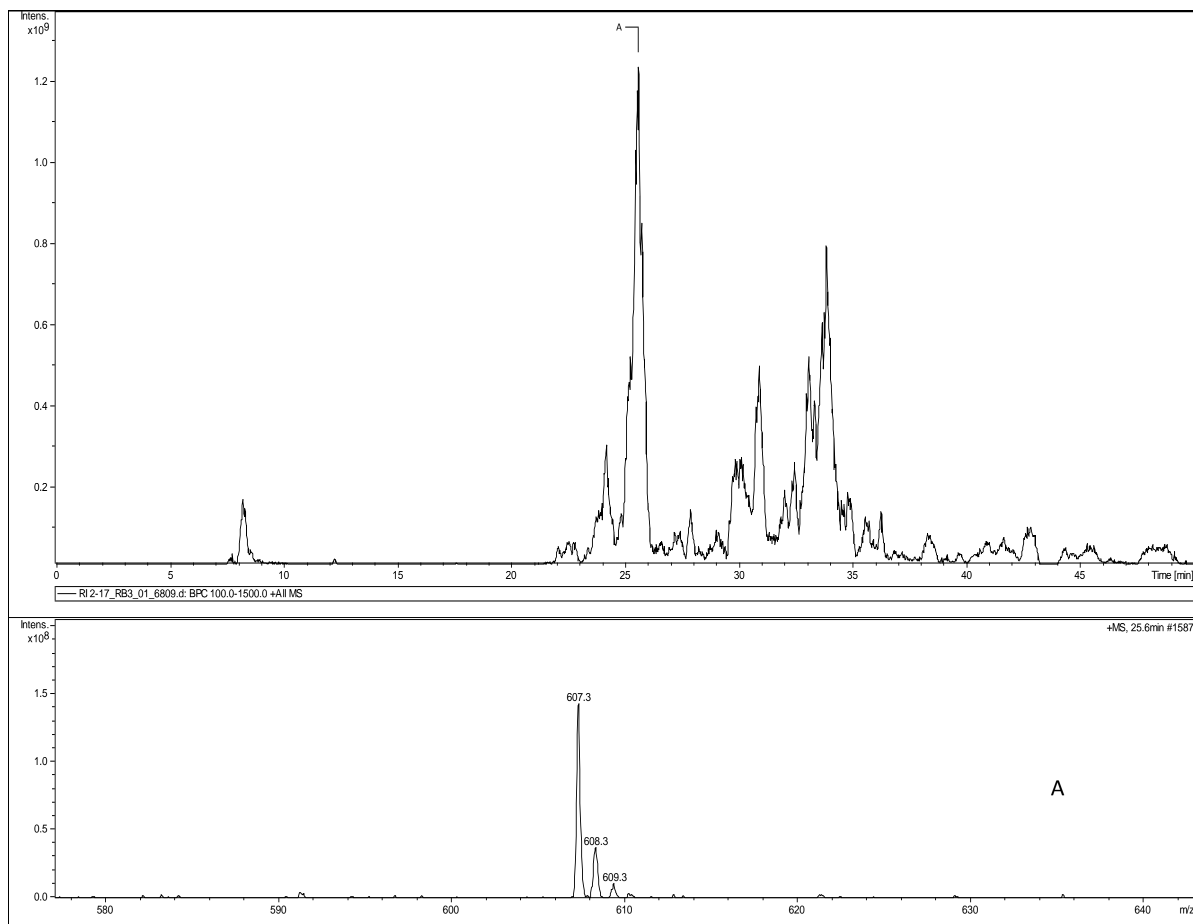


Figure 78: Base peak chromatogram of RI 2-17 *Ircinia sp.* (horny sponge) with mass spectra of the metabolite of interest

References

1. Hanson, J., *Natural Products the Secondary Metabolites* 2003, Milton Road, Cambridge U.K: The Royal Society of Chemistry.
2. Drew, S.W. and A.L. Demain, *Effect of Primary Metabolites on Secondary Metabolism. Annual Review of Microbiology*, 1977. **31**: 343-356.
3. Selvin, J. and A.P. Lipton, *Biopotentials of secondary metabolites isolated from marine sponges. Hydrobiologia*, 2004. **513**: 231-238.
4. Bhat, S., Nagasampagi, B.A. and Meenaksi, S, *Natural Products Chemistry and Applications,* 2009, India: Alpha Science.
5. Clardy, J. and C. Walsh, *Lessons from natural molecules. Nature*, 2004. **432**: 829-837.
6. Sykes, R., *Penicillin: from discovery to product*. Vol. 79. 2001. 778-9.
7. Wallace, R.W., *Drugs from the sea: harvesting the results of aeons of chemical evolution. Molecular Medicine Today*, 1997. **3**: 291-295.
8. Proksch, P., A. Putz, S. Ortlepp, J. Kjer, and M. Bayer, *Bioactive natural products from marine sponges and fungal endophytes. Phytochemistry Reviews*, 2010. **9**: 475-489.
9. Rahman, H., B. Austin, W.J. Mitchell, P.C. Morris, D.J. Jamieson, D.R. Adams, A.M. Spragg, and M. Schweizer, *Novel Anti-Infective Compounds from Marine Bacteria. Marine Drugs*, 2010. **8**: 498-518.
10. Berlinck, R.G.S., E. Hajdu, R.M. da Rocha, J.H.H.L. de Oliveira, I.L.C. Hernández, M.H.R. Seleghim, A.C. Granato, É.V.R. de Almeida, C.V. Nuñez, G. Muricy, S. Peixinho, C. Pessoa, M.O. Moraes, B.C. Cavalcanti, G.G.F. Nascimento, O. Thiemann, M. Silva, A.O. Souza, C.L. Silva, and P.R.R. Minarini, *Challenges and Rewards of Research in Marine Natural Products Chemistry in Brazil. Journal of Natural Products*, 2004. **67**: 510-522.
11. Torres-Ramos, M.A. and M.B. Aguilar, *Recent Advances in Cnidarian Neurotoxin Research. Comments on Toxicology*, 2003. **9**: 161.
12. Simmons, T.L., E. Andrianasolo, K. McPhail, P. Flatt, and W.H. Gerwick, *Marine natural products as anticancer drugs. Molecular Cancer Therapeutics*, 2005. **4**: 333-342.

13. Hart, S., *Cone Snail Toxins Take off. Bioscience*, 1997. **47**: 131-134.
14. Paul, V.J., P. Ciminiello, and W. Fenical, *Diterpenoid feeding deterrents from the pacific green alga Pseudochlorodesmis furcellata. Phytochemistry*, 1988. **27**: 1011-1014.
15. Pawlik, J.R., *The Chemical Ecology of Sponges on Caribbean Reefs: Natural Products Shape Natural Systems. Bioscience*, 2011. **61**: 888-898.
16. Faulkner, D.J., *Marine natural products. Natural Product Reports*, 1986. **3**: 1-33.
17. Blunt, J.W., B.R. Copp, R.A. Keyzers, M.H.G. Munro, and M.R. Prinsep, *Marine natural products. Natural Product Reports*, 2012. **29**: 144-222.
18. Rocha, J., L. Peixe, N.C. Gomes, and R. Calado, *Cnidarians as a source of new marine bioactive compounds--an overview of the last decade and future steps for bioprospecting. Marine Drugs*, 2011. **9**: 1860-86.
19. Grimmelikhuijzen, C.J.P., M. Williamson, and G.N. Hansen, *Neuropeptides in cnidarians. Canadian Journal of Zoology*, 2002. **80**: 1690-1702.
20. Knowlton, N., *Coral reefs. Current Biology*, 2008. **18**: R18-R21.
21. Hines, D. and J. Pawlik, *Assessing the antipredatory defensive strategies of Caribbean non-scleractinian zoantharians (Cnidaria): is the sting the only thing? Marine Biology*, 2012. **159**: 389-398.
22. El-Gamal, A.A.H., C.-Y. Chiang, S.-H. Huang, S.-K. Wang, and C.-Y. Duh, *Xenia Diterpenoids from the Formosan Soft Coral Xenia blumi. Journal of Natural Products*, 2005. **68**: 1336-1340.
23. Šuput, D., *In vivo effects of cnidarian toxins and venoms. Toxicon*, 2009. **54**: 1190-1200.
24. Castañeda, O. and A.L. Harvey, *Discovery and characterization of cnidarian peptide toxins that affect neuronal potassium ion channels. Toxicon*, 2009. **54**: 1119-1124.
25. Reimão, J., A. Migotto, M. Kossuga, R.S. Berlinck, and A. Tempone, *Antiprotozoan activity of Brazilian marine cnidarian extracts and of a modified steroid from the octocoral Carijoa riisei. Parasitology Research*, 2008. **103**: 1445-1450.

26. Neuber, H., *Leishmaniasis. JDDG: Journal der Deutschen Dermatologischen Gesellschaft*, 2008. **6**: 754-765.
27. Urbina, J.A., *Chemotherapy of Chagas Disease. Current Pharmaceutical Design*, 2002. **8**: 287-95.
28. Croft, S.L., S. Sundar, and A.H. Fairlamb, *Drug resistance in leishmaniasis. Clin Microbiol Rev*, 2006. **19**: 111-26.
29. *mollusc*, in *Philip's Encyclopedia,* 2008, Philip's.
30. Cimino, G., S. De Rosa, S. De Stefano, R. Morrone, and G. Sodano, *The chemical defense of nudibranch molluscs: Structure, biosynthetic origin and defensive properties of terpenoids from the dorid nudibranch dendrodoris grandiflora. Tetrahedron*, 1985. **41**(6): p. 1093-1100.
31. Renault, T. and B. Novoa, *Viruses infecting bivalve molluscs. Aquatic Living Resources*, 2004. **17**: 397-409.
32. Defer, D., N. Bourgougnon, and Y. Fleury, *Screening for antibacterial and antiviral activities in three bivalve and two gastropod marine molluscs. Aquaculture*, 2009. **293**: 1-7.
33. Cutignano, A., D. Blihoghe, A. Fontana, G. Villani, G. d'Ippolito, and G. Cimino, *Fusaripyrones, novel polypropionates from the Mediterranean mollusc Haminoea fusari. Tetrahedron*, 2007. **63**: 12935-12939.
34. Subasinghe, N.L., M.J. Wall, M.P. Winters, N. Qin, M.L. Lubin, M.F.A. Finley, M.R. Brandt, M.P. Neeper, C.R. Schneider, R.W. Colburn, C.M. Flores, and Z. Sui, *A novel series of pyrazolylpiperidine N-type calcium channel blockers. Bioorganic & Medicinal Chemistry Letters*, 2012. **22**: 4080-4083.
35. Garber, K., *Peptide leads new class of chronic pain drugs. Nature Biotechnology*, 2005. **23**: 399-399.
36. Lyseng-Williamson, K.A.C., *Ziconotide. CNS Drugs*, 2006. **20**: 331.
37. Shubina, L.K., S.N. Fedorov, A.I. Kalinovskiy, A.S. Dmitrenok, J.O. Jin, M.G. Song, J.Y. Kwak, and V.A. Stonika, *Four new chamigrane sesquiterpenoids from the opistobranch mollusk Aplysia dactylomela. Russian Chemical Bulletin*, 2007. **56**: 2109-2114.

38. Dias, T., I. Brito, L. Moujir, N. Paiz, J. Darias, and M. Cueto, *Cytotoxic Sesquiterpenes from Aplysia dactylomela. Journal of Natural Products*, 2005. **68**: 1677-1679.
39. Schmitz, F.J. and F.J. McDonald, *Marine natural products: Dactyloxene-B, a sesquiterpene ether from the sea hare, Aplysia dactylomela. Tetrahedron Letters*, 1974. **15**: 2541-2544.
40. González, A.G., J.D. Martín, M. Norte, R. Pérez, V. Weyler, S. Rafii, and J. Clardy, *A new diterpene fromAplysia dactylomela. Tetrahedron Letters*, 1983. **24**: 1075-1076.
41. Manzo, E., M. Gavagnin, G. Bifulco, P. Cimino, S. Di Micco, M.L. Ciavatta, Y.W. Guo, and G. Cimino, *Aplysiols A and B, squalene-derived polyethers from the mantle of the sea hare Aplysia dactylomela. Tetrahedron*, 2007. **63**: 9970-9978.
42. Bone, Q., C. Carré, and P. Chang, *Tunicate feeding filters. Journal of the Marine Biological Association of the United Kingdom*, 2003. **83**: 907-919.
43. Wieczorek, D., *The Tunicates are coming*, in *Underwater Naturalist,* 2009. 26.
44. Schmidt, E.W., M.S. Donia, J.A. McIntosh, W.F. Fricke, and J. Ravel, *Origin and Variation of Tunicate Secondary Metabolites. Journal of Natural Products*, 2012. **75**: 295-304.
45. Miyata, Y., T. Diyabalanage, C.D. Amsler, J.B. McClintock, F.A. Valeriote, and B.J. Baker, *Ecdysteroids from the Antarctic Tunicate Synoicum adareanum. Journal of Natural Products*, 2007. **70**: 1859-1864.
46. Kurtzberg, L.S., S.D. Roth, R.G. Bagley, C. Rouleau, M. Yao, J.L. Crawford, R.D. Krumbholz, S.M. Schmid, and B.A. Teicher, *Bone marrow CFU-GM and human tumor xenograft efficacy of three tubulin binding agents. Cancer Chemotherapy and Pharmacology*, 2009. **64**: 1029-38.
47. Cooper, E.L. and D. Yao, *Diving for drugs: tunicate anticancer compounds. Drug Discovery Today*, 2012. **17**: 636-648.
48. Molinski, T.F., D.S. Dalisay, S.L. Lievens, and J.P. Saludes, *Drug development from marine natural products. Nature Reviews. Drug Discovery*, 2009. **8**: 69-85.

49. Plaza, M., A. Cifuentes, and E. Ibáñez, *In the search of new functional food ingredients from algae. Trends in Food Science & Technology*, 2008. **19**: 31-39.

50. Bano, S., N. Bano, V.U. Ahmad, M. Shameel, and S. Amjad, *Marine Natural Products: 3-Formylindole from the Red Algae Botryocladia leptopoda. Journal of Natural Products*, 1986. **49**: 549-549.

51. Chowdhury, B.K. and D.P. Chakraborty, *3-Formylindole from Murraya exotica. Phytochemistry*, 1971. **10**: 481-483.

52. Hodges, R. and A.L. Porte, *The structure of loliolide : A terpene from lolium perenne. Tetrahedron*, 1964. **20**: 1463-1467.

53. Valdes, L.J., *Loliolide from Salvia divinorum. Journal of Natural Products*, 1986. **49**: 171-171.

54. Percot, A., A. Yalçın, V. Aysel, H. Erduğan, B. Dural, and K.C. Güven, *Loliolide in marine algae. Natural Product Research*, 2009. **23**: 460-465.

55. *Sponges*, in *Animals: A Visual Encyclopedia,* 2008, Dorling Kindersley Publishing, Inc.

56. Braekman, J.-C. and D. Daloze, *Chemical and biological aspects of sponge secondary metabolites. Phytochemistry Reviews*, 2004. **3**: 275-283.

57. Esteves, A.I.S., M. Nicolai, M. Humanes, and J. Goncalves, *Sulfated Polysaccharides in Marine Sponges: Extraction Methods and Anti-HIV Activity. Marine Drugs*, 2011. **9**: 139-153.

58. Sipkema, D., M.R. Franssen, R. Osinga, J. Tramper, and R. Wijffels, *Marine Sponges as Pharmacy. Marine Biotechnology*, 2005. **7**: 142-162.

59. Erdogen, I., J. Tanaka, T. Higa, and B. Sener, *Two new hydroquinone derivatives from two sponge species of the Aegean Sea. Journal of the Chemical Society of Pakistan*, 2000. **22**: 200-204.

60. Orhan, I., B. Şener, M. Kaiser, R. Brun, and D. Tasdemir, *Inhibitory Activity of Marine Sponge-Derived Natural Products against Parasitic Protozoa. Marine Drugs*, 2010. **8**: 47-58.

61. Palermo, J.A., M.F. Rodríguez Brasco, and A.M. Seldes, *Storniamides A-D: Alkaloids from a Patagonian sponge Cliona sp. Tetrahedron*, 1996. **52**: 2727-2734.

62. Ebel, H., A. Terpin, and W. Steglich, *A concise synthesis of storniamide A nonamethyl ether [1]. Tetrahedron Letters*, 1998. **39**: 9165-9166.

63. Boger, D.L., C.W. Boyce, M.A. Labroli, C.A. Sehon, and Q. Jin, *Total Syntheses of Ningalin A, Lamellarin O, Lukianol A, and Permethyl Storniamide A Utilizing Heterocyclic Azadiene Diels−Alder Reactions. Journal of the American Chemical Society*, 1998. **121**: 54-62.

64. Andersen, R.J., *Tetracetyl clionamide, a 6-bromotryptophan derivative from the sponge Cliona celata. Tetrahedron Letters*, 1978. **19**: 2541-2544.

65. Yang, L. and R.J. Andersen, *Absolute Configuration of the Antiinflamatory Sponge Natural Product Contignasterol. Journal of Natural Products*, 2002. **65**: 1924-1926.

66. Shen, Y. and D.L. Burgoyne, *Efficient Synthesis of IPL576,092: A Novel Anti-Asthma Agent. The Journal of Organic Chemistry*, 2002. **67**: 3908-3910.

67. Belarbi, E.H., A. Contreras Gómez, Y. Chisti, F. Garcia Camacho, and E. Molina Grima, *Producing drugs from marine sponges. Biotechnology Advances*, 2003. **21**: 585-598.

68. Kehr, J.C., D. Gatte Picchi, and E. Dittmann, *Natural product biosyntheses in cyanobacteria: A treasure trove of unique enzymes. Beilstein journal of organic chemistry*, 2011. **7**: 1622-1635.

69. El Gamal, A.A., *Biological importance of marine algae. Saudi Pharmaceutical Journal*, 2010. **18**: 1-25.

70. Liu, L. and K.S. Rein, *New Peptides Isolated from Lyngbya Species: A Review. Marine Drugs*, 2010. **8**: 1817-1837.

71. Leao, P.N., N. Engene, A. Antunes, W.H. Gerwick, and V. Vasconcelos, *The chemical ecology of cyanobacteria. Natural Product Reports*, 2012. **29**: 372-391.

72. Abed, R.M.M., S. Dobretsov, and K. Sudesh, *Applications of cyanobacteria in biotechnology. Journal of Applied Microbiology*, 2009. **106**: 1-12.

73. Nunnery, J.K., E. Mevers, and W.H. Gerwick, *Biologically active secondary metabolites from marine cyanobacteria. Current Opinion in Biotechnology*, 2010. **21**: 787-793.

74. Teruya, T., H. Sasaki, H. Fukazawa, and K. Suenaga, *Bisebromoamide, a Potent Cytotoxic Peptide from the Marine Cyanobacterium Lyngbya sp.: Isolation, Stereostructure, and Biological Activity. Organic Letters*, 2009. **11**: 5062-5065.
75. Brückner, H. and N. Fujii, *Free and Peptide-Bound D-Amino Acids in Chemistry and Life Sciences. Chemistry & Biodiversity*, 2010. **7**: 1333-1336.
76. Fisher, G.H., *Biologically active D-amino acids. Amino Acids*, 2007. **32**: 1.
77. Balunas, M.J., R.G. Linington, K. Tidgewell, A.M. Fenner, L.-D. Ureña, G.D. Togna, D.E. Kyle, and W.H. Gerwick, *Dragonamide E, a Modified Linear Lipopeptide from Lyngbya majuscula with Antileishmanial Activity. Journal of Natural Products*, 2009. **73**: 60-66.
78. Tan, L.T., *Bioactive natural products from marine cyanobacteria for drug discovery. Phytochemistry*, 2007. **68**: 954-979.
79. Luesch, H., W.Y. Yoshida, R.E. Moore, V.J. Paul, and T.H. Corbett, *Total Structure Determination of Apratoxin A, a Potent Novel Cytotoxin from the Marine Cyanobacterium Lyngbya majuscula. Journal of the American Chemical Society*, 2001. **123**: 5418-5423.
80. Chen, J. and C.J. Forsyth, *Total Synthesis of Apratoxin A. Journal of the American Chemical Society*, 2003. **125**: 8734-8735.
81. Liu, Y., B.K. Law, and H. Luesch, *Apratoxin a reversibly inhibits the secretory pathway by preventing cotranslational translocation. Molecular Pharmacology*, 2009. **76**: 91-104.
82. Matthew, S., P.J. Schupp, and H. Luesch, *Apratoxin E, a Cytotoxic Peptolide from a Guamanian Collection of the Marine Cyanobacterium Lyngbya bouillonii. Journal of Natural Products*, 2008. **71**: 1113-1116.
83. Chen, Q.-Y., Y. Liu, and H. Luesch, *Systematic Chemical Mutagenesis Identifies a Potent Novel Apratoxin A/E Hybrid with Improved in Vivo Antitumor Activity. ACS Medicinal Chemistry Letters*, 2011. **2**: 861-865.
84. Linington, R.G., B.R. Clark, E.E. Trimble, A. Almanza, L.-D. Ureña, D.E. Kyle, and W.H. Gerwick, *Antimalarial Peptides from Marine Cyanobacteria: Isolation and Structural Elucidation of Gallinamide A. Journal of Natural Products*, 2008. **72**: 14-17.

85. Yoo, H.-D. and W.H. Gerwick, *Curacins B and C, New Antimitotic Natural Products from the Marine Cyanobacterium Lyngbya majuscula. Journal of Natural Products*, 1995. **58**: 1961-1965.
86. Wipf, P., J.T. Reeves, and B.W. Day, *Chemistry and Biology of Curacin A. Current Pharmaceutical Design*, 2004. **10**: 1417-37.
87. Rahbari, R., T. Sheahan, V. Modes, P. Collier, C. Macfarlane, and R.M. Badge, *A novel L1 retrotransposon marker for HeLa cell line identification. Biotechniques*, 2009. **46**: 277.
88. Hutchins, G.M., B.P. Lucey, and W.A. Nelson-Rees, *Henrietta Lacks, HeLa cells, and cell culture contamination. Archives of Pathology & Laboratory Medicine*, 2009. **133**: 1463.
89. Hyman, A.H. and K. Simons, *The new cell biology: Beyond HeLa cells. Nature*, 2011. **480**: 34-34.
90. Qiang, S., A. Keiko, J.L. Vandeberg, and W. Xing Li, *Passage-Dependent Changes in Baboon Endothelial Cells—Relevance to In Vitro Aging. DNA & Cell Biology*, 2004. **23**: 502-509.
91. Momtaz, S., A.A. Hussein, S.N. Ostad, M. Abdollahi, and N. Lall, *Growth inhibition and induction of apoptosis in human cancerous HeLa cells by Maytenus procumbens. Food and Chemical Toxicology*, 2013. **51**: 38-45.
92. Uhlig, S., H. Wisløff, and D. Petersen, *Identification of Cytotoxic Constituents of Narthecium ossifragum Using Bioassay-Guided Fractionation. Journal of Agricultural and Food Chemistry*, 2007. **55**: 6018-6026.
93. Li, Z.-B., J.-Y. Wang, B. Jiang, X.-L. Zhang, L.-J. An, and Y.-M. Bao, *Benzobijuglone, a novel cytotoxic compound from Juglans mandshurica, induced apoptosis in HeLa cervical cancer cells. Phytomedicine*, 2007. **14**: 846-852.
94. Liu, Q.-Y. and B.H. Tan, *Dietary fish oil and vitamin E enhance doxorubicin effects in P388 tumor-bearing mice. Lipids*, 2002. **37**: 549-556.
95. Szmigielska-Kaplon, A., E. Ciesielska, L. Szmigiero, and T. Robak, *Anthracyclines potentiate activity against murine leukemias L1210 and*

P388 in vivo and in vitro. European Journal of Haematology, 2002. **68**: 370-375.

96. Balis, F.M., *Evolution of Anticancer Drug Discovery and the Role of Cell-Based Screening. Journal of the National Cancer Institute*, 2002. **94**: 78-79.
97. Kondoh, M., T. Usui, T. Nishikiori, T. Mayumi, and H. Osada, *Apoptosis induction via microtubule disassembly by an antitumour compound, pironetin. Biochemical Journal*, 1999. **340**: 411-6.
98. Dias, L.C., L.G. de Oliveira, and M.A. de Sousa, *Total Synthesis of (−)-Pironetin†. Organic Letters*, 2003. **5**: 265-268.
99. Liu, W.M. and A.G. Dalgleish, *MTT assays can underestimate cell numbers. Cancer Chemotherapy and Pharmacology*, 2009. **64**: 861-2.
100. Stockert, J.C., A. Blázquez-Castro, M. Cañete, R.W. Horobin, and Á. Villanueva, *MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histochemica*, 2012. **114**: 785-796.
101. Peng, L., B. Wang, and P. Ren, *Reduction of MTT by flavonoids in the absence of cells. Colloids and Surfaces B: Biointerfaces*, 2005. **45**: 108-111.
102. Zhukova, N.V. and N.A. Aizdaicher, *Fatty acid composition of 15 species of marine microalgae. Phytochemistry*, 1995. **39**: 351-356.
103. W. Gribble, G., *The diversity of naturally occurring organobromine compounds. Chemical Society Reviews*, 1999. **28**: 335-346.
104. Prinsep, M.R., *Chemistry 514 Special Topics Lecture Notes*, page 10, 2010: University of Waikato.
105. Teruya, T., H. Sasaki, K. Kitamura, T. Nakayama, and K. Suenaga, *Biselyngbyaside, a Macrolide Glycoside from the Marine Cyanobacterium Lyngbya sp. Organic Letters*, 2009. **11**: 2421-2424.
106. Tan, L.T., B.P.L. Goh, A. Tripathi, M.G. Lim, G.H. Dickinson, S.S.C. Lee, and S.L.M. Teo, *Natural antifoulants from the marine cyanobacterium Lyngbya majuscula. Biofouling*, 2010. **26**: 685-695.
107. Matthew, S., V.J. Paul, and H. Luesch, *Tiglicamides A–C, cyclodepsipeptides from the marine cyanobacterium Lyngbya confervoides. Phytochemistry*, 2009. **70**: 2058-2063.

108. Beer, S., W. Spencer, G. Holbrook, and G. Bowes, *Gas exchange and carbon fixation properties of the mat-forming cyanophyte Lyngbya birgei G.M. Smith. Aquatic Botany*, 1990. **38**: 221-230.

109. Xavier, J.R., P.G. Rachello-Dolmen, F. Parra-Velandia, C.H.L. Schönberg, J.A.J. Breeuwer, and R.W.M. van Soest, *Molecular evidence of cryptic speciation in the "cosmopolitan" excavating sponge Cliona celata (Porifera, Clionaidae). Molecular Phylogenetics and Evolution*, 2010. **56**: 13-20.

110. Suh, S.-J., C.-H. Kwak, K.-H. Song, K.-M. Kwon, T.-W. Chung, S.-H. Cho, Y.-K. Kim, H.-D. Yoon, Y.-C. Lee, D.-S. Kim, S.-J. Park, M. Na, J.-K. Son, H. Chang, and C.-H. Kim, *Triple Inhibitory Activity of Cliona celata Against TNF-α-Induced Matrix Metalloproteinase-9 Production Via Downregulated NF-κB and AP-1, Enzyme Activity, and Migration Potential. Inflammation*, 2012. **35**: 736-745.

111. Chaves-Fonnegra, A., L. Castellanos, S. Zea, C. Duque, J. Rodríguez, and C. Jiménez, *Clionapyrrolidine A—A Metabolite from the Encrusting and Excavating Sponge Cliona tenuis that Kills Coral Tissue upon Contact. Journal of Chemical Ecology*, 2008. **34**: 1565-1574.

112. *Octocorallia (Alcyonaria)*, in *McGraw-Hill Concise Encyclopedia of Science and Technology,* 2006, McGraw-Hill.

113. Erwin, P.M., S. López-Legentil, R. González-Pech, and X. Turon, *A specific mix of generalists: bacterial symbionts in Mediterranean Ircinia spp. FEMS Microbiology Ecology*, 2012. **79**: 619-637.

114. Ellis, G., *P388 (MTT) Assay*, 2001, University of Canterbury.

115. Rai, A.N., E. Söderbäck, and B. Bergman, *Tansley Review No. 116. New Phytologist*, 2000. **147**: 449-481.