Novel Brominated Alkaloids from the Bryozoan *Amathia citrina*

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Abstract

Sixteen bryozoan species from the United Kingdom and New Zealand were surveyed for the presence of brominated secondary metabolites by Liquid Chromatography tandem Mass Spectrometry (LCMS) analysis. Brominated metabolites were detected in six of these samples. Collections of the species *Amathia citrina* and *Amathia verticillata* were determined as ideal candidates for a natural products study due to the presence of potentially novel brominated secondary metabolites in reasonable abundance.

The sample with the greatest number and seemingly highest concentration of brominated secondary metabolites, of all species surveyed, was the species *Amathia citrina* (previously *Bowerbankia citrina*) from Swansea, Wales, and so was selected as the candidate for this study.

Eight related metabolites were detected in *A. citrina*, speculated to be a family of brominated alkaloids. Attempts were made to isolate and purify two members of this family of compounds by reversed-phase and size exclusion chromatography, but were unable to be obtained in a pure state. Nevertheless, they were then partially characterised by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy to be discovered as a new family of brominated indole alkaloids, tentatively named the amathamines.

Acknowledgements

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List of Abbreviations

AIDS   Acquired Immune Deficiency Syndrome

BPC   Base Peak Chromatogram

CD$_3$OD   Deuterated Methanol

COSY   Correlation Spectroscopy

DCM   Dichloromethane

g   Gram/s

HIV   Human Immunodeficiency Virus

HPLC   High Performance Liquid Chromatography

HMBC   Heteronuclear Multiple Bond Correlation

HRMS   High Resolution Mass Spectrometry

HSQC   Heteronuclear Single Quantum Coherence

Hz   Hertz

IC$_{50}$ Concentration at 50% of Growth Inhibition

IR   Infrared

LCMS   Liquid Chromatography Mass Spectrometry

MeOH   Methanol

MID   Minimum Inhibitory Dose

NCI   National Cancer Institute

NOE   Nuclear Overhauser Effect

NMR   Nuclear Magnetic Resonance

NOESY   Nuclear Overhauser Effect Spectroscopy

SPE   Solid Phase Extraction

UV   Ultraviolet

Chapter One

Introduction

1.1   Natural Products

Natural products have been the best source of mankind's lifesaving medicines. The treatment of infectious diseases, cancer and immunological disorders have all had breakthroughs due to natural products. The metabolites of a living cell are classified two-fold: primary metabolites, which are used by cells for their own sustenance and growth such as carbohydrates, lipids and amino acids, and secondary metabolites, which are biosynthesised from primary metabolites for other purposes. It is these secondary metabolites that have pharmaceutical potential and make up the lead chemicals extracted in a natural products study. Plants, bacteria, fungi and marine invertebrates are the major foci of these studies.[1]

Historically, terrestrial plants have been the source of most medicines for the treatment of diseases, and were administered as crude or semi-pure extracts. Not until the purification and characterisation of individual components from these extracts, in the early nineteenth century, were the effects of these mixtures attributed to the pure compounds responsible. Then began a new era of medicine where drugs were purified and administered in precise dosages. In 1817, Friedrich Seturner reported colourless crystals, possessing the narcotic properties of opium,

from precipitation of a hot water extraction of the plant *Papaver somniferum* (Opium Poppy), the first ever alkaloid to be reported from a plant.[2] A pharmacist named Emanuel Merck industrialized and marketed this extraction of opium in 1827, developing the first commercial and pure natural product, morphine.[3] Examination of terrestrial fungi extracts in the early twentieth century led to the discovery that microorganisms are also important sources of pharmacologically relevant natural products. In 1929, Alexander Fleming discovered the penicillins, from the fungus *Penicillium chrysogenum*,[4] the most active being penicillin G, which later became the most famous life-saving natural product-based drug of recent history.[5]

From the middle of the twentieth century onwards, the field of marine chemical ecology matured into a well-developed science. Benthic communities in tropical and temperate waters were found to be rich in valued secondary metabolites.[6] From these benthic communities, a significant number of cytotoxic and antibiotic compounds have been discovered. Cytarabine (**1**), a C-nucleoside isolated from the sponge *Cryptotheca crypta* in the 1950s, was the first marine-derived drug used clinically.[7] However, this success did not correspond to an influx of new drug candidates, due to the decreased interest by the pharmaceutical industry in natural products research during the middle and late twentieth century. Instead, such companies focused on combinatorial chemistry for drug discovery.[8] Yet, the higher success rate of marine natural products in drug discovery (1 in every 3140), over that of combinatorial chemistry (1 in every 5000-10,000), has led to a renewed interest in marine natural products research for the purposes of drug discovery.[9] Narrowing down the focus to natural products from marine

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invertebrates has been proven to be an effective strategy for discovering drug-like leads. Since invertebrates use secondary metabolites as chemical defences to ward off predators (possible cytotoxic effects) and microfouling organisms (possible anti-infective effects) these compounds have already been attested by nature and so have been optimised to be bioactive through evolutionary selection.[10]
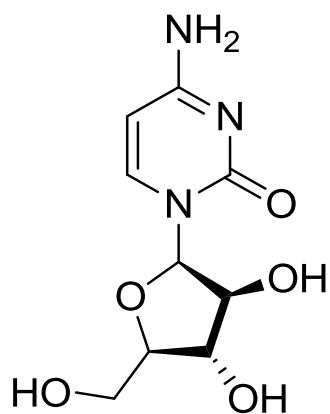
(1)

The majority of marine natural products research has been focused on invertebrate phyla other than Bryozoa. In recent years (2011-2013), the annual ''Marine Natural Products reviews'' in the journal *Natural Product Reports* have noted a sum total of 2887 citations referring to compounds isolated from marine microorganisms and phytoplankton, green, brown and red algae, bryozoans, tunicates, cnidarians, sponges, molluscs, echinoderms, mangroves and other intertidal plants and microorganisms, reporting 3169 new compounds. Of the 2887 citations, only 25 concern bryozoan research, with only 16 novel compounds and one revised structure reported. In comparison to the phylum Porifera (Sponges) for which there were a sum total of 526 citations, reporting 655 novel structures, Bryozoa remains a minimally explored phylum.[11-13]

The volume of bryozoan natural products research is unjustifiably small. Many novel compounds isolated from bryozoans (or a dependant symbiont) hold potent biological activities. Bryostatin 1 **(2)**, isolated from the bryozoan *Bugula neritina*, has been shown to reduce malignant melanoma, lymphoma, and ovarian carcinoma tumours in Phase I trials.[14,15] Bryostatin 1 has also been through Phase II trials for treatment against non-Hodgkin's lymphoma and chronic lymphocytic leukaemia.[16] Additionally, treatment with bryostatin 1 and analogues shows promise to efficiently overcome the largest obstacle to the eradication of the Human Immunodeficiency Virus (HIV), presence of latent proviral reservoirs. Bryosatin 1 and analogues have been shown to modulate the signalling pathways associated with HIV viral reactivation in order to help lower the proviral pool of HIV in Acquired Immune Deficiency Syndrome (AIDS) patients, thus allowing antiviral medicines and natural immunological processes to work on the active virus. Currently, the time taken to potentially cure AIDS patients is expected to take many decades in patients only treated with the leading antiviral drugs.[17,18]

(2)

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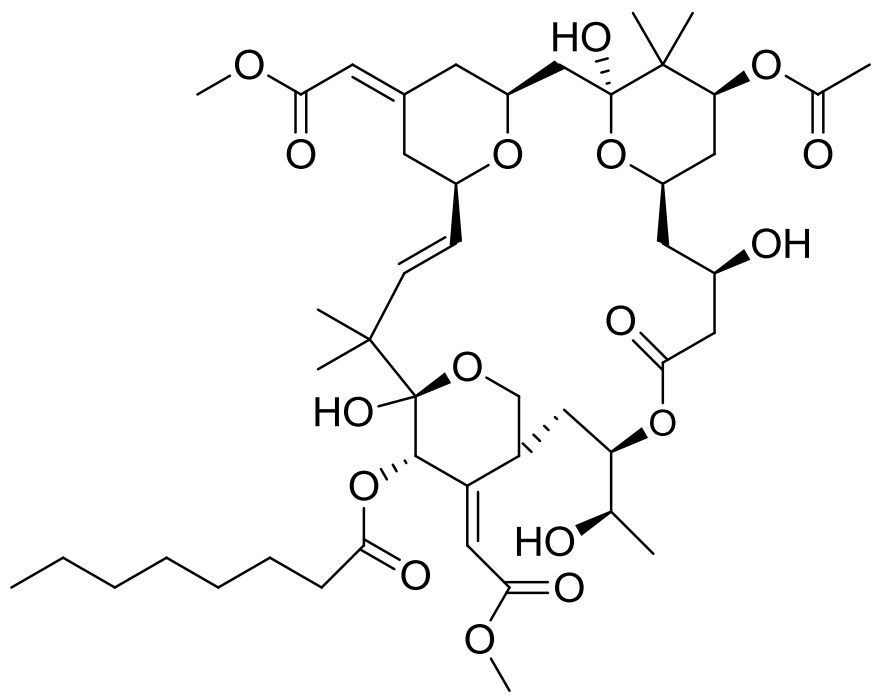
1.2 Biohalogenation

An organism's ability to modify chemical functionality of secondary metabolites directly modifies their bioactivity. One such example is nature's ability to halogenate these secondary metabolites with specific and non-specific halogenases. A plentiful number of halogenated natural products have been isolated in recent years. As of 2010, the number exceeded 4700 with approximately 2100 discovered from marine organisms.[19] The concentration of chloride in the oceans is approximately 19,000 mg/L, which far exceeds the concentration of bromide at about 65 mg/L. Despite this, bromination is, in the majority, a distinct functionality of marine natural products whilst chlorination is instead favoured by terrestrial organisms. Marine organisms are more readily able to oxidise bromine, and so it can be more easily incorporated into a molecule.[20,21]

Nature utilises enzymes to halogenate molecules. Bromoperoxidase is an enzyme commonly used to brominate molecules. There are two classes of halogenating enzymes; the highly specific halogenases, which require oxygen, or the less specific haloperoxidases that require peroxide, of which bromoperoxidase is an example. Haloperoxidases are divided into two classes; haem iron-dependent peroxidases and vanadium-dependent peroxidases. These enzymes generate the electrophilic halogenating species hypohalous acid. The mechanism of these haloperoxidases requires electrophilic activation by reaction of the halide with peroxide, under the assistance of the enzyme. The nucleophile to be halogenated reacts with the released hypohalous acid, which can result in multiple halogenations.[22] On account of this, it can be argued that these less specific peroxidases are not primarily halogenating enzymes, as halogenated natural

products hold specific regiospecificity. Instead, they can be used so when taken out of their native context.[19] However, controlled haloperoxidase reactions have been reported by the bioprocess department of Merck in the synthesis of Indinavir, and by Kaysser and co-workers for the synthesis of merochlorins A–D, among others.[23,24]

The more specific halogenases that use oxygen are classified into two categories; those that utilise flavin (the flavin-dependent halogenases) and those that utilise iron (the non-haem iron-dependent halogenases). Halogens are oxidised in the flavin-dependent halogenases by oxygen via reaction with flavin. The hypohalous acid is released and travels to the substrate which is bound to the enzyme at another site. This enzymatic process can halogenate electron-rich, or activated sites on indoles and pyrroles.[25,26] Non-haem iron-dependent halogenases do not require electron-rich substrates, instead the oxo-ferryl intermediate formed is sufficiently reactive to transfer halogens by removal of a hydrogen to form a reactive radical substrate.[27]

1.3 Phylum Bryozoa

 Bryozoans are sessile, colonial invertebrates of benthic marine, and sometimes freshwater, environments. They are filter feeding animals and have been commonly referred to as moss mats or moss animals. Bryozoans were historically miss-identified as plants due to some species having seemingly plant like appearances.[28] The phylum Bryozoa has three classes, Phylactolaemata, Stenolaemata and Gymnolaemata.[29] A taxanomic classification is outlined in figure 1.1 below.
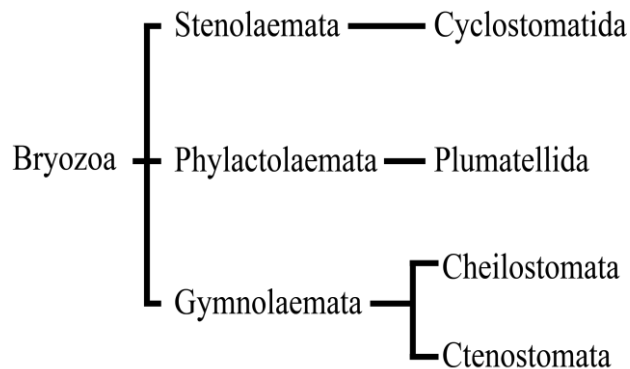
Figure 1.1. Bryozoa taxa. Orders of Bryozoa. (Adapted from reference 29).

Bryozoans live in colonies called zoaria. An individual is termed a zooid, and some contain specialised morphology. An autozooid, depected in figure 1.2, is the basic organizational unit of a colony, which bears the lophophore and digestive parts, some zooids specialise into brood chambers or interconnecting packing cells, generally termed heterozooids. A colony arises from a single ancestral individual, which buds repeatedly to form a colony of connected zooids.[29] Bryozoans contain excreted exoskeletons called zoecia. The composition of a zoecium can differ among species. It can be gelatinous, such as in the freshwater species, *Pectinatella magnifica*, found in the Mississippi river, United States of

America. This outer covering can also be chitinous, such as that found in species of Phylactolaemata and Ctenostomata, or can be calcareous, as in species of Stenolaemata and Cheilostomata.[30]
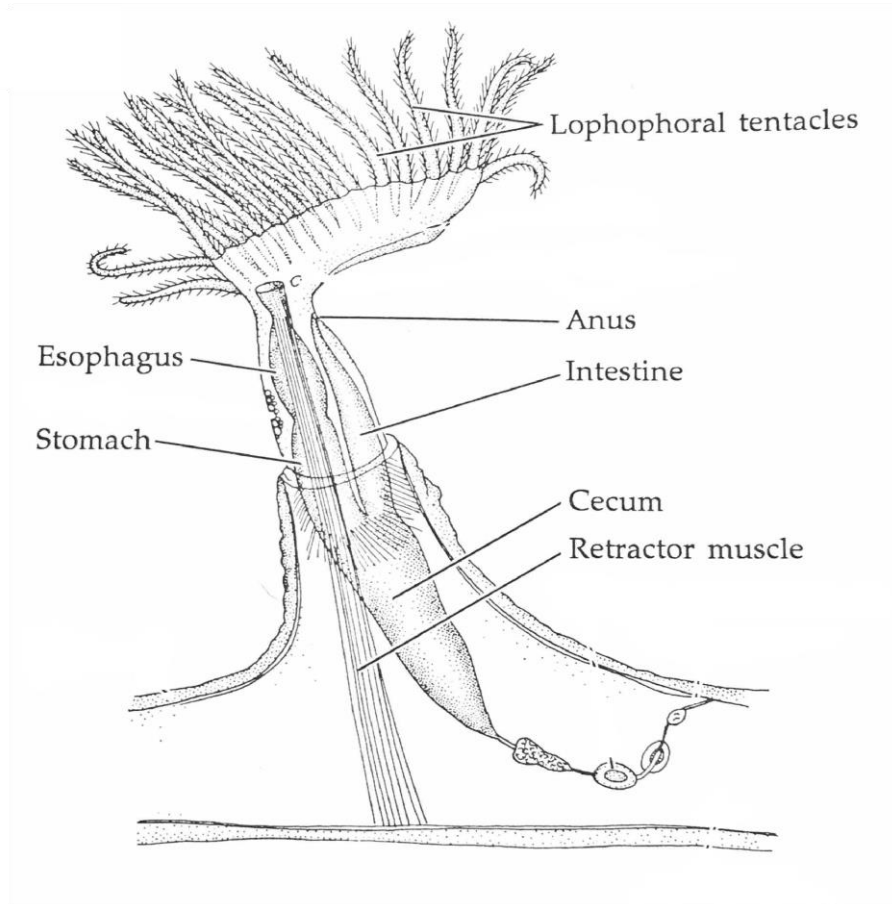
Figure 1.2. Typical autozooid morphology. "A zooid of *Plumatella* (cutaway view)." (Adapted from reference 29).

Bryozoans contain a lophophore for feeding which is a ciliated crown of tentacles for the purpose of catching debris from the surrounding water. Additional characteristic morphology of a bryozoan is a unidirectional U-shaped gut comprised of an esophagus, stomach, cecum, intestine and an ectoproct, an anus outside of the lophophore. A Lophophore can be withdrawn for protection. Species of the Stelnolaemates family utilise a membranous sac that decompresses by expelling fluid into another cavity in order to pull down the lophophoral

tentacles which are attached by ligands. Species of the class Ctenostomata are examples of bryozoans that use simple retractor muscles for lophophore extraction. The ability of bryozoans to retract their lophophores is characteristic among lophophorates.[30]

Of the three taxonomic classes of Bryozoa, Stenolamata and Gymnolaemata are the only orders to contain marine bryozoans. Phylactolaemata is comprised only of freshwater Bryozoa. No natural products have yet been reported from any freshwater species. Gymnolaemata is predominantly comprised of marine Bryozoa with the majority of natural products being isolated from this class.[28]

1.4 Natural Products Isolated from Bryozoans

1.4.1 Introduction

Families of alkaloids with at least a single compound containing bromine functionality are reported herein. All of the bryozoans reported to contain these compounds are from the orders Cheilostomata and Ctenostomatida. This is, perhaps, due to these orders belonging to the same class, Gymnolaemata, which has the greatest number of species.[29] The majority of natural products isolated from species of *Amathia* (additionally *Zoobotryon*, now *Amathia*)[31] are brominated alkaloids. The Genus *Amathia* is seemingly one of the most extensively investigated genera of bryozoans in natural products research. Many species of *Amathia* reported below produce a range of brominated amino acid-like compounds based on tryptophan, proline, leucine and tyrosine and it is likely that many more additional natural products isolated from species of *Amathia* will follow this trend.

1.4.2 Survey of Brominated Alkaloids Isolated from Bryozoans

Studies on *Flustra foliacea* were among the first on a marine source which yielded structurally and pharmacologically interesting alkaloids. The *cis*-fused dipyrrolidine brominated alkaloids, flustramines A (**3**) and B (**4**), were extracted from *F. foliacea*. The structures are reminiscent of physostigmine alkaloids of the Calabar bean, however they contain the additional feature of bromination. Based on the Ultraviolet (UV) absorption spectrum, the extract was purified by standard chromatographic methods. Characterisation was also achieved by standard methods, namely mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy.[32,33]

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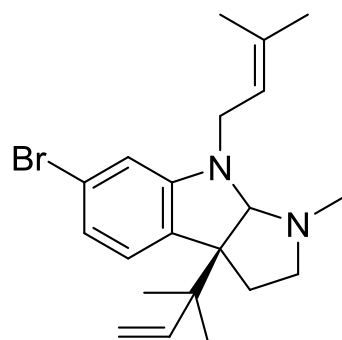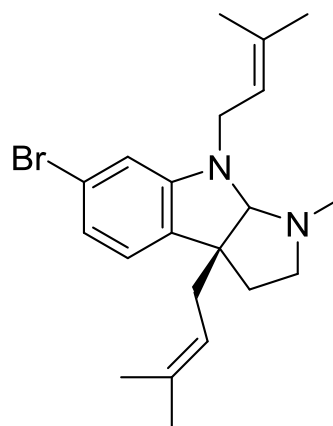
(3)   **(4)**

Flustramine C (**5**) and the flustraminols A (**6**) and B (**7**) were also isolated from *F. foliacea*. Like the flustramines, flustraminols A and B possess a physostigmine skeleton with bromination exclusively at C-6. Another consistent moiety within flustramines and flustraminols is the presence of at least one isoprene unit, whether it be a 3-methyl-2-butenyl, or the unusual 2-methyl-3-buten-2-yl substituent, which is an inverted form of the former. Isolation of compounds (**3**), (**4**), and (**5**) was carried out from an ethanol extract using common methods of fractionation and chromatography and characterisation was achieved by standard methods.[34] Methylation at N_1 remains consistent within the flustramines and flustraminols, whether the isolation procedure used methanol or not, so it is improbable that the N_1 methyl amide is an isolation artefact.
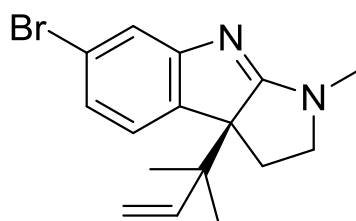
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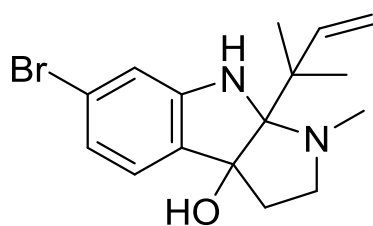
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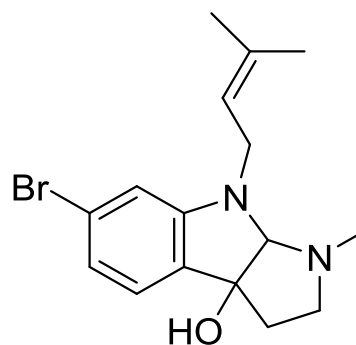
(7)

Flustrabromine **(8)**, flustramide A **(9)** and the brominated tryptamine, 6-bromo-*Nb*-methyl-*Nb*formyltryptamine **(10)** were also isolated from *F. foliacea*,[35,36] as well as the first reported naturally occuring bromo-substituted quinoline, 7-bromo-4-(2-ethoxyethyl)-quinoline **(11)**. Common extraction and isolation methods were used for the flustramines.[37] Dihydroflustramine C **(12)** was isolated from a dichloromethane extract of *F. foliacea* and this protonated form of flustramine C showed potent antibacterial activity against *Bacillus subtilis*.[38]

(8)

(9)

(10)

(11)

(12)

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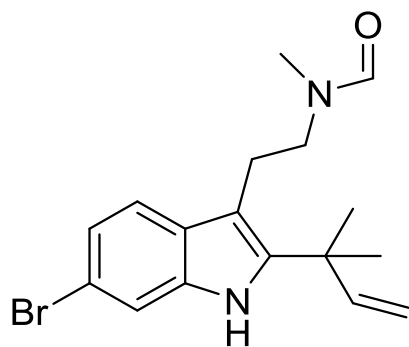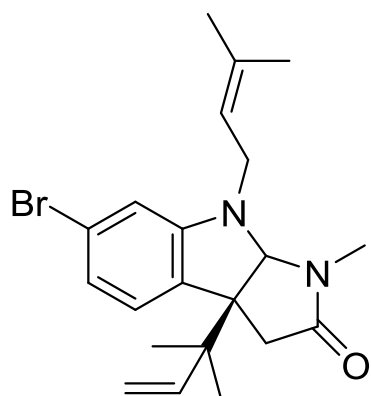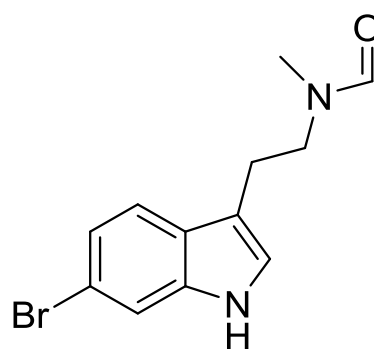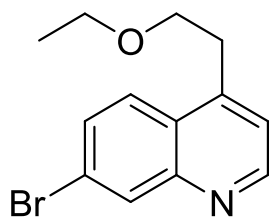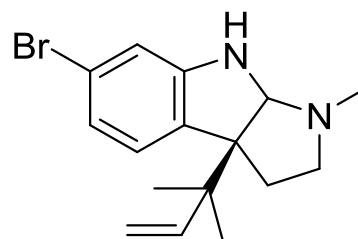
Flustramide B **(13)** and flustrarine B **(14)** were isolated from a Scandinavian collection of *F. foliacea*. A petroleum ether and an ethyl acetate (EtOAc) extract were fractionated by High performance Liquid Chromatography (HPLC). Characterisation of flustramide B was achieved by comparison of Infrared (IR), UV, and NMR spectroscopic data to thoes of flustramide A **(9)**. Flustrarine B had similar spectroscopic properties to that of flustramine B **(13)**, however the mass spectrum showed the addition of an oxygen to the structure. Structural elucidation was achieved by the usual methods.[39]
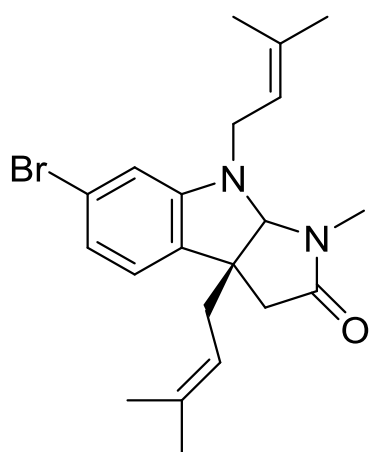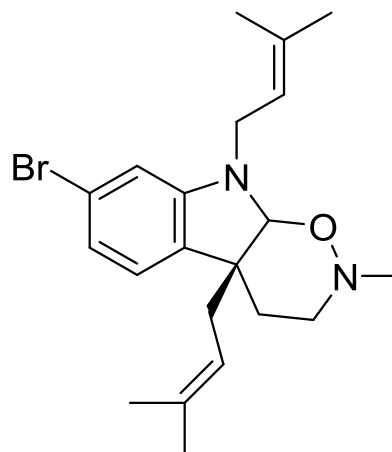
| **(13)** | **(14)** |

From a methanol extraction of a Nova Scotian collection of *F. foliacea*, dihydroflustramine C **(12)** has also been found. However, flustramine C **(5)**, the likely precursor to this potent antibacterial compound could not be detected in this

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extract. Flustramine D (**15**), flustramine D *N*-oxide (**16**), isoflustramine D (**17**) and dihydroflustramine C *N*-oxide (**18**) were also isolated from this sample. The crude extracts were chromatographed on a pad of silica gel, then subjected to rotating-disc adsorption chromatography and HPLC, piror to characterisation by usual methods. [40] Flustramine E (**19**) and debromoflustramine B (**20**) have been reported from a North Sea sample of *F. foliacea*,.[41]
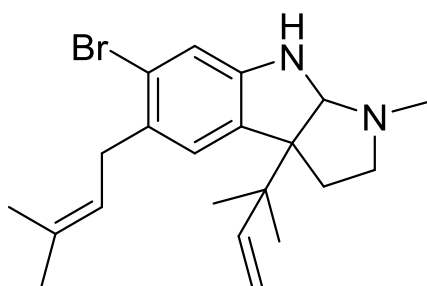
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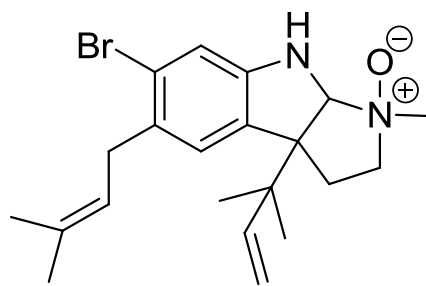
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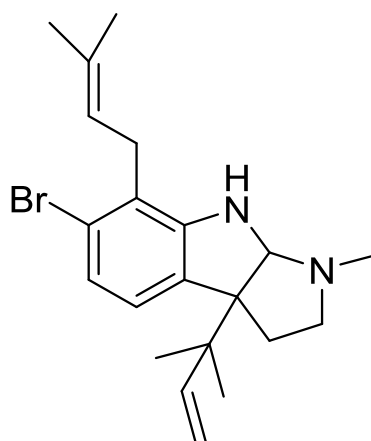
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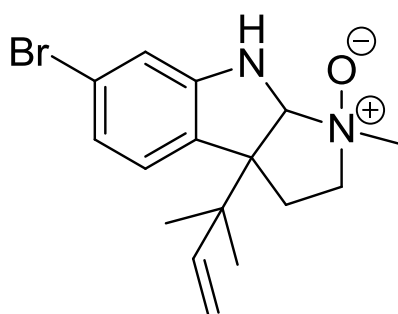
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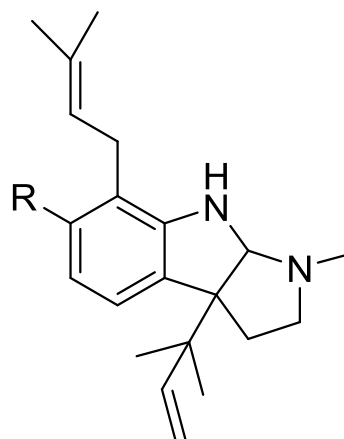
(19) R=Br
(20) R=H

Fifteen years after the discovery of flustramine E, eleven new flustramines, F-P **(21-31),** were isolated in a 2009 study from dichloromethane extracts of both the New Brunswick and Nova Scotian strains of *F. foliacea*.[8] The apparent increase in the number of secondary metabolites isolated in one study is contributed, in part, to the increased sensitivity of chromatographic and spectroscopic instruments available. Unlike in previous studies of the flustramines, two-dimensional NMR spectroscopy was utilised for the determination of heteronuclear correlations.[42]
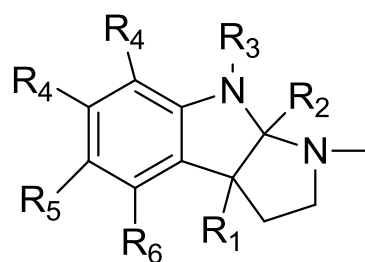
(21) R_1 = Ac; R_2 = Y; R_3, R_4, R_6 = H; R_5 = Br
(22) R_1, R_3, R_6 = H; R_2 = Y; R_4, R_5 = Br
(23) R_1, R_4, R_5 = H; R_2 = X; R_3 = OH; R_6 =Br
(24) R_1, R_4, R_5 = H; R_2 = X; R_3 = OH; R_6 = Br
(25) R_1, R_5 = H; R_2 = Z; R_3 = OH; R_4, R_6 = Br
(26) R_1, R_5 = H; R_2 = A; R_3 = OH; R_4, R_6 = Br
(27) R_1, R_5 = H; R_2, R_4 = A; R_3 = OH; R_6 = Br

X=   Y=   Z=

(28)

(29)

(30)

19
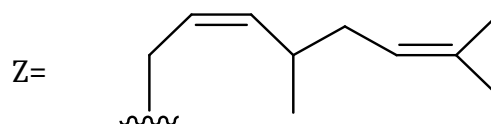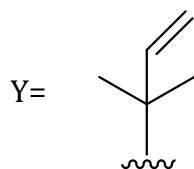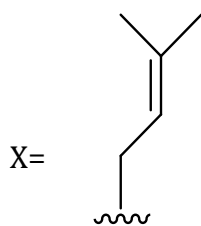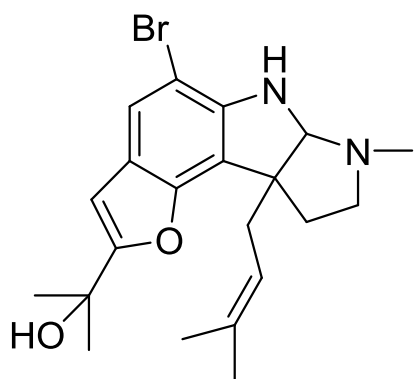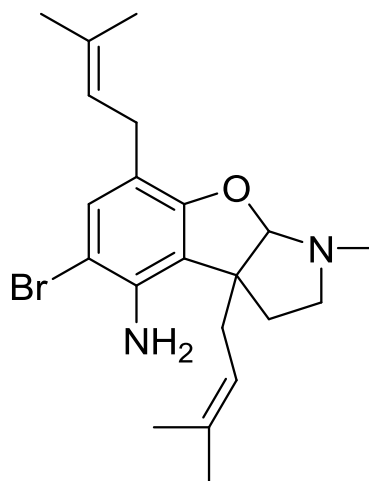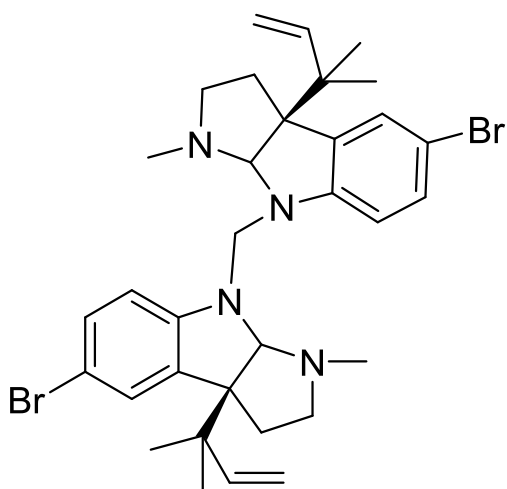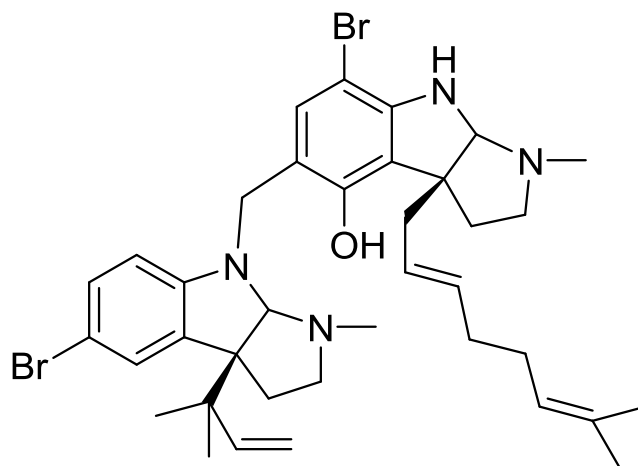
(31)

The majority of brominated alkaloids isolated from bryozoan species belong to the order Cheilostomata such as the *Bugulidae*, *Flustridae* and *Catenicellidae* families. The bromopyrrole alkaloids, aspidostomides A-H **(32-39)**, aspidazide A **(40)** and 9-*O*-ethyl aspidostomide C **(41),** which were isolated from the Patagonian bryozoan *Aspidostoma giganteum*, were the first obtained from the *Aspidostomatidae* family, and further extended the brominated alkaloids known from bryozoans of the order Cheilostomata. Aspidostomides A-H **(32-39)** were extracted then fractionated using silica gel chromatography then purified by preparative HPLC. Techniques used during the characterisation of these alkaloids reflect the usual analytical tools applied in modern structural elucidation studies. Two-dimensional NMR spectroscopy, [1]H-[1]H Correlation Spectroscopy (COSY), [1]H-[13]C Heteronuclear Single Quantum Coherence (HSQC), [1]H-[13]C Heteronuclear Multiple Bond Correlation (HMBC) and Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were utilised, as well as High Resolution Mass Spectrometry (HRMS) and chemical transformations. Apidostomide E **(36)**

showed activity against the 786-O renal carcinoma cell line. Aspidazide A **(40)** contains a rare N-N linkage between two lactams, yet this could not be unambiguously characterised by correlation NMR spectroscopy. Mass spectral analysis and NOE correlations between protons of the different parts of the molecule gave evidence for the existence of a diacylazide dimeric structure in Aspidazide A **(40)**, made up by the union of two $C_{13}H_5Br_4N_2O_2$ moieties. The compound was reduced with sodium hydride and methyl iodide in dichloromethane in an attempt to confirm the presence of the diacylazide bond. Two products, the corresponding monomers with *N*-methylation and *O*-methylation of the phenol functionalities were isolated and characterised unambiguously by standard methods.[43]
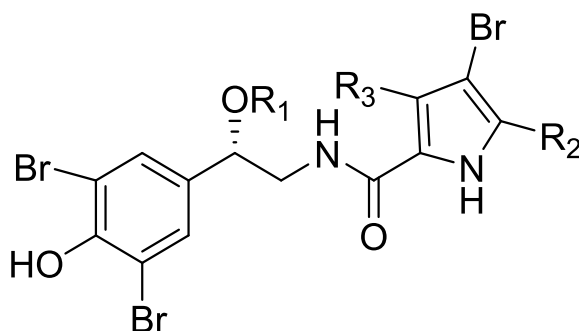
(32) $R_1=R_2=H$; $R_3=Br$
(33) $R_1=R_3=H$; $R_2=Br$
(34) $R_1=H$; $R_2=R_3=Br$
(41) $R_1=Et$; $R_2=R_3=Br$

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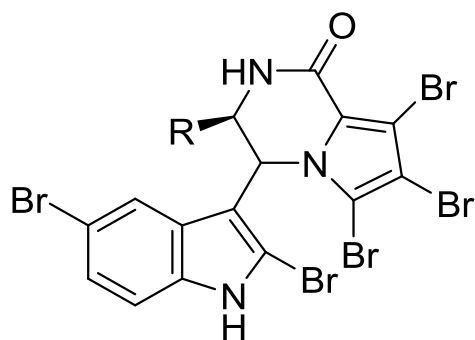
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(36) R=OMe
(37) R=H
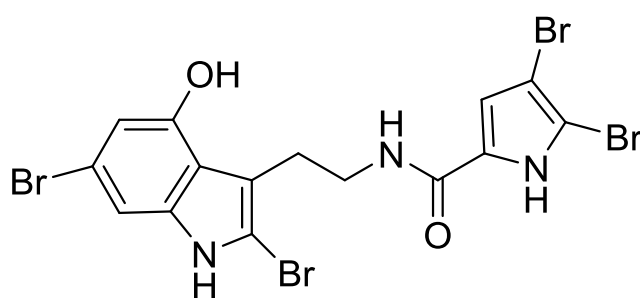
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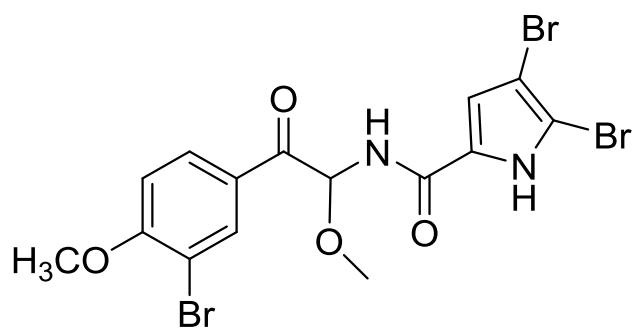
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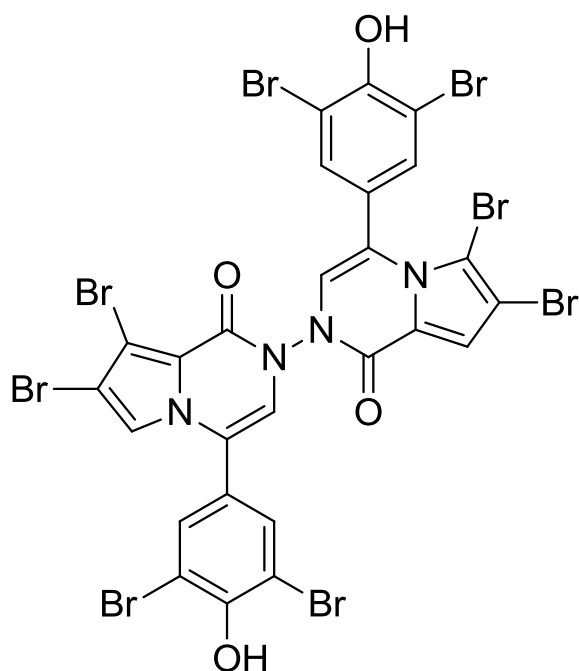
(40)

From a San Diego collection of *Amathia verticillata* (previously *Zoobotryon verticillatum*), the indole derived metabolites 2,5,6-tribromo-*N*-methylgramine **(42)** and its corresponding *N*-oxide **(43)** have been reported. Pure samples of compounds **(41)** and **(43)** were obtained by standard chromatographic methods. Characterisation was achieved by HRMS and NMR spectroscopy. The structures were confirmed by comparing the melting point and spectroscopic data of the natural products to thoes of the synthesised compounds.[44] Interestingly compound **(42)** had already been synthesised before being isolated from a natural source.[45]

A debrominated form of **(42)**, 2,6-dibromo-*N*-methylgramine **(44)** has been reported from collections of *A. verticillata* from Cabo Frio and Porto Belo, Brazil, as well as from Little Jim and Coon Island, Florida, from a single study. Standard

chromatographic and structural analysis methodologies were utilised. Feeding experiments were undertaken to establish if the widespread invasion of *A. verticillata* into tropical to warm-temperate waters was due to chemical defences against predation. No correlation between concentrations of (42) in samples of *A. verticillata* and feeding deterrence by fishes belonging to Chaetodontidae, Haemulidae, Labridae and Pomacentridae families was observed. Every sample of *A. verticillata* containing (42) tested did not deter feeding. This study suggests the success of invasion by *A. verticillata* is not due to chemical defences deterring predation.[46]
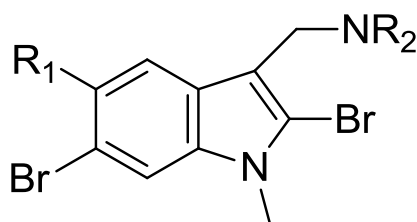
(42) R_1=Br, R_2=(CH$_3$)$_2$
(43) R_1=Br, R_2=O(CH$_3$)$_2$
(44) R_1=H, R_2=(CH$_3$)$_2$

2,5,6-Tribromo-*N*-methylindole-3-carbaldehyde (45) was also isolated from *A. verticillata*, however from collections off the Spanish coast, and underwent standard chromatographic methods to yield a pure sample of compound (45), as well as compounds (41) and (43). Characterisation was carried out by standard analytical methodologies. The origin of compound (45) has been hypothesized to

be from acetylation of compound **(43)**. This was experimentally shown to occur *in vitro* by reaction of **(43)** with acetic anhydride in pyridine, which yielded compound **(45)** and was proposed to occur after two successive rearrangements, firstly the Polonovski rearrangement, then a rearrangement of the acetoxy moiety to acetaldehyde, to give **(45)**. Cell division in sea urchin eggs was potently inhibited by compounds **(41)** and **(43)**. While compound **(45)** only delayed metamorphosis within cell division.[47] Pharmacological applications of crude extracts from *A. verticillata* sourced off the coast of India showed anti-inflammatory and central nervous system stimulation comparable to the positive controls of pentazocine and caffeine respectively.[48]
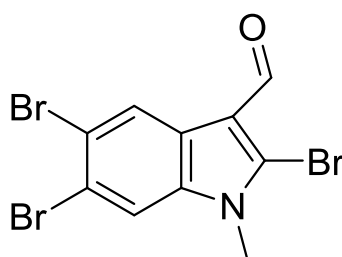
(45)

The bromotryptamine derived alternatamides A-D **(46-49)** were extracted from a sample of *Amathia alternata*. The extract was purified by standard methods. Structures were elucidated by one- and two-dimensional NMR spectroscopy and HRMS.[49] Like the flustramines, bromination occurs predominantly at the same sites upon the aromatic ring for alternatamides A-D **(46-49)** as for the flustramines. The flustramine compounds **(4**, **5**, **7-11**, **13-15)** all exhibit bromination at C-6, as do the alternatamides A **(46),** B **(47)**, and D **(49)** This is a

site commonly brominated in tryptamine/tryptophan-like natural products, possibly signifying a relatable metabolic function within marine invertebrates.[15]
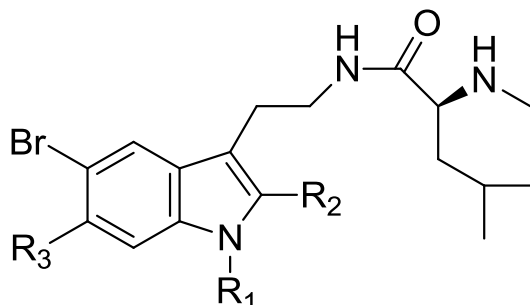
(46) R_1=Me; R_2=R_3=Br
(47) R_1=H; R_2=R_3=Br
(48) R_1=R_3=H; R_2=Br
(49) R_1=R_2=H; R_3=Br

From the Australasian species *A. wilsoni* Kirkpatrick, collected off the coast of Tasmania, the amathamides A-H, **(50-57)**, tri-, di- and monobrominated proline-derived compounds have been discovered. All of the amathamides were isolated from *A. wilsoni* except for amathamide G which was isolated from a Tasmanian collection of *A. convoluta*. A methanol extract of *A. wilsoni* was purified by standard chromatographic methods to afford the amathamides A-G. Amathamide H was isolated using the unusual method of packing sample adsorbed silica beads into a HPLC column then subjecting the sample to multiple HPLC isolation steps. Amathamide H was not very soluble in dichloromethane and so remained undiscovered in previous studies. The amathamides were characterised by standard spectroscopic methods. [50-53]
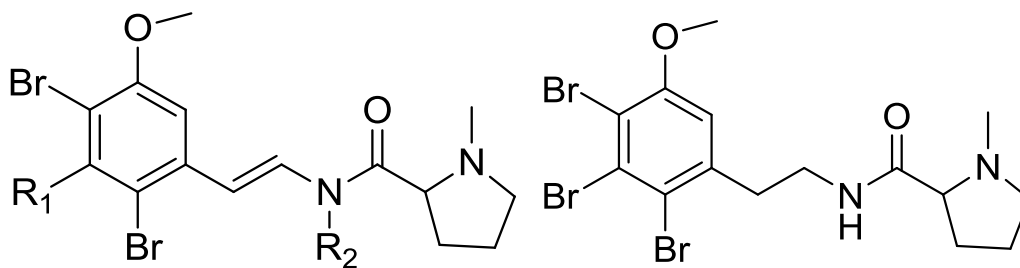
(50) R₁=R₂=H
(52) R₁=Br; R₂=Me
(54) R₁=Br; R₂; =H
(56) R₁=OCH₃; R₂; =CH₃

(51) R=H
(55) R=Br
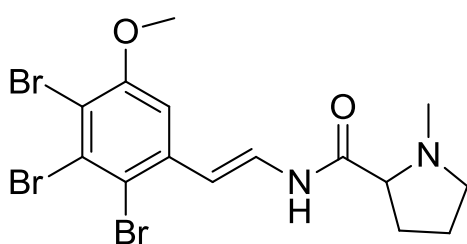
(53)
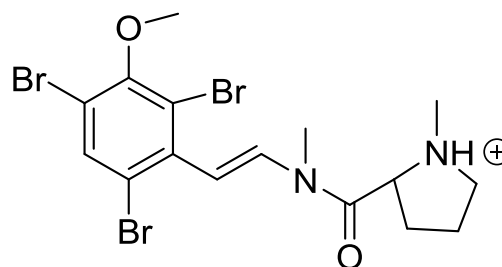
(57)

In addition to amathamide H, the wilsoniamines A **(58)** and B **(59)**, possessing a hexahydropyrrolo[1,2-*c*]imidazol-1-one scaffold previously unreported in nature, were isolated from *A. wilsoni*. Extraction and isolation methods were identical to those mentioned for amathamide H. Amathamide C was rediscovered by Carrol and co-workers, and found to contain a 2,4,6-tribromo-3-methoxyphenyl moiety instead of the 2,3,4-tribromo- 5-methoxyphenyl moiety previously reported.[51,52] Researchers advised that the structures proposed for amathamides C – F **(52-55)**

should be revised so they contain instead the 2,4,6-tribromo-3-methoxyphenyl moiety. Wilsoniamines A and B were found to have no antimalarial and antitrypanosomal activity, unlike amathamides C **(52)** and H **(57)**, which showed modest activity. It was proposed that a lack of cell permeability influenced the wilsoniamines lack of activity. [53]
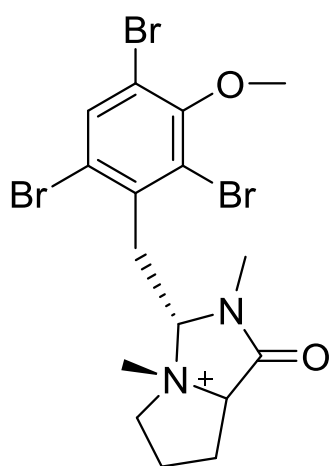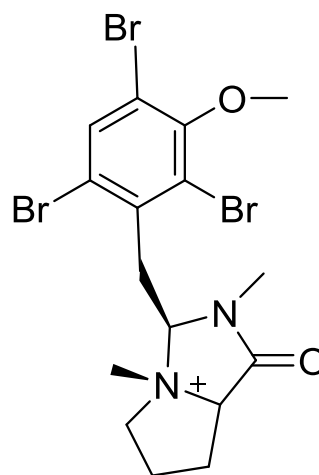
(58)   **(59)**

Another family of brominated alkaloids, the amathaspiramides A-F, **(60-65)** respectively, were isolated from a collection of *A. wilsoni* from Wellington, New Zealand. Data from single-crystal X-ray diffraction of amathaspiramide A was used for structural conformations and absolute configuration determinations. Amathaspiramide E **(64)** showed strong antiviral activity against the type 1 *Polio* virus. It was demonstrated that no amathamides were present in the New Zealand strain. This could be due to genetic variation or different environmental conditions. Levels of amathaspiramides

were found to be consistent within different populations of *A. wilsoni* collected at the same site.[54]

It is possible that the differences in brominated alkaloid content between Tasmanian and Wellington *A. wilsoni* populations could be due to differing symbiotic bacteria. It has become well known that secondary metabolites from marine invertebrates are, at least in part, due to symbiotic bacteria.[55] As the Tasmanian and Wellington *A. wilsoni* populations differ and marine bacteria have been shown to produce brominated alkaloids, this lends weight to the hypothesis that the origin of the amathamides and amathaspiramides is, at least in part, due to bacteria. A bacterium, *Pseudomonas bromoutillis*, has been found to contain a 2,3,4-tribromo5(1' hydroxy,2',4' dibromophenyl)pyrrole, a compound similar in structure to the amathamides.[56]
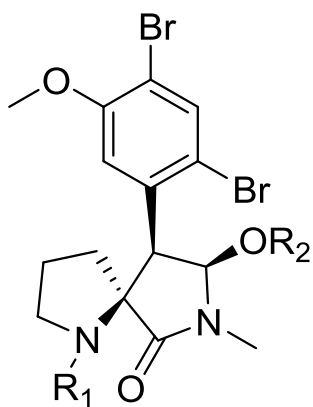
(60) R$_1$=Me; R$_2$=H
(62) R$_1$=R$_2$=H
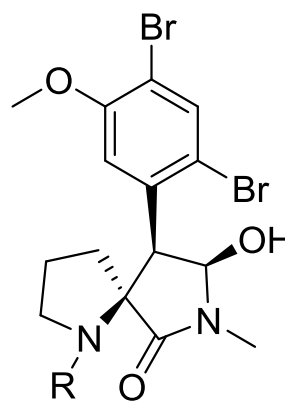
(61) R=Me
(63) R=H
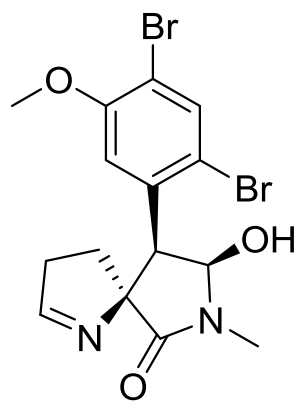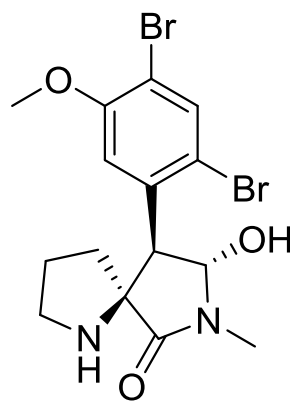
(64)   **(65)**

The leucine and tyrosine derived volutamides A-E (**66-70**), were isolated from the Atlantic species *A. convoluta*, collected from Morehead City Port, North Carolina. The samples were isolated by standard methods, guided by bioactivity of a hydroid larvae (*Eudendrium carneum*) settling bioassay. The structures were determined by standard spectroscopic methods as well as by chemical methods. Volutamide B (**67**), and C (**68**) effectively deterred feeding by predators and volutamide B (**67**) and D (**69**) were toxic towards larvae of the aforementioned hydroid. Therefore these metabolites likely form the basis of the bryozoan's chemical defence.[57]
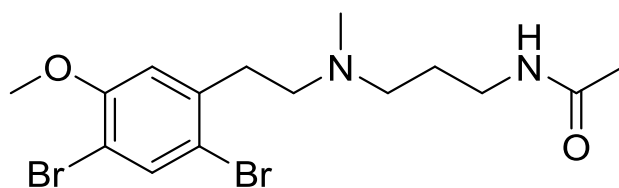
(66)
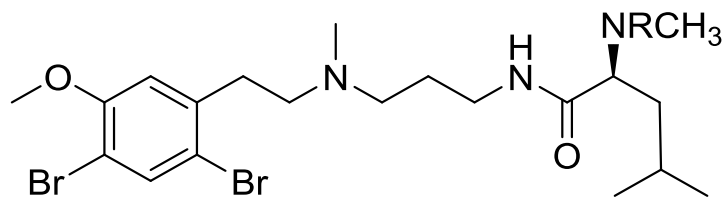
(67) R=H
(68) R=CH₃
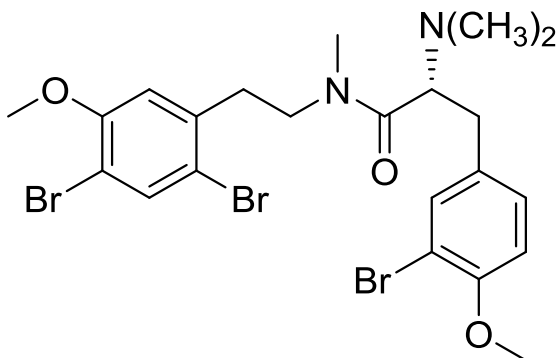
(69)
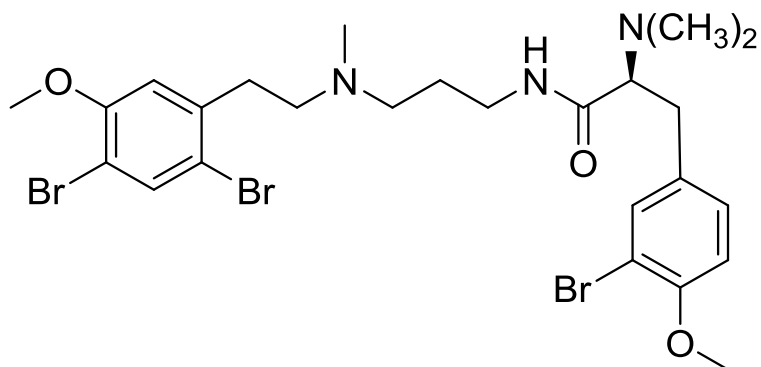
(70)

The convolutamines A-J **(71-80)** are beta-phenylethylamine containing metabolites found in *A. convoluta* and *A. tortusa*. Convolutamines A-D **(71-74)**, were isolated from a collection of *A. convoluta* from off the coast of Florida. Convolutamines A-C **(71-73)** are monobrominated whilst convolutamine D **(74)** is dibrominated.[58] Convolutamines E-G **(75-77)** were obtained from another

collection of *A. convoluta* off the coast of Florida, and are also dibrominated.[59] Convolutamine H **(78)** was extracted from an Australian strain of *A. convoluta* collected off the coast of Tasmania along with a tribrominated indole-based compound, convolutindole A **(81)**. Convolutamine H **(78)** and convolutindole A **(81)** were found to be potent nematocidal agents.[60] Convolutamines I **(79)** and J **(80)** were extracted from another *Amathia* species, *A. tortusa*, which was also sourced from Tasmania, and both showed toxicity towards the parasite that causes Human African trypanosomiasis, *Trypanosoma brucei brucei*.[61]
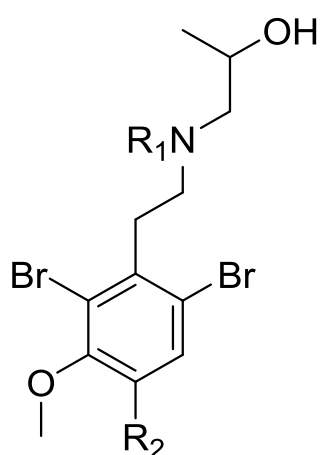
(71) R_1=Me; R_2=Br
(72) R_1=Me; R_2=H
(73) R_1=H; R_2=Br
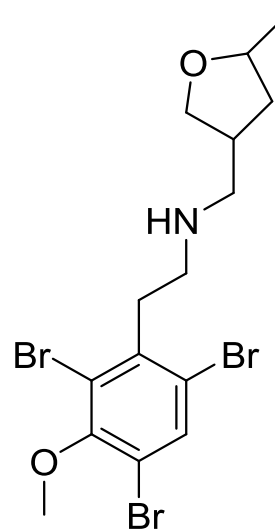
(74)
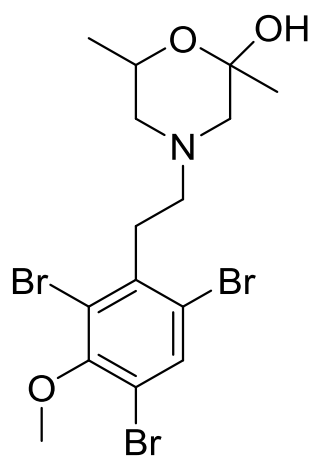
(75)
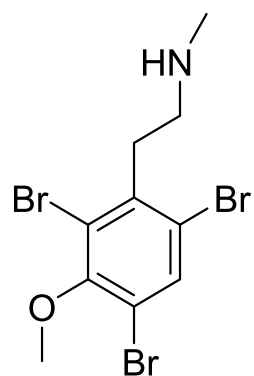
(76)
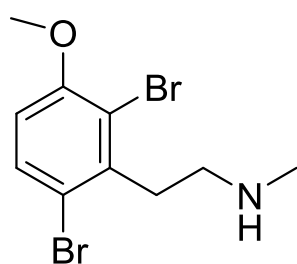
(77)
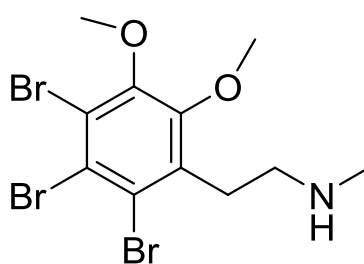
(78)
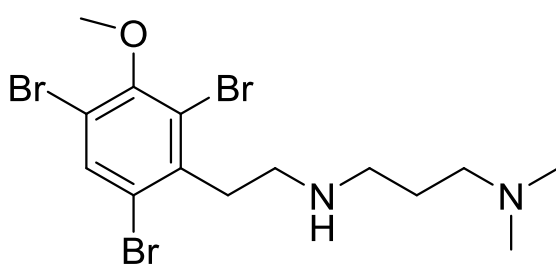
(79)
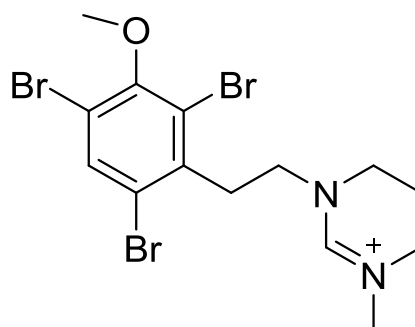
(80)
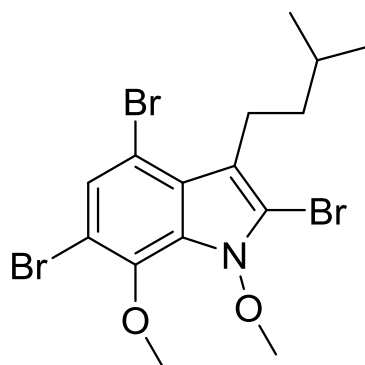
(81)

The dibrominated convolutamides A-F, **(82-87)**, which contain an *N*-acyl-γ-lactam moiety, have also been isolated from the bryozoan *A. convoluta*. A collection of *A. convoluta* from the Gulf of Mexico afforded the convolutamides as mixtures of pure compounds.[62] Also extracted from a collection of *A. convoluta* sourced from the Gulf of Mexico, were the convolutamydines A-E. The convolutamydines **(88-92)**, are based on indole alkaloids with bromination at C-6, thus resemble convolutindole A **(81)**.[59,63,64]

(82) R= $CH_3(CH_2)_{11}$

(83) R= $CH_3(CH_2)_{13}$

(84) R= $CH_3(CH_2)_{15}$

(85) R= $(CH_2)_6CH=CH(CH_2)_5$

(86) R= $(CH_2)_6CH=CH(CH_2)_7$

(87) R= $(CH_2)_5CH=CH(CH_2)_{10}$

(88)

(89)

35
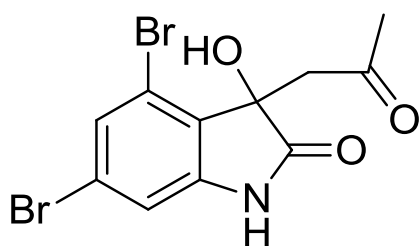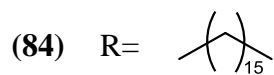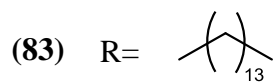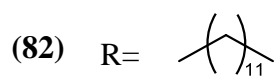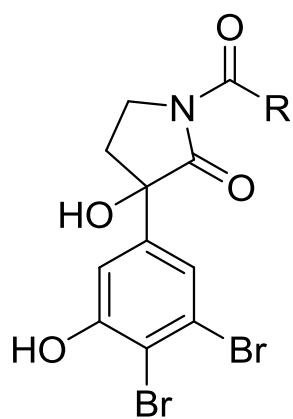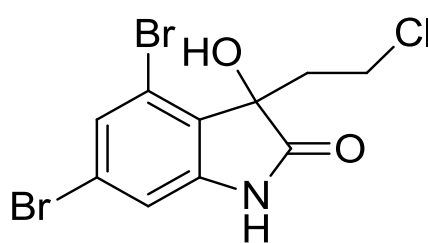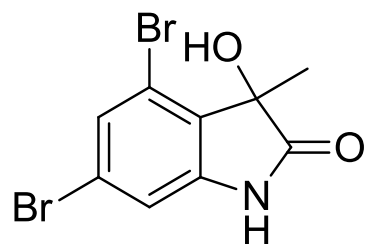
(90)
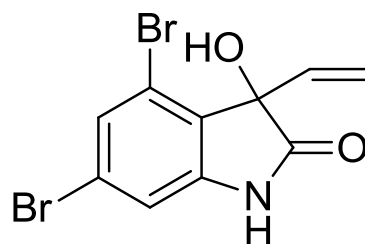
(91)
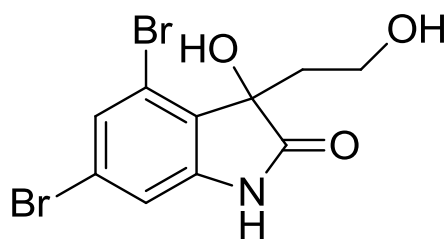
(92)

With renewed interest in bryozoans, researchers at Griffith University, Australia, have embarked on a programme to investigate the chemistry of temperate and subtropic bryozoans of Australia. Previously described compounds discovered from this campaign were the wilsonamines A and B and amathamide H. Researchers at Griffith University have also discovered new tribrominated indole-based compounds, kororamide A **(93)** and B **(94)**. Kororamide A was isolated from *A. tortuosa* using the same methods mentioned as for the isolation of Amathamide H. The structure of kororamide A was elucidated by standard spectroscopic methods. Convolutamine F was also isolated in addition to kororamide A, and both were tested for activity against chloroquine-sensitive and resistant strains of the parasite *Plasmodium falciparum* which causes Malaria. Both compounds were marginally active against both the chloroquine-sensitive and resistant strains.[65]
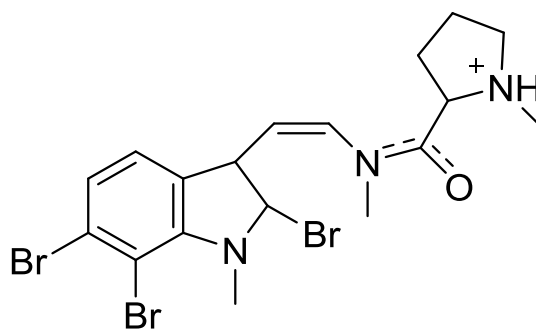
(93)

The isolation and structural elucidation of kororamide B **(94)**, isolated from *A. tortuosa*, highlights the recent advancements in NMR and MS based dereplication procedures. Small scale extractions of *A. tortuosa* were mixed and purified by solid phase extraction (SPE), and HPLC. The presence of three known brominated alkaloids were detected by their MS and NMR features, kororamide A **(93)**, convolutamine J **(80)** and convolutamine I **(79)**, as well as that of a previously unidentified brominated alkaloid. A large scale extraction of *A. tortuosa* following the same methods as above was carried out to isolate enough of the unknown alkaloid for comprehensive structural elucidation. Researchers found this unknown metabolite to be kororamide B **(94)** a tryptophan-based metabolite. Kororamide B also contained a hexahydropyrrolo[1,2-c]imidazole-1-one moiety as present in wilsonamines A and B.[66]
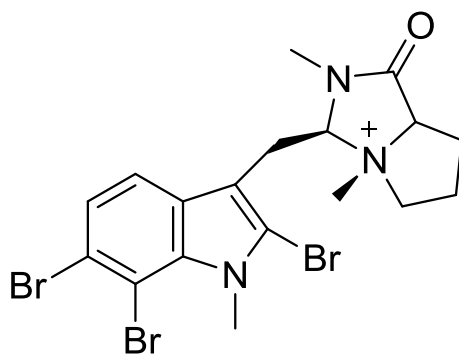
(94)

Pterocella vesiculosa collected from the Alderman Islands, off the North Island of New Zealand, yielded the compounds 5-bromo-8-methoxy-1-methyl-beta-carboline **(95)**, and 7-bromo-1-ethyl-beta-carboline **(96)**. Purification and structural elucidation was achieved by standard methods. Compound **(95)** showed activity against *Bacillus subtilis* as well as two fungi with Minimum Inhibitory Dose (MID) values in the low µg/mL range as well as moderate activity, with a concentration at 50% growth inhibition (IC$_{50}$) value of 5089 ng/mL, against the P388 murine leukaemia cell line.[67] Data found in a structure-activity relationship study of mono-substituted beta-carboline alkaloids from bryozoans suggests that the bromine substituent at C-5 enhances activity.[68,69]
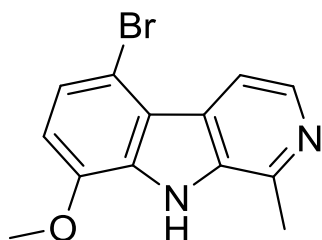
(95)
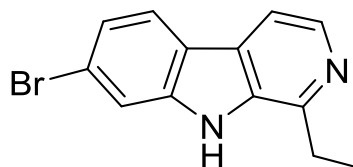
(96)

From the Australasian species *Euthyroides episcopalis*, the interesting family of brominated quinone methides, the euthyroideones A-C, **(97-99)**, have been reported. A collection of the bryozoan from Fiordland, off the South Island of New Zealand afforded the euthyroideones which was then structurally elucidated by standard methods including single-crystal X-ray diffraction crystallography. The euthyroideones show little or no activity against the P388 murine leukaemia cell line nor antiviral or antimicrobial activity.[70]

(97)   **(98)**   **(99)**
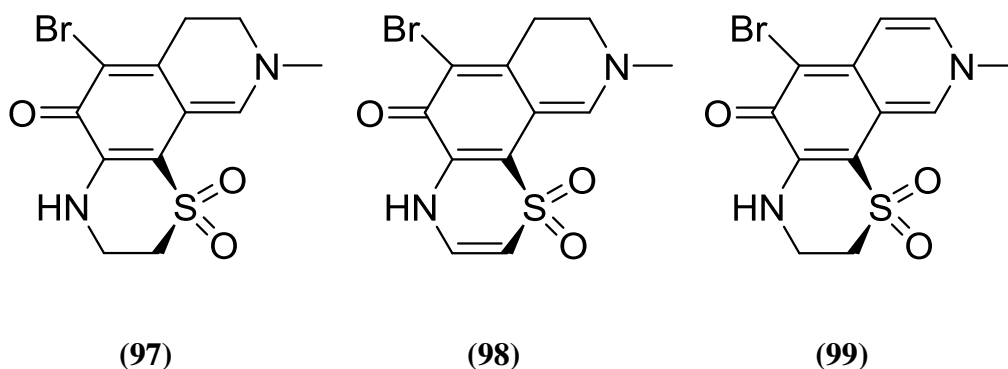
The bipyrrole alkaloids, tambjamines have been isolated from the bryozoans, *Sessibugula translucens*,[71] and *Bugula dentata*. Tambjamines A-D,[72] (**100-103**), were isolated from methanol extracts of the bryozoan *Sessibugula translucens* and its predatory nudibranchs *Tambje eliora* and *T. abdere*. A carnivorous nudibranch *Roboastra tigris*, that preys on the *Tambje* nudibranchs was repelled by a yellow mucus produced by *T. abdere* which was found to contain a high level of the tambjamines. The nudibranch *T. abdere* sequesters secondary metabolites for

39

defense, taken from *S. translucens*.[71] Tambjamines E **(104)** and F **(105),** together with tambjamines A **(100)** and C **(102)**, were not found in a bryozoan, but were instead isolated from an ascidian *Atapozoa sp*.[72]

Additional tambjamine alkaloids, tambjamines G-K **(105-110)** have been obtained from multiple studies on Tasmania, Australia collections of *Bugula dentata*. One collection of *Bugula dentata* afforded tambjamines G-J **(106-109)** as well as the previously reported tambjamines C **(102)** and E **(104)**. Tambjamines E **(104)**, G **(106)** and I **(108)** were found to kill larvae of the brine shrimp *Artemia salina*.[73] Tambjamine K **(110)** was discovered in another study on a Tasmanian collection of *B. dentata,* as well as tambjamines A **(100)** and B **(101)**.[74] Tambjamines J **(109)** was also found from the bryozoan's predator, the nudibranch mollusk *Tambje ceutae*. Tambjamine K **(110)** displayed potent cytotoxicity against human tumour and non-tumour cell lines CaCo-2 (human epithelial colorectal adenocarcinoma cells) abd HeLa (human cervical cancer cells) as well as against the non-human cell lines C6 (rat glioma cells), H9c2 (rat cardiac myoblast cells) and 3T3-L1 (murine fibroblasts).[74]
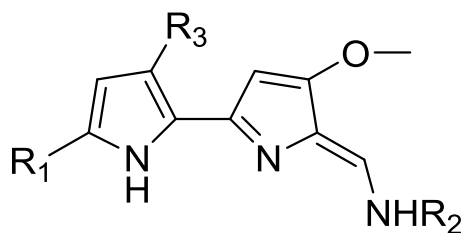
(100) $R_1 = R_2 = R_3 = H$
(101) $R_1 = Br; R_2 = R_3 = H$
(102) $R_1 = R_3 = H; R_2 = CH_2CH(CH_3)_2$
(103) $R_1 = H; R_2 = CH_2CH(CH_3)_2; R_3 = Br$
(104) $R_1 = H; R_2 = CH_2CH_3; R_3 = Br$
(105) $R_1 = R_3 = H; R_2 = CH_2CH_2Ph$
(106) $R_1 = Br; R_2 = CH_2CH_3; R_3 = H$
(107) $R_1 = Br; R_2 = CH_2CH_2CH_3; R_3 = H$
(108) $R_1 = Br; R_2 = CH_2CH(CH_3)_2; R_3 = H$
(109) $R_1 = Br; R_2 = CH_2CH(CH_3)_2; R_3 = H$
(110) $R_1 = Br; R_2 = CH_2CH(CH_3)CH_2CH_3; R_3 = H$
(111) $R_1 = Br; R_2 = CH_2CH_2CH(CH_3)_2; R_3 = H$

The chartellines A-C, **(112-114)**, are unusual beta-lactam-imidazole alkaloids found in the bryozoan *Chartella papyracea* collected off the coast of Roscoff, France. Chartelline A was first to be isolated using common chromatographic methods.[75] After the initial investigation of *C. papyracea*, two further members of the family, chartellines B **(113)** and C **(114)**, as well as a chlorinated methoxy analogue of chartelline A **(112)**, methoxy-dechlorochartelline A **(115)**, an artefact of isolation, were obtained from an ethyl acetate extraction. The chartellines contain a beta-lactam ring condensed with a ten-membered heterocyclic ring which contains three degrees of unsaturation. The chartellines differ amongst themselves in the positioning and extent of halogenation.[76]
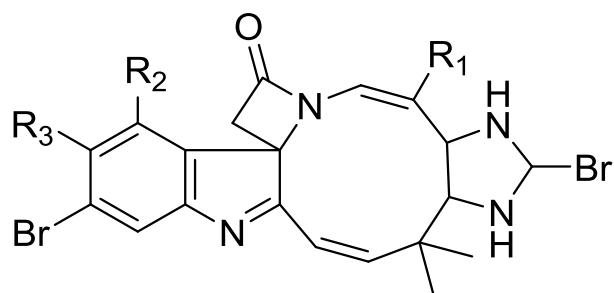
(112) R₁ = Cl; R₂ = R₃ = Br
(113) R₁ = Cl; R₂ = Br R₃ = H
(114) R₁ = Cl; R₂ = R₃ = H
(115) R₁ = OCH₃; R₂ = R₃ = Br

Further brominated alkaloids, the chartellamides A **(116)** and B **(117)** were isolated from *C. papyracea*. Like the chartellines, compounds **(116)** and **(117)** are beta-lactam-imidazole alkaloids, however they contain a complex hexacyclic skeleton.[77]
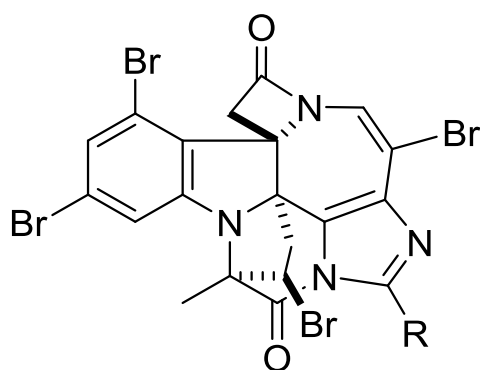
(116) R=H
(117) R=Br

Another family of brominated beta-lactam alkaloids have been reported from a bryozoan belonging to the same family (Flustridae) as *C. papyracea*. A North Sea collection of *Securiflustra securifrons* afforded the securamines A-D, **(118-121)**. The general conformation of the securamines is similar to that of the previously

reported beta-lactam alkaloids from *C. papyracea*. Securamines B (**119**) and C (**120**) are brominated analogues of securamines A (**118**) and D (**121**).[78] The other members of the family were isolated from a separate study on *S. securifrons*. Additionally, structural variants of tambjamine C (**102**) were found, securamines E-G, (**122-124**) respectively.[79] In the initial study of *S. securifrons*, the isolation of securines A (**125**) and B (**126**) were reported. Researchers observed that the securines were present in equilibrium with securamides A and B respectively, when securamine A and B were dissolved in deuterated dimethyl sulphoxide-d_6 (DMSO-d_6).
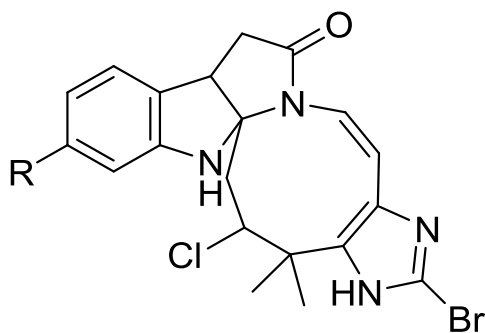
(**118**) R = H
(**119**) R = Br
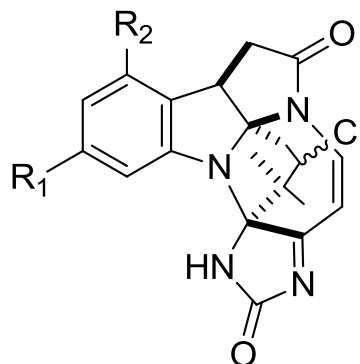
(**120**) R$_1$=R$_2$=H
(**121**) R$_1$=Br; R$_2$=H
(**122**) R$_1$=R$_2$=Br
(**123**) R$_1$=Br; R$_2$=H

(**124**)

(**125**) R=H
(**126**) R=Br

43
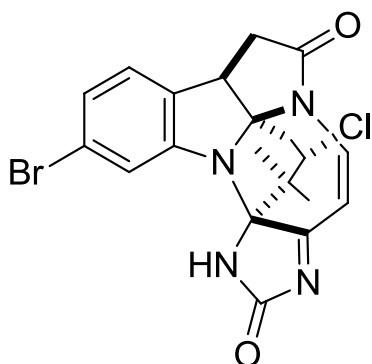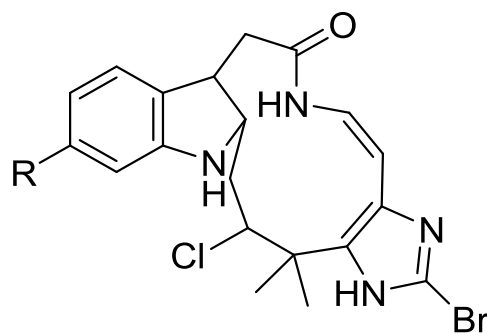
An extract of Indo-Pacific collections of *Caulibugula intermis* showed cytotoxicity in a National Cancer Institute (NCI) 60 cell line antitumour screen, prompting further investigation of the extract. Six new compounds, caulibugulones A-F, **(127-132)**, were isolated from bioactivity guided fractionation of a cytotoxic aqueous extract. The caulibugulones are isoquinoline, quinones, or iminoquinone-based metabolites but caulibugulone B **(128)** is the only brominated member of the family. The caulibugulones showed potent cytotoxicity, with the highest activity being reported for caulibugulone E **(130)**, which had an IC_{50} of 0.03 $\mu g/mL^{-1}$ against the murine IC-2WT Cell Line. Bromination did not seem to have a substantial effect on the cytotoxicity of the caulibugulones as caulibugulone B **(128)** had an IC_{50} of 0.22 $\mu g/mL$ against the same cell line whereas calibugulone A **(127)**, a debrominated form of caulibugulone B had an IC_{50} of 0.34 $\mu g/mL$.[80]
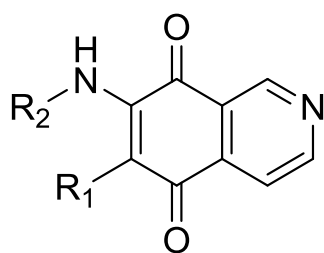
(**127**) R_1 = H; R_2 = CH_3
(**128**) R_1 = Br; R_2 = CH_3
(**129**) R_1 = Cl; R_2 = CH_3
(**130**) R_1 = H; R_2 = CH_2CH_3OH
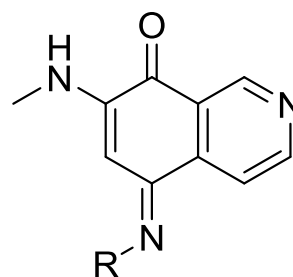
(**131**) R = H
(**132**) R = CH_2CH_2OH

Chapter Two

Survey of Bryozoan Species for Brominated

Natural Products

2.1 Introduction

As discussed in chapter one, bromination is a common feature of marine
metabolites in general, and is especially prevalent in bryozoan metabolites.
Sixteen bryozoan samples have been surveyed for the presence of bromine
containing compounds, by LCMS, with the intention to discover possible novel
compounds of significance. Small scale, crude organic extracts of bryozoans,
collected from the United Kingdom and New Zealand, were analysed for
characteristic bromine isotope patterns. From the sixteen samples of bryozoans
surveyed, six species contained ions representative of the presence of at least one
brominated compound. The data of only one compound, from the species *Amathia
verticillata* (formerly *Zoobotryon*), could be matched with previously isolated
metabolites, indicating that five samples surveyed most likely contain novel
brominated metabolites.

2.2 Survey by Liquid Chromatography-Mass spectrometric Analysis

The natural isotopic abundance of bromine, ^{79}Br:^{81}Br 1:1, gives brominated compounds very distinctive isotopic peak patterns in mass spectrometry. For example, bromine in monobrominated compounds contributes two peaks with equal intensities two mass units apart. In dibrominated compounds, bromine contributes three peaks with intensities of 1:2:1, every peak being two mass units apart. LCMS chromatograms of small scale, crude extracts of sixteen bryozoan species collected in 2013 and 2014 were analysed by manual peak picking to generate a mass spectrum for each significant peak. The resulting mass spectra were examined for evidence of bromination, indicated by characteristic isotope patterns. A complete species list with collection data as well as their LCMS traces with mass spectra of each significant peak is recorded in Appendix A. Bromine containing compounds were identified in six of the sixteen bryozoan species investigated (Table 2.1).

Table 2.1. Species surveyed containing brominated natural products

Species	Brominated compounds, M+H$^+$ (*m/z*) (lowest monoisotopic peak)	Number of bromine atoms
Bugula neritina	321.9	2
	452.8	3
Orthoscuticella, *Costaticella* (or another *Catenicellidae* species)	261.0, 303.1 304.0	1
Orthoscuticella, *Costaticella* (or another *Catenicellidae* species)	221.9, 239.0	1
Amathia verticillata (Formerly *Zoobotryon verticillatum*)	208, 222.0	1
	299.9, 285.9, 344.9	2
	377.8, 422.8	3
Amathia citrina	359.03, 436.9, 482.9, 468.9, 498.9, 479.9	2
	514.9	3
	592.7	4
Amathia imbricata	316.0, 252.9, 208.0	1
	436.9	2

The two species of *Orthoscuticella* or *Costaticella* (or another *Catenicellidae* species) analysed as well as *Bugula neritina* contained what appeared to be minute quantities of brominated compounds based on mass spectrometric analysis. The intensity of signals for brominated compounds found LCMS analysis of these species were low, approximately 1×10^6-5×10^7. In contrast, equivalently brominated compounds observed in *A. citrina* and *A. verticillata* had intensities of at least one magnitude greater, generally 1×10^8 to 1×10^9. Bromine containing compounds are generally easily ionisable, thus small intensities suggest very small concentrations of brominated compounds exist, therefore *A. citrina* and *A. verticillata* should have the greatest concentrations of brominated compounds of all species surveyed.

A. citrina contained the greatest number of compounds with bromine functionality, as well as the only tetrabrominated compound observed. The chromatograms obtained from LCMS analysis including the mass spectra of the *A. citrina* and *A. verticillata* extracts are displayed in Figures 2.1 and 2.2 respectively.
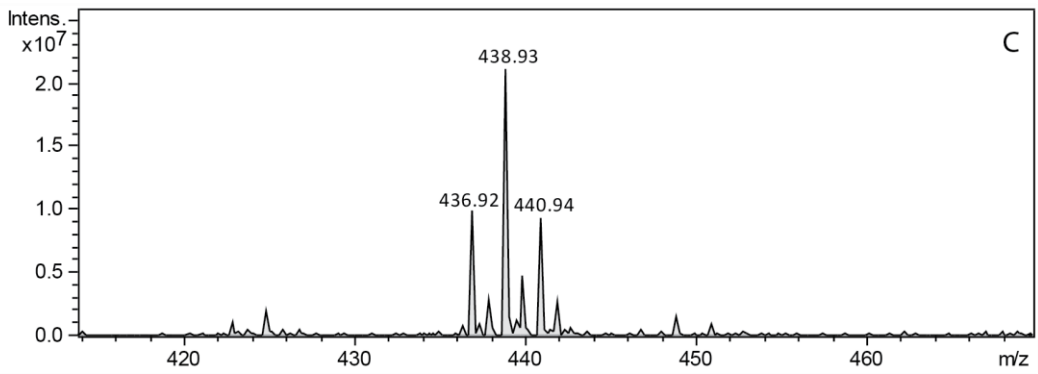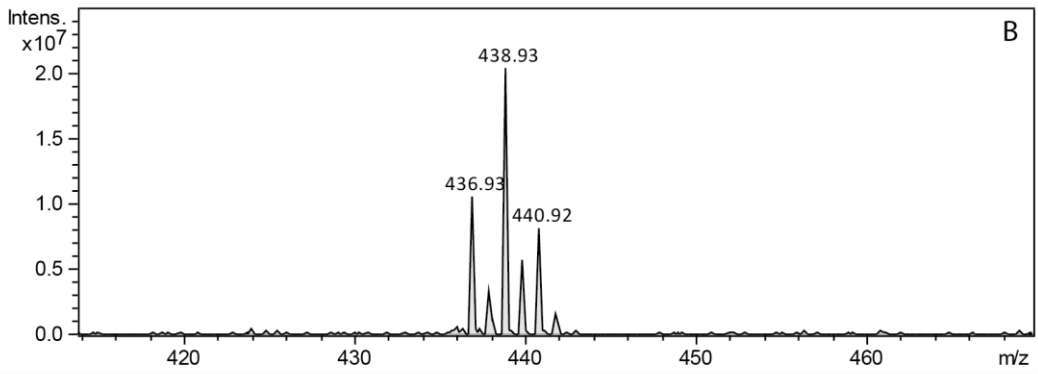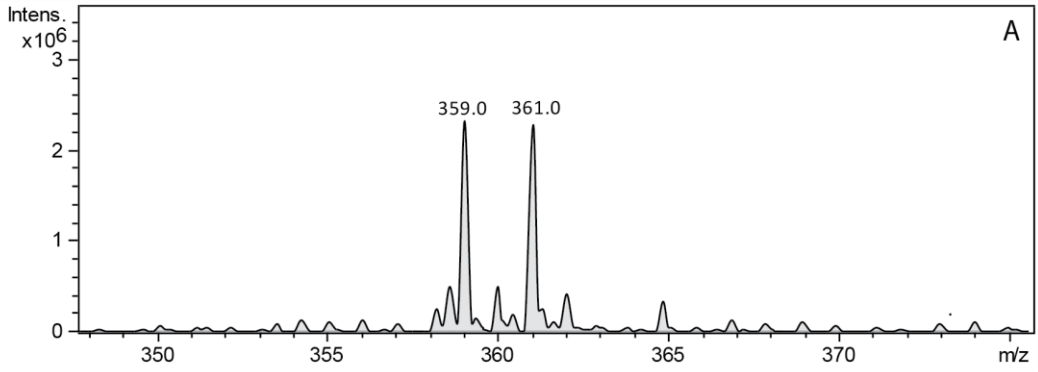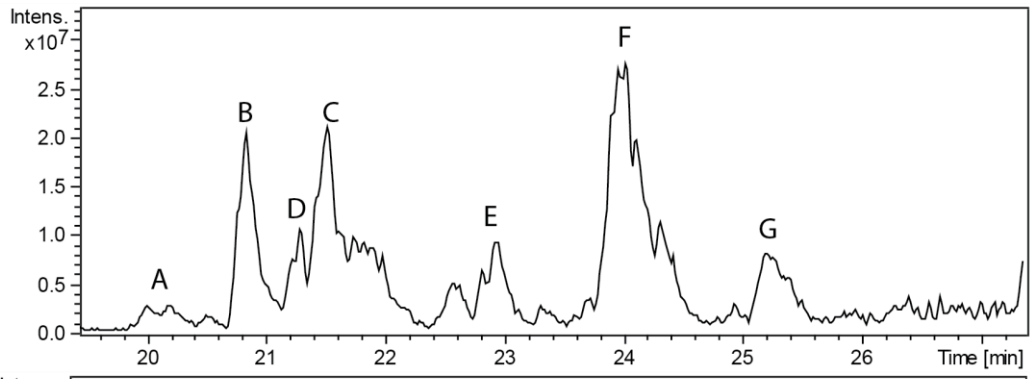
(Figure 2.1 continued)
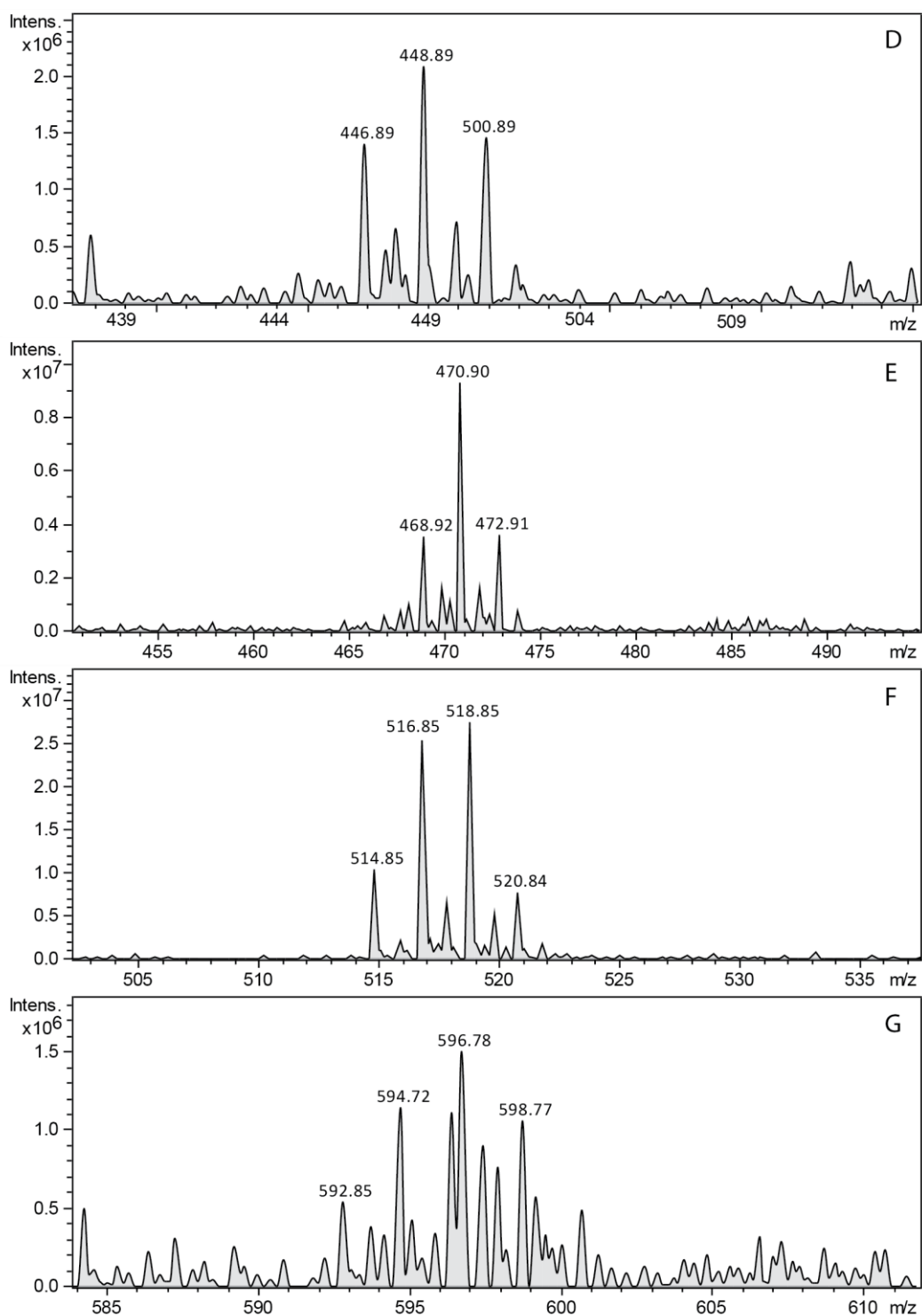
Figure 2.1. Chromatogram and mass spectra of *Amathia citrina* extract from LCMS analysis
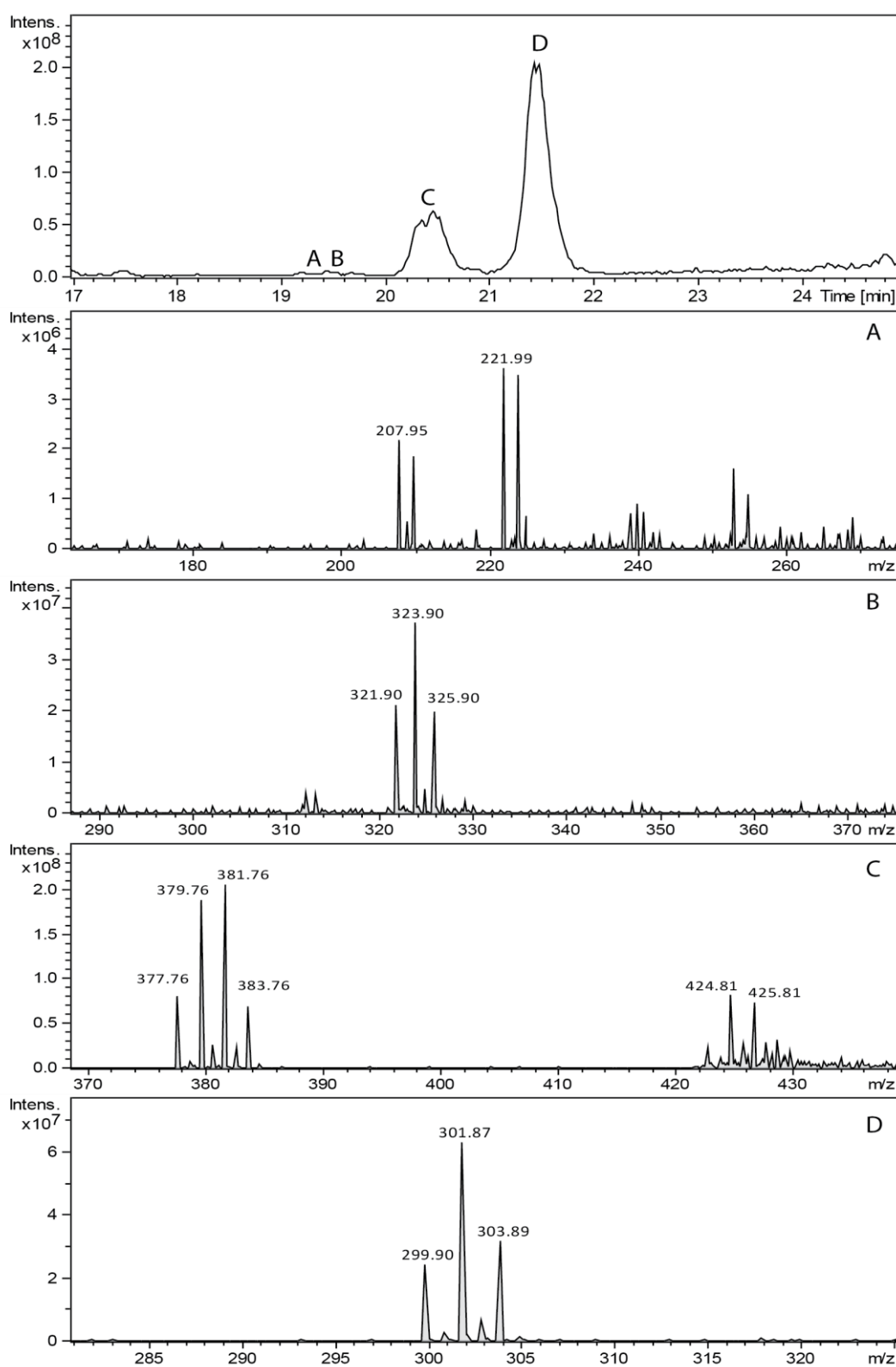
Figure 2.2. Chromatogram and mass spectra of *Amathia verticillata* extract from LCMS analysis

Dereplication is an important preliminary step in natural products research to prevent rediscovery of known compounds. LCMS analysis of crude extracts and database searches of observed masses served to provide a quick dereplication step. The comprehensive marine natural products literature database MarinLit,[81] was used to search for brominated compounds observed in the surveyed species, based on mass (nominal, ±0.6 g/mol), bromine functionality and taxonomy. Of the twenty six brominated compounds observed from the six bryozoan species, fifteen matched known compounds by mass and bromination patterns, however the known compounds originate from different taxa, mostly sponges and algae (see Table 2.2).

Only two surveyed compounds observed in the survey appeared to match known compounds originating from comparable taxonomy (genus or species) based on mass and bromination patterns. The compounds with nominal masses of 422 ($[M+H]^+ = m/z$ 422.8) and 344 ($[M+H]^+ = m/z$ 344.9) from the species *A. verticillata* matched 2,3,5-tribromo-*N*-methylgramine **(41)** and 2,6-dibromo-*N*-methylgramine **(45)** reported from collections of *A. verticillata* from USA[44] and Brazil.[46] The other surveyed compounds are most likely novel. *B. neritina* collected from New Zealand waters did not contain bryostatins, consistent with previous analysis of New Zealand samples of this species by our group and others.[82]

Table 2.2. Compounds from surveyed species with mass and bromination patterns matching known compounds and their sources

Surveyed Species	Compound mass (nominal ± 0.6 g/mol)	Source species of previously isolated compound
B. neritina	320	*Amathia convoluta*[59]
Orthoscuticella, Costaticella or *Catenicellidae* sp.	238	*Mycale fibrexilis,*[83] *Didemnum candidum*[84]
Orthoscuticella, Costaticella or *Catenicellidae* sp.	260	*Rhodomela confervoides,*[85] *Alglaophenia pluma linnaeus*[86]
A. verticillatum	344	*Plocamium cartilagineum,*[87] *Ochtodes crockeri,*[88] *Aplysia kupodai,*[89] *Snenosongia echina*[90]
	330	*Polyfibrospongia maynardii*[91]
A. citrina	436	*Ritterella rubra,*[92] *Laurencia tenera,*[93] *Psammaplysilla purpurea*[94]
	422	*Psammalplysilla purpurea,*[95] *Laurencia obtusa*[96]
	468	*Polysiphonia lanosa,*[97] *Laurencia glandulifera,*[98] *Pharmacelocarpus abillardieri*[99]
	498	*Hexadella* sp.[100] (sponge)
	480	*Laurencia irieii,*[101] *Suberea clavata*[102]
	464	*Aplysia depilans,*[103] *Sphaerococcus coronopifolis,*[104] *Aplysia punctata,*[105] *leathesia nana*[106]
	592	*Ritterella rubra*[92]
A. imbricata	315	*Pseudoceratina crassa,*[107] *Rhodomela confervoides*[108]
	252	*Pseudosuberites hyalinus,*[109] *paramuricea clavata*[110]

Convolutamine G, a beta-phenylethylamine compound, from the bryozoan *Amathia convoluta*[59] matched the mass (320 g/mol) and bromination pattern of a compound observed in the New Zealand collection of *B. neritina*. No other members of the convolutamines were observed. Convoultamines have thus far only been observed in two species of *Amathia*, not *Bugula*, thus it is unlikely that this compound actually is convolutamine G.

From all species surveyed, *A. citrina* was the most promising candidate for a natural products study. Due to the many brominated compounds present with high ion intensities in LCMS, the unlikely possibility of rediscovering already known compounds and a large collection available (583.35g, lyophilised mass), *A. citrina* was selected as the candidate for this natural products study.

Chapter Three

Amathamines: Brominated Indole Alkaloids

from *Amathia citrina*

3.1 Introduction

Among the many brominated compounds observed from *A. citrina* two compounds were investigated in most detail. Compounds with nominal masses of 514 and 468 g/mol were targeted for isolation to afford samples of adequate purity for structural analysis. The compounds with the nominal masses of 514 and 468 g/mol, tentatively called amathamines A (**133**) and B (**134**) respectively, were partially characterised by MS and NMR spectroscopy A putative structure for other members of this family of alkaloids are proposed based on tandem MS data, called amathamines B (**135**) and C 498 (**136**).
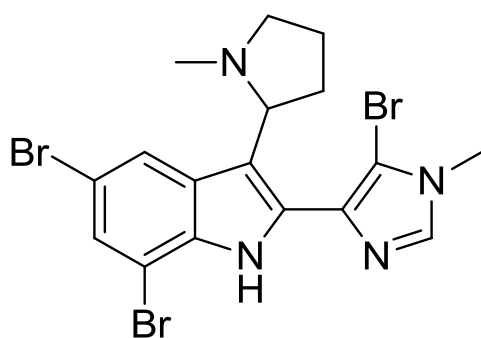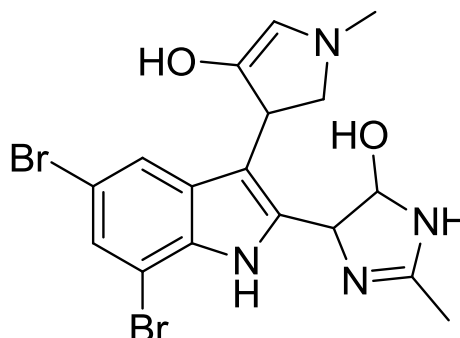
| (133) | (134) |
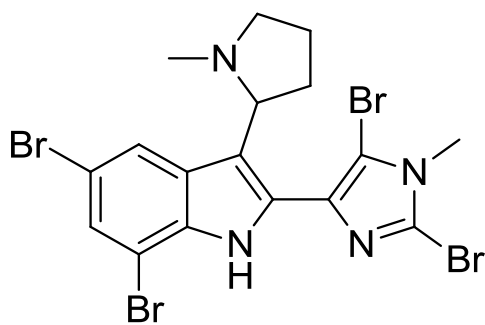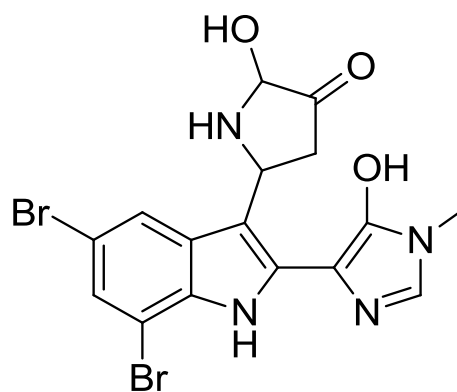
(135)   (136)

A sample of amathamine A had been isolated previously by Chris Lockley, a former group member of our Natural Products Group, from a collection of *A. citrina,* without structural elucidation.[111] This sample was used for characterisation, in conjunction with a sample of amathamine A **(133)** isolated during the course of work described in this thesis. Spectral differences were observed between purified samples of amathamine A despite these compounds having the same mass of 514 g/mol.

Heteronuclear ^{1}H-^{13}C connectivity NMR experiments HSQC and HMBC coupled with computer-generated predictive NMR spectral data for the proposed structures were invaluable during structural elucidation. The NMR and tandem MS data for amathamine A is recorded in Appendices B and C respectively.

3.2 Isolation and characterisation amathamine A

3.2.1 Introduction

Based on analysis of MS and NMR data the structure of amathamine A (**133**) was determined to be a tri-brominated indole alkaloid with *N*-methyl pyrrolidine and brominated imidazole moieties (**133**). LCMS guided fractionation of the crude extract by reversed-phase and size exclusion chromatographic steps afforded a semi-pure sample.
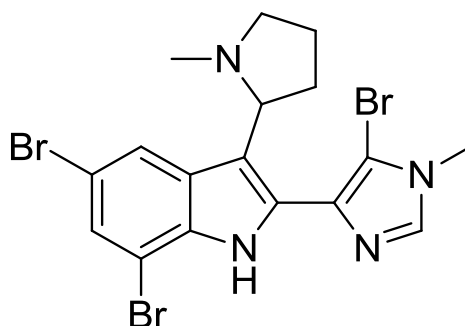
(**133**)

3.2.2 Isolation of Amathamine A

From LCMS data on the crude extract of *A. citrina* the compound having the nominal mass of 514 g/mol was identified as tri-brominated by its characteristic isotopic peak pattern, a quartet, with each peak two mass units apart (Figure 3.1). This compound analysed for the molecular formula of $C_{17}H_{17}N_4Br_3$ (error: 8.1 ppm) by HRMS analysis with an accurate monoisotopic mass of 513.9118 g/mol ($^{79}Br_3$). The isotopic peak profile matched closely that of the theoretical profile.
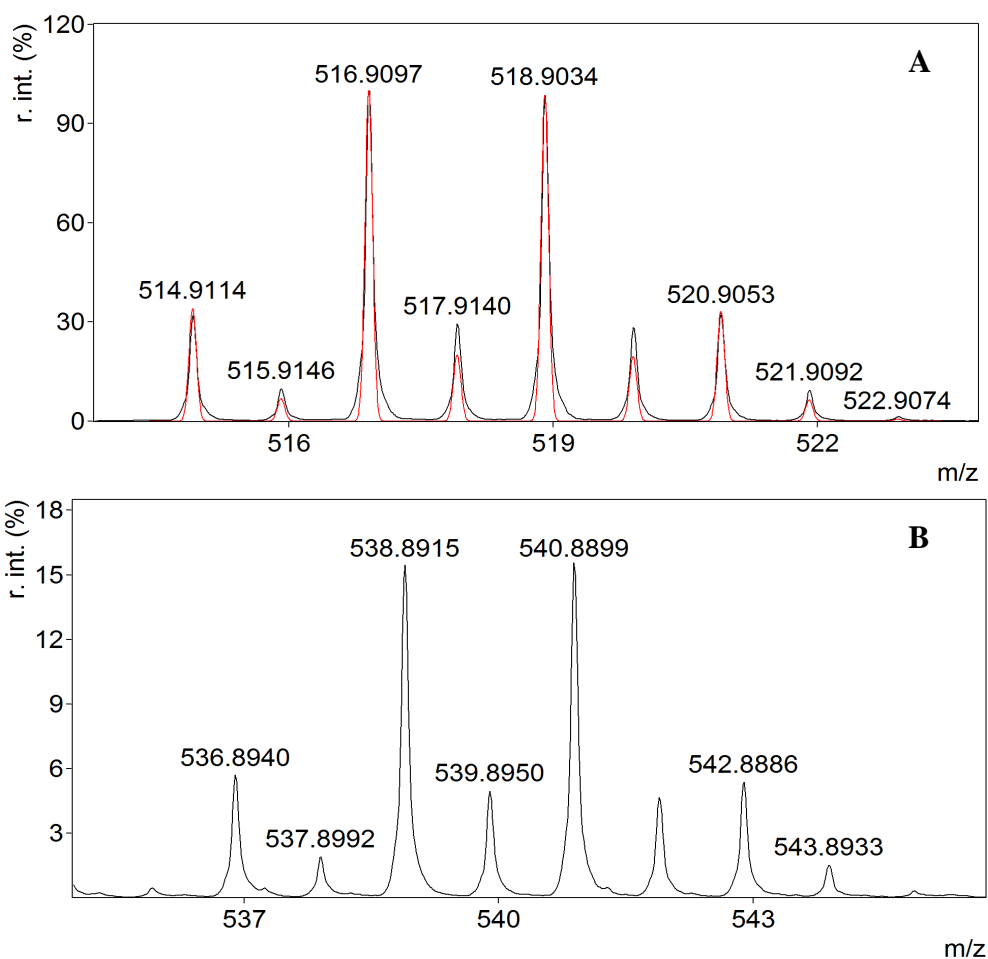
Figure.3.1. (**A**): HRMS spectrum of amathamine A [M+H]$^+$. (Black) and Predicted Mass Spectrum of [C$_{17}$H$_{17}$N$_4$Br$_3$+H]$^+$ (Red). (**B**) [514+Na]$^+$, presence of sodium adduct confirmed molecular mass.

This compound, tentatively called amathamide A, eluted in MeOH fractions of crude extract processed on reversed-phase flash column chromatography. The MeOH fractions were combined and extensively subjected to chromatographic techniques, guided by LCMS analysis. An eleven step fractionation method was utilised, comprised of multiple reversed-phase and size exclusion chromatographic steps (see Appendix H). Amathamine A proved extremely difficult to isolate because of closely eluting contaminates, most notably a compound with a nominal mass of 340.9 g/mol. After isolation, sample purity was

deemed adequate for NMR spectroscopic analysis by visual observation of Base Peak Chromatogram (BPC) traces in LCMS (Figure 3.2). The sample was insoluble in deuterated chloroform ($CDCl_3$) but readily soluble in deuterated methanol (CD_3OD). NMR analysis was only utilised for structural elucidation, not purity determination because of the budget constraints imposed by the use of CD_3OD.
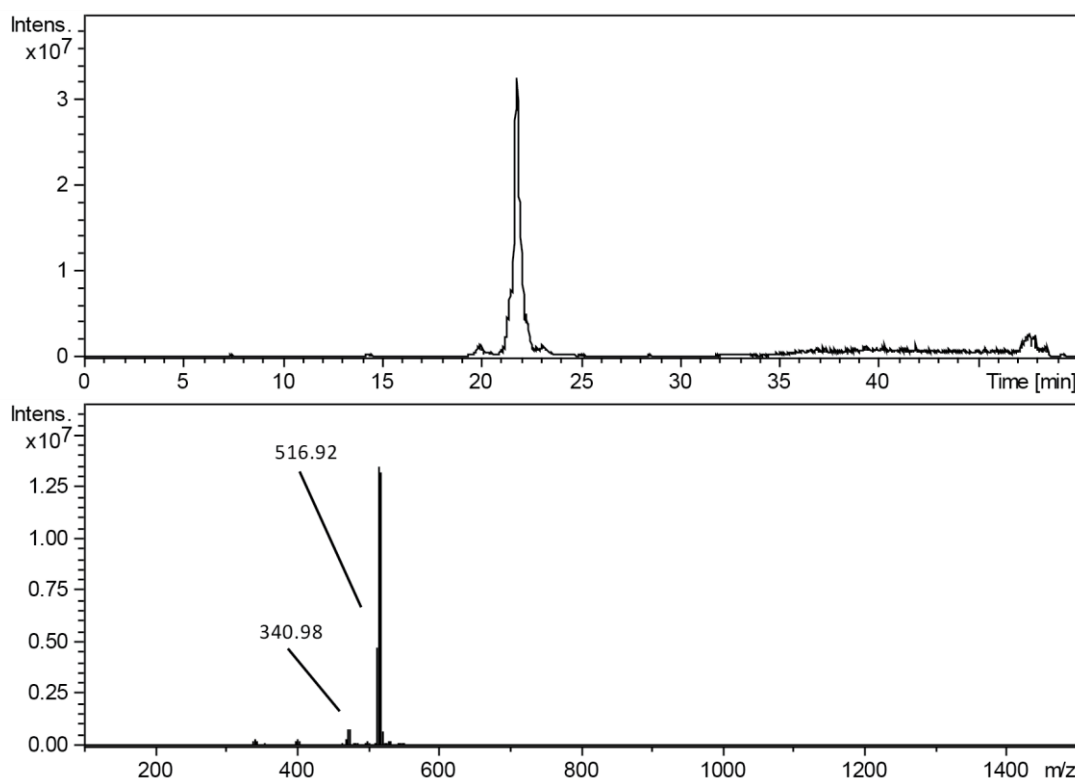
Figure 3.2. (Above) BPC trace of sample containing amathamine A isolated during the work described in this thesis. **(Below)** averaged mass spectrum.

A sample of a tribrominated compound with the nominal mass of 514 g/mol, reported to have the same molecular formulae of $C_{17}H_{17}N_4Br_3$, had been previously isolated from a smaller sample of *A. citrina* by Chris Lockley but was not characterised. Determining *A. citrina* was still the best candidate for analysis, isolation of more amathamine A, from a bigger collection of *A. citrina*, for the purpose of structural elucidation was the major goal of this thesis. The time constraints imposed by extensive chromatographic fractionation necessary to isolate amathamide A and B did not leave enough time for further purification and characterisation after discovering the samples were insufficiently purified for NMR analysis.

The ^{1}H NMR spectrum of amathamine A purified during the work described in this thesis vaguely resembled that of the sample previously isolated. However, some characteristic signals were absent. Additionally, no signal observed had matching chemical shift values with the ^{1}H NMR spectrum of the previously isolated sample. Thus it was concluded NMR spectroscopy data generated for the previous sample should also be utilised for characterisation, fearing insufficient time for rigorous spectroscopic analysis of the sample isolated during the work described in this thesis. Additionally, this sample is potentially an unnatural structural variant of amathamine A, formed during isolation.

3.2.3 Characterisation of Amathamine A

The following characterisation of amathamine A was established using the tandem MS and NMR data generated by Chris Lockley. The ^{1}H NMR spectrum (Figure 3.3.) showed three aromatic signals, a singlet at δ_H 7.86 and two doublets at δ_H 7.55 (J=1.7) and 7.34 (J=1.7). Additionally, two N-methyl resonances, singlets at δ_H 2.51 and 3.67, four downfield aliphatic signals at δ_H 3.25, 3.15, a complex proton band at approximately 2.8 (integrating for three hydrogens) and a methine singlet at δ_H 3.92 (J=7.0, 6.9) was observed. The ^{13}C NMR spectrum (Figure 3.2.) revealed the presence of eight downfield quaternary carbons at δ_C 146.6, 138.7, 135.6, 132.3, 112.8, 109.2, 106.0 and 104.6, three aromatic carbons at δ_C 139.9, 126.88 and 121.2, two N-methyl carbons at δ_C 43.2 and 34.2 and four downfield aliphatic carbons at δ_C 61.8, 49.9, 32.2 and 20.0.

The aromatic and downfield aliphatic carbons had correlations in a HSQC experiment (Appendix B.1), assigning all protons to their directly attached carbons. Six downfield aliphatic protons were witnessed to correlate to three carbons at δ_C 49.9, 20.0 and 32.2, two proton correlations per carbon, δ_H at 3.24/2.84, 2.80/2.74, and 3.15/2.81 respectively, indicating the presence of three methylene carbons. The coupling constant between the two aromatic doublets of 1.7 Hz indicated meta-positioning.
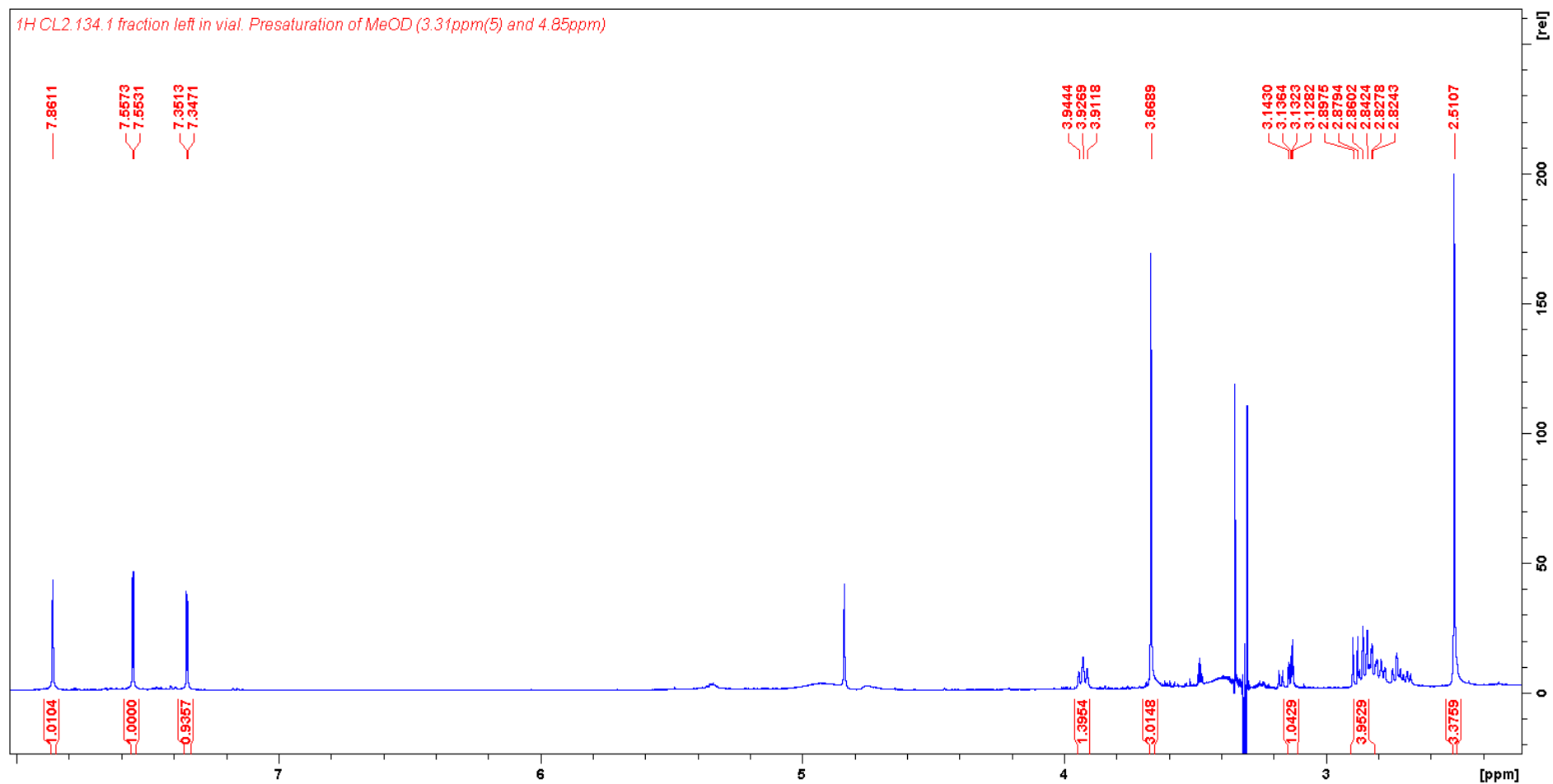
Figure 3.3. [^1]H NMR spectrum (400MHz) of amathamine A in CD₃OD
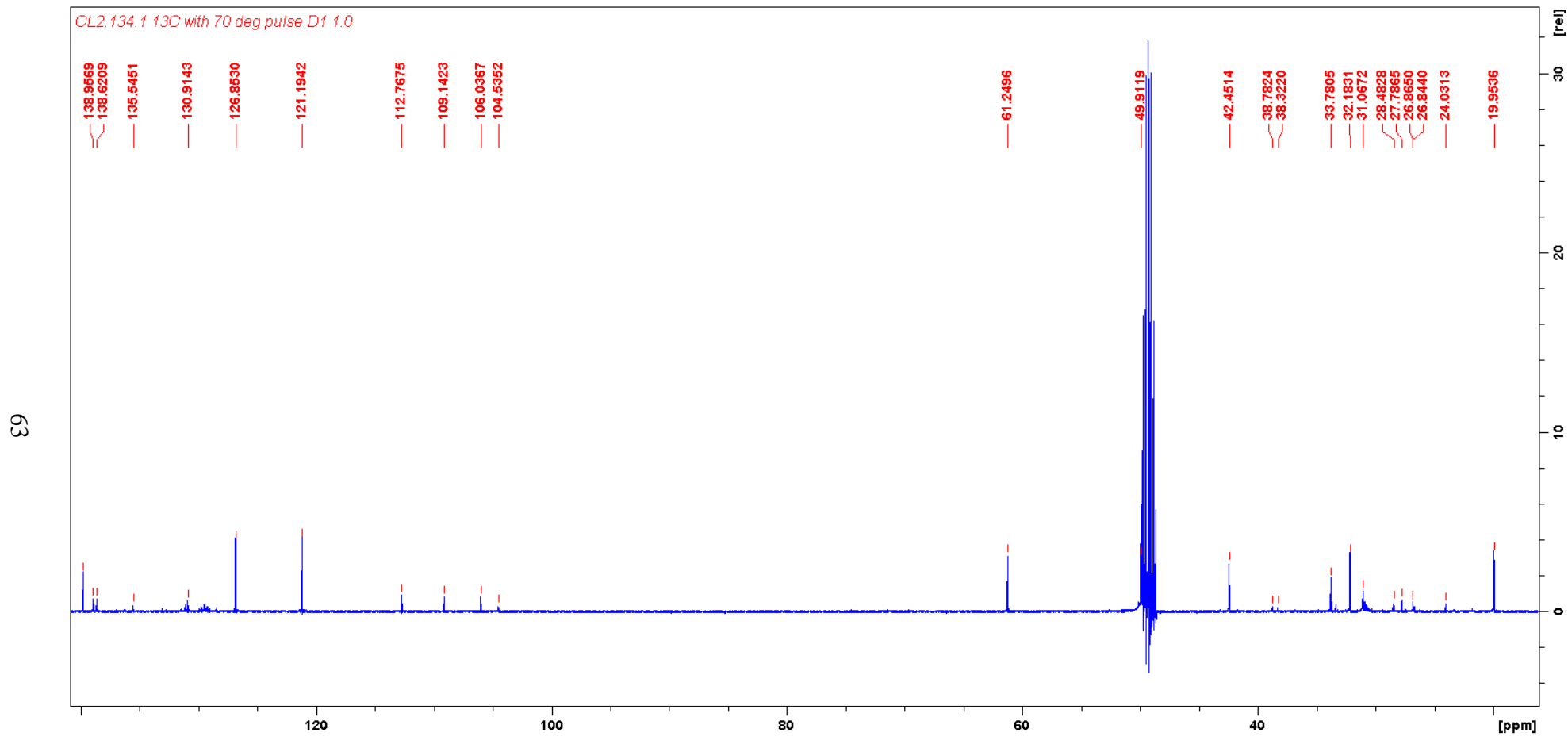
Figure 3.4. ^{13}C NMR spectrum (400MHz) of amathamine A in CD_3OD

The COSY NMR spectrum (Appendix B.2) indicated that the methine and methylene protons exist as a separate spin system, a –CHCH$_2$CH$_2$CH$_2$- moiety. HMBC experiment (Appendix B.3) further proved evidence for these fragments. The N-methyl protons, δ_H 2.51, showed correlations to two carbons at δ_C 49.9 and 61.2. Carbons at δ_C 49.1, 32.2, 20.0 and 61.2 were confirmed as three methylenes and a methine respectively, by a DEPT-135 experiment (Appendix B.4). These data thus indicated the molecule contains a pyrrolidine ring (see figure 3.4).

No coupling constants could be measured in the complex proton band around δ_H 2.8 on account of overlap, however splitting of a doublet of doublets at δ_H 3.15 could be measured. The larger splitting of 14.8 Hz observed is probably due to geminal coupling to proton δ_H 2.81. The smaller splitting, 5.8 Hz, is likely from vicinal coupling to a proton of the adjacent methylene not the methine proton. The coupling constants for the methine proton, a doublet of doublets are 7.0 and 6.9 Hz. The splitting expected of proton δ_H 3.15 is a doublet of doublets of doublets of doublets, however this pattern was not witnessed. No truly representative examples of N-methyl pyrrolidine coupling constants were found in the literature, however similar coupling values were observed in a fused pyrrolidine moiety of the tetra-cyclic alkaloid spirotryprostatin A.[112] A more in-depth analysis of these scalar coupling interactions require further NMR spectroscopic analysis than carried out for structural analysis. An HMBC experiment established an indole moiety as outlined in Table 3.1 and Figure 3.4. The proposed structures closely follow predicted δ_C values generated by ChemDraw® software.[113]

Table 3.1. NMR data of amathamine A in CD_3OD

Position	Amathamine A			
	δ_C	δ_H (J, Hz)	HMBC (from ^1H)	Predicted δ_C
1-N	-	-	-	-
2	138.7	-	-	132.8
3	109.2	-	-	108.8
3a	132.3	-	-	131.8
4	121.2	7.55, d (1.7)	7a, 6, 5, 3	120.0
5	112.8	-	-	119.2
6	126.9	7.34, d (1.7)	7a, 7, 5, 4	126.0
7	106.0	-	-	102.2
7a	135.6	-	-	134.5
8	61.2	3.91, dd, (7.0,6.9)	-	71.4
9-N	-	-	-	-
10	43.2	2.51	8,11	43.3
11	49.9	3.24, m/2.85, m	12,13	60.3
12	20.0	2.80, m/2.74, m	11,12,8	23.0
13	32.2	3.15, dd (14.8,5.8)/ 2.81, m	8,11,12	35.8
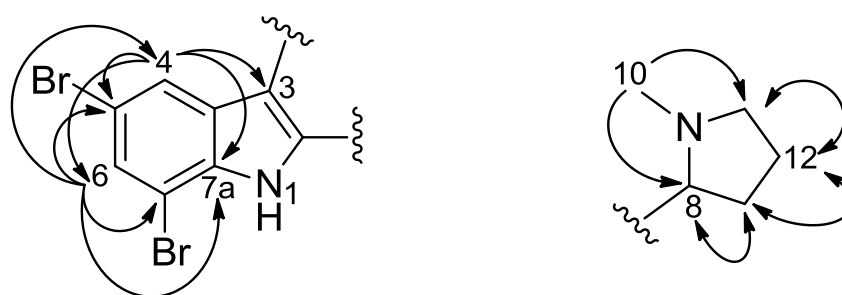
Figure 3.5. Key HMBC and COSY connectivities establishing the 5,7-dibromoindole and *N*-methyl pyrrolidine moieties. Single headed arrows indicate $^{2-3}J_{CH}$ correlations. double headed arrows indicate COSY correlations.

The protons H-4 and H-6 showed strong correlations to quaternary carbons C-5 and C-7a. H-4 had the only correlation to C-6, while H-5 had the only correlation to C-3 and a very weak correlation to C-3a. The positioning of the bromine atoms on the benzene ring was determined to be at C-5 and C-7, thus establishing the presence of a 5,7-dibromoindole moiety.

Tandem MS analyses yielded fragment masses detailed in Table 3.2. Two fragmentation pathways are witnessed for tandem MS of amathamine A.

Table 3.2. Tandem MS analysis of amathamine A.

Parent Ion $[M+H^+]$ (m/z)	MS^2 Observed Fragments (m/z)	Mass loss to form MS^2 fragment (mass units)	MS^3 Observed Fragments (m/z)	Mass loss to form MS^2 fragment (mass units)
515	343	172	302	41
			287	56
			262	81
			220	123
	472	43	393	79
			378	94
			351	121
			173	299

Fragmentation of the parent ion, m/z 515, to ion m/z 472 required the loss of 43 mass units. Mass change is from an even nominal mass to an odd nominal mass, 514 to 471. Applying the nitrogen rule, this indicates the loss of an uneven number of nitrogen atoms, one or three. Loss of three nitrogen atoms (42 mass units) in a fragment of 43 mass units is impossible. So the loss of 43 mass units must contain a single nitrogen. The remainder of the mass lost, 29 mass units, is accountable by C_2H_5, giving the molecular formula of the loss of 43 likely to be

C_2H_5N. Upon establishing the presence of the 5,7-dibromoindole core by NMR spectroscopy, it became apparent that the loss of C_2H_5N represents the fragmentation of the *N*-methyl pyrrolidine moiety, as shown by the fragmentation pathway detailed in Figure 3.6.
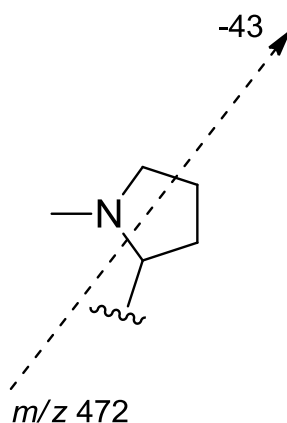
Figure 3.6. Proposed fragmentation of the *N*-methyl pyrrolidine moiety.

A separate constellation of ^3J ^1H-^{13}C connectivities was witnessed in the HMBC spectrum, with no correlations to the 5,7-dibromoindole core or to the *N*-methyl pyrrolidine moiety, and indicated the presence of a bromo-indole moiety as shown in figure 3.7, showing these connectivities.
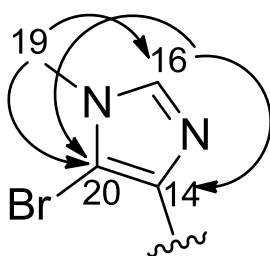
Figure 3.7. Key HMBC correlations establishing the imidazole moiety.

This separate constellation contained correlations between the aromatic proton H-16 to two quaternary carbons C-14 and C-20 and the *N*-methyl group C-19. The

N-methyl protons H-19 showed correlations to C-20 and C-16. This separate constellation was proposed to be the other structural moiety, besides the *N*-methyl pyrrolidine moiety, off the 5,7-dibromoindole core.

Both 15 and 79 mass unit losses were witnessed in the MS^3 analysis of *m/z* 515>471. These masses are indicative of the loss of a methyl and a bromine group respectively and are consistent with the structural characteristics of the imidazole moiety proposed. The imidazole moiety must be completely lost in the MS^2 fragmentation of the parent ion, *m/z* 515 > 343.

MS^2 fragmentation of the parent ion, *m/z* 515, to ion *m/z* 343 requires the loss of 172 mass units, an even mass unit loss. Following the nitrogen rule, this requires a loss of an even number of nitrogen, further suggesting the presence of the remaining nitrogen is amongst this separate constellation, the proposed imidazole moiety. A degree of unsaturation principle value of three ($C_4H_4N_2Br$ = 3.5/$C_4H_5N_2Br$ = 3) additionally indicated a brominated imidazole ring. No COSY correlations were observed, as expected, additionally a SELROESY (Appendix B.5) experiment further confirmed the connection of this moiety.

Establishment of the complete structure of amathamine A, the placement of the pyrrolidine and imidazole moieties upon the indole core could not be confidently assigned by NMR spectroscopy alone. This is due to seemingly erroneous HMBC connectvities of the pyrrolidine protons H-8 and H-12 to carbons C-2, C-3 and C-20. Two substituents upon the indole moiety remain un-assigned, at the two

quaternary carbons C-2 and C-3, thus the positions of the pyrrolidine and imidazole moieties. As observed in Figure 3.8, the HMBC spectrum of amathamine A focused upon the connectivities of the pyrrolidine protons at δ_H 3.91 and 3.15, there appears to be strong correlations to carbons at δ_C 138.66 and 104.58.
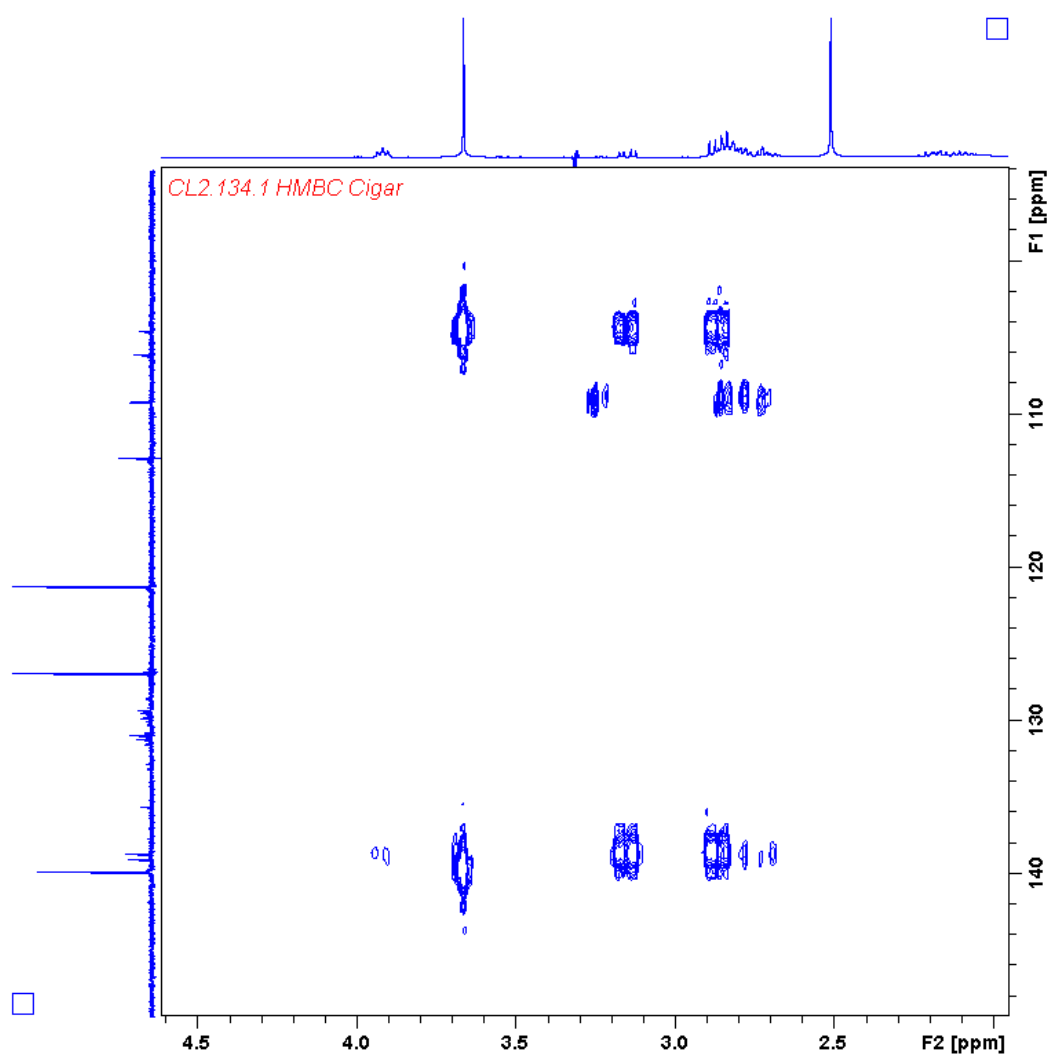
Figure 3.8. HMBC spectrum of amathamine A focused upon the connectivities of the pyrrolidine protons at δ_H 3.91 and 3.15.

These carbons, at δ_C 138.66 and 104.58, were assigned as the quaternary and brominated carbons C-14 and C-20 of the imidazole moiety respectively, indicating these are potentially very long-range correlations (n J_{CH} n>3) or artefacts. The structural changes necessary to satisfy these as $^{2-3}J_{CH}$ directly contradicts the establishing COSY, HMBC and NOE evidence for the pyrrolidine and imidazole moieties. Furthermore, Figure 3.8 shows the lack of connectivities between the pyrrolidine protons and the carbons C-3 and C-3a of the indole ring, vital for confident assignment.

The best evidence connecting the pyrrolidine and imidazole moieties to their proposed positions upon the indole core is a comparison of ChemDraw® software[113] predicted and observed chemical shift values, particularly the effect on C-4 by the substituent upon C-3. The predicted chemical shift value of H-4 with the imidazole moiety at C-3 is 125.0, whilst the predicted shift value with the pyrrolidine moiety at C-3 is 120.0. Positioning of the pyrrolidine at C-3 is more consistent with the observed chemical shift value of C-4, δ_C = 121.24. Additionally, the predicted chemical shift values of C-3 and C-3a are closer for this permutation as shown by the differences in the predicted and observed chemical shift data in Table 3.3, these structural variations are visualised in Figure 3.9.

Table 3.3. Predicted chemical shift values of two permutation of amathamine A.

| | Amathamine A | | | | |
Position	Observed chemical shift values (Obs)	Predicted chemical shift values, pyrrolidine at C-3 (Pred$_{pyrrolidine}$)	Δ(Obs-Pred$_{pyrrolidine}$)	Predicted chemical shift values, imidazole at C-3 (Pred$_{imidazole}$)	Δ(Obs-Pred$_{imidazole}$)
2	138.66	131.9	6.76	134.4	4.26
3	109.23	108.8	0.43	99.3	9.93
3a	132.30	131.8	0.5	132.8	-0.5
4	121.24	120.0	1.24	125.0	5
5	112.81	119.2	-6.39	119.2	-6.39
6	126.88	126.0	0.88	126	0.88
7	106.07	102.2	3.87	102.2	3.87
7a	135.56	134.5	1.06	133.2	2.36
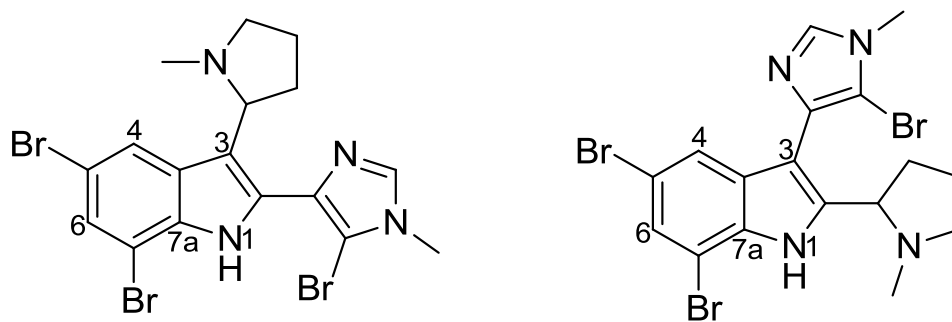
Figure 3.9. Permutations of amathamine A.

3.2.4 Spectral Differences between Samples of Amathamine A

A comparison of ^{1}H NMR spectra (figure 3.10) showed a loss of the imidazole hydrogen at δ_H 7.86 in the sample of amathamine A isolated in this study. Based on the interpretation of a HMBC experiment (Figure 3.11) the 5,7 di-bromo indole core remains. Loss of the imidazole hydrogen signal at δ_H 7.86 was postulated to occur due to the substitution of the imidazole *N*-methyl group and this hydrogen. However, this was unable to be proved due to poor resolution in the HMBC experiment.
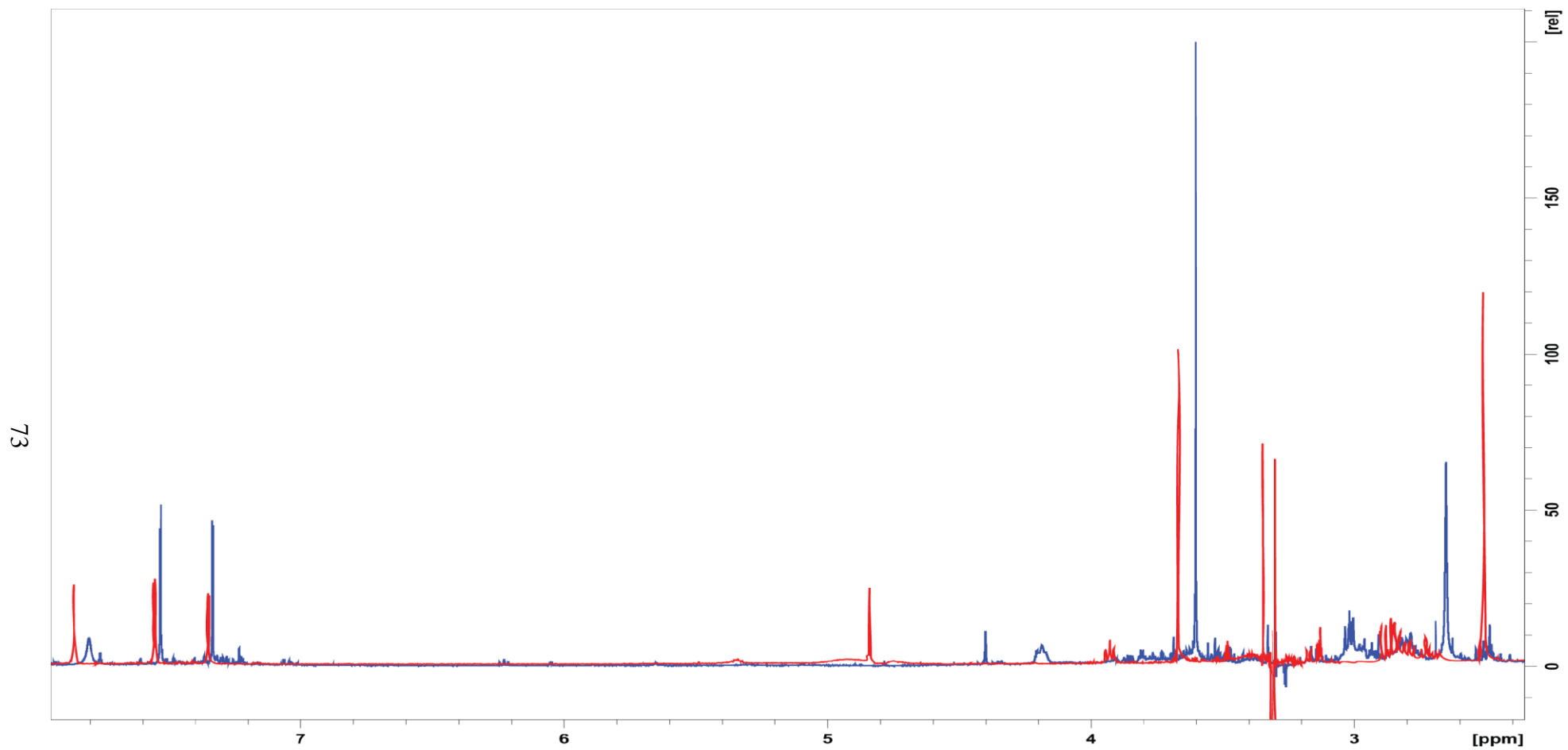
Figure 3.10. Comparison of ¹H NMR spectra of amathamine A isolated during the work described in this thesis **(red)**, and the previously isolated sample of amathamine A **(blue)**
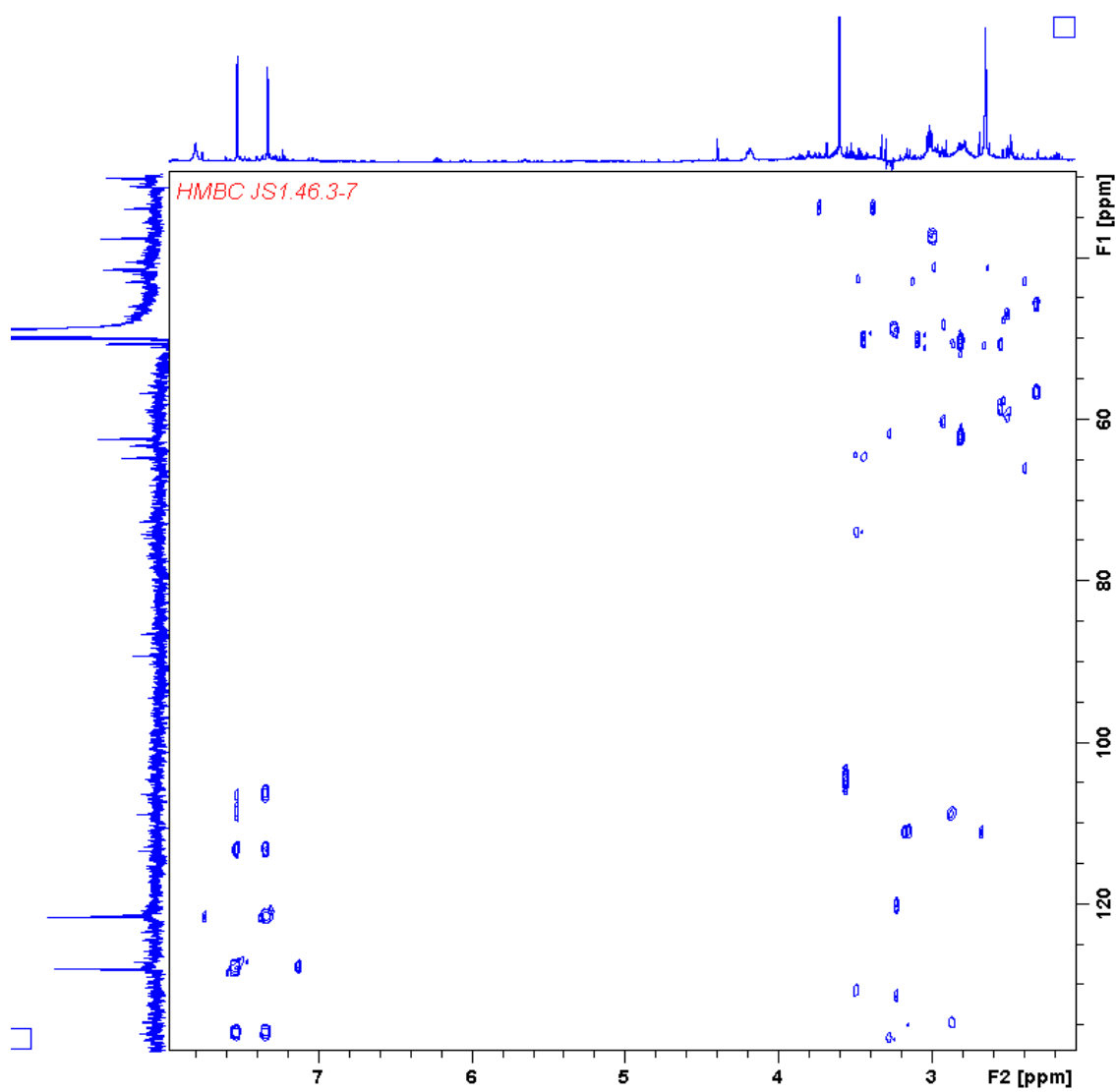
Figure 3.11. HMBC NMR spectra of amathamine A isolated during the work described in this thesis

3.3   Characterisation of Amathamine B

3.3.1 Introduction

Utilising the same method as employed for the structural elucidation of amathamine A, Amathamine B was proposed to be a di-brominated indole alkaloid with an *N*-methyl hydroxy-pyrrole moiety **(134)**. Characteristic chemical shift frequencies and ^{1}H-^{13}C heteronuclear connectivities established the presence of a di-brominated indole core such as in amathamine B. Development of the core structure led to the understanding of an *N*-methyl-dihydro-hydroxy-pyrrole moiety by two-dimensional NMR spectroscopy experiments, supported by fragmentation patterns in tandem MS. The sample utilised for analysis was not pure enough for full structural elucidation. Further purification steps were not undertaken due to time constraints. NMR spectroscopic data and tandem MS data for amathamine B are recorded in Appendices D and E respectively.
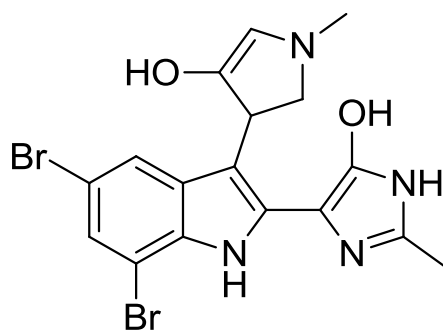
(134)

3.3.2 Isolation of Amathamine B

From the crude extract LCMS trace the compound having the nominal mass of 468 g/mol was identified as dibrominated by its characteristic isotopic peak pattern, a triplet, peaks two mass units apart. HRMS established the accurate monoisotopic mass to be 467.9882 g/mol (^{79}Br$_2$) and analysed for $C_{17}H_{18}N_4Br_2O_2$ (error: 2.5ppm) (Figure 3.12).
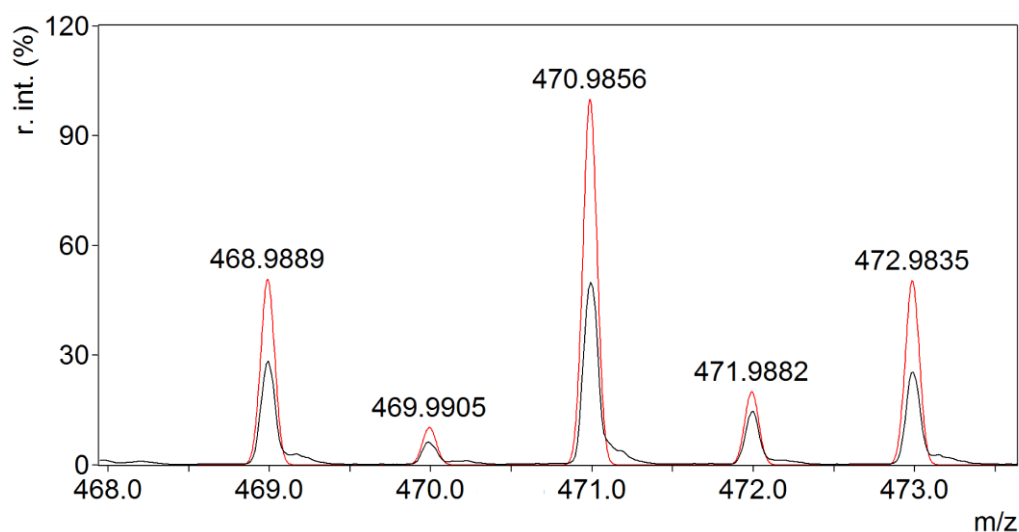
Figure 3.12. HRMS spectrum of amathamine B [M+H]$^+$. **(Black)** and Predicted Mass Spectrum of $[C_{17}H_{17}N_4Br_2O_2+H]^+$ **(Red)**.

This compound, tentatively called amathamide B, eluted in H_2O:MeOH (3:7) fractions of crude extract processed on reversed-phase flash column chromatography. These fractions were combined and extensively subjected to chromatographic techniques, guided by LCMS analysis. A five step fractionation method was utilised, comprised of reversed-phase and size exclusion chromatographic steps (Appendix G.1 and 3). Amathamine B also proved extremely difficult to isolate because of closely eluting contaminates. After isolation, sample purity was also deemed adequate for NMR spectroscopic

analysis by visual observation of BPC trace in LCMS (see Figure 3.13). Amathamine B had a longer retention time than amathamine A in reversed-phase chromatography, indicating amathamine B is more polar.
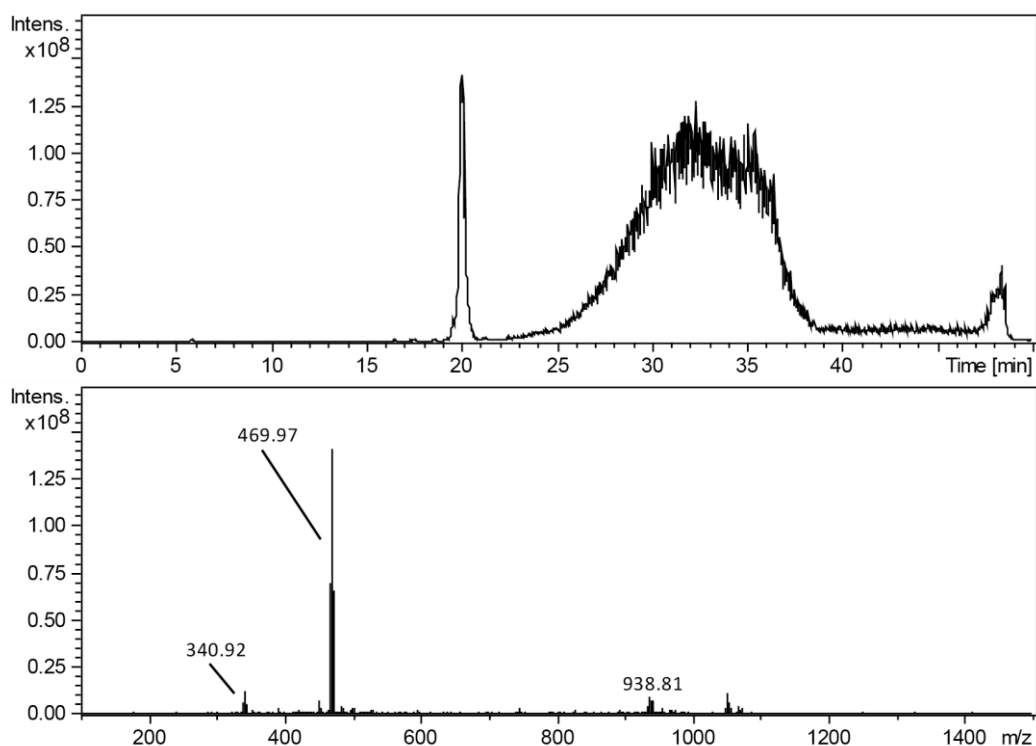
Figure 3.13. (Above) BPC of a sample containing amathamide B used during characterisation. **(Below)** averaged mass spectra. (Note: rising base line, ~25-40 minutes was determined to be liquid phase contamination.)

3.3.3 Characterisation of Amathamine B

The ^{1}H NMR spectrum (Figure 3.14) indicated the sample utilised for analysis contained contaminants. Additional signals not belonging to amathamine B were observed. Integration of proton signals in the ^{1}H NMR spectrum and HMBC connectivities indicated the signals of amathamide B. two large and four minor proton signals were witnessed in the ^{1}H NMR spectrum. The major and minor

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signals showed correlations in a HSQC experiment to differing carbons. In the ^{1}H NMR spectrum, coupling values throughout the aromatic region were inconsistent with values expected of true couplings around an aromatic ring. Additionally, the proton spectrum shows protons at δ_H 7.40 and 7.32 integrate to approximately one. Therefore, it was established only these two signals in the aromatic region were from amathamine B.

The ^{1}H-NMR spectrum also contained two downfield methine protons at δ_H 5.24 and 4.76, a *N*-methyl signal at δ_H 2.97, a methyl signal at δ_H 2.84 and two downfield aliphatic multiplets at δ_H 3.99 and 3.78 as well as other unassigned signals. The ^{13}C-NMR spectrum (Figure 3.15) contained two aromatic methines at δ 120.3 and 126.0, two quaternary carbons at δ 106.2 and 127.1, and other carbons at δ 93.5, 66.79 and 55.9, as well as other signals unassigned. The coupling of the aromatic protons in a HMBC experiment, most notably to C3a C7a, as well as to C5 and C7 was similar to that of the correlations in the analysis of amathamine A (figure 3.10). This led to the establishment of a 5,7-dibromoindole moiety. HMBC connectivities established the presence of the remaining indole carbons, the quaternary carbons at δ 138.5 and 119.7. The tandem MS analyses of amathamine B are listed in Table 3.4. Two fragmentation pathways are witnessed in the tandem MS experiments of 469, resulting in the loss of 41/57 then 128/112 mass units, respectively.

Table 3.4. Tandem MS of amathamine B

Parent Ion (m/z) [M+H$^+$]	MS^2 Fragments Observed (m/z)	Mass loss to form MS^2 fragment (mass units)	MS^3 Fragments Observed (m/z)	Mass loss to form MS^3 fragment (mass units)
4**69**	412	57	397	15
			300	112
			221	191
	341	128	300	41
			221	120

Figure 3.14. ^{1}H NMR spectrum (400MHz) of amathamine B in CD$_3$OD
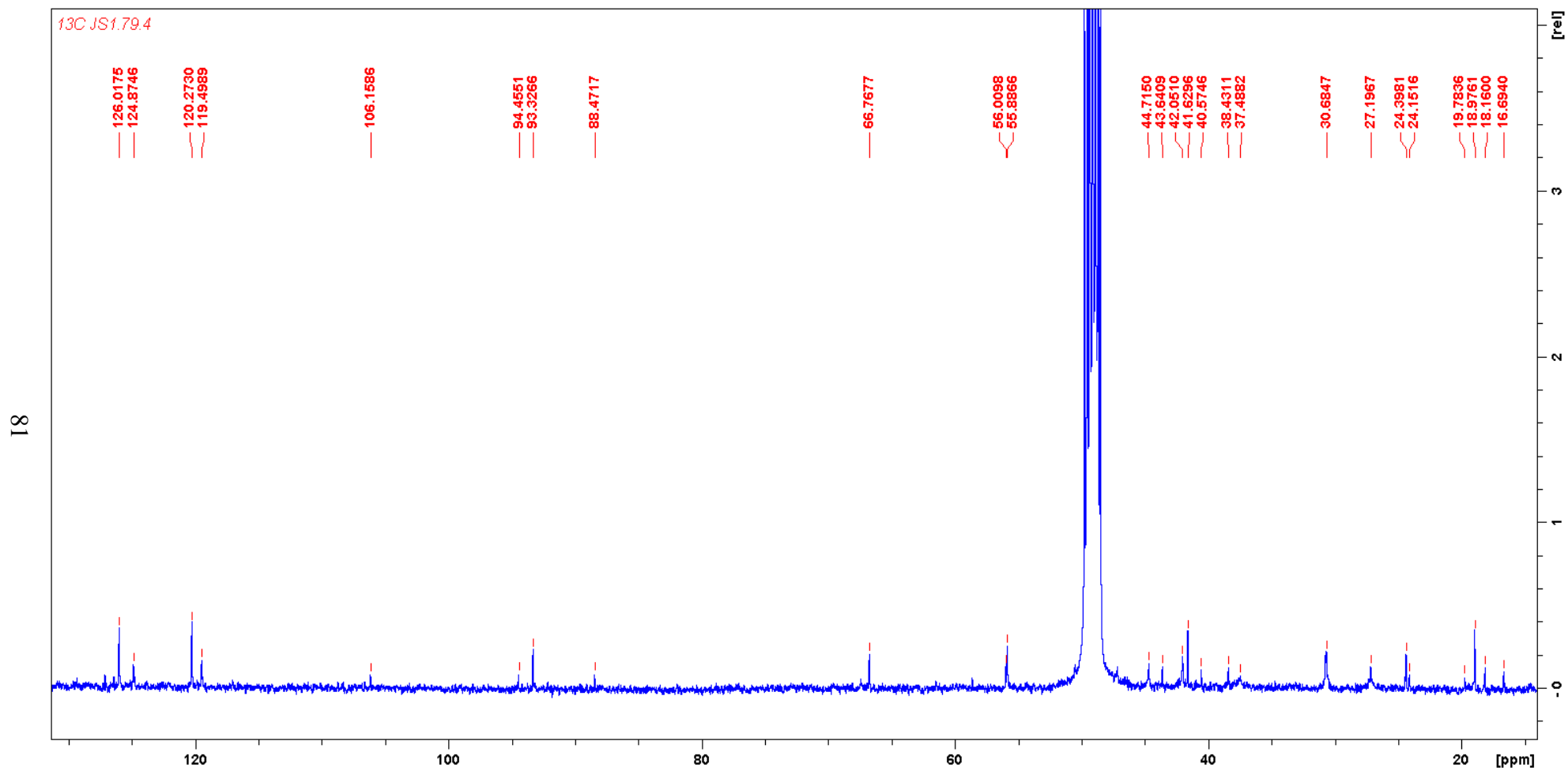
Figure 3.15. ^{13}C NMR spectrum (400MHz) of amathamine B in CD_3OD

HMBC (Appendix D.1) analysis indicated the presence of an *N*-methyl hydroxy-pyrrole moiety at C-3 as shown in Figure 3.16, outlining all key HMBC and COSY correlations. (see Table 3.5).
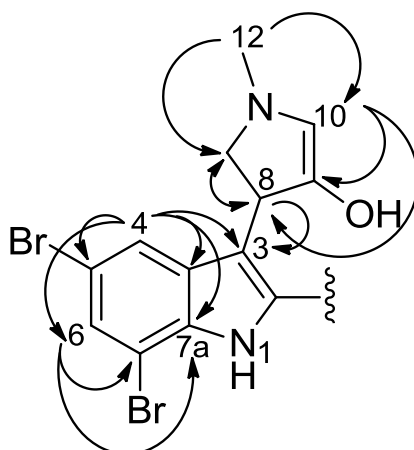
Figure 3.16. Key HMBC and COSY correlations establishing the 5,7 di-bromo indole core and the *N*-methyl hydroxy-pyrrole moiety at C-3. Single headed arrows indicate HMBC correlations, whilst double headed arrows indicate COSY correlations.

Table 3.5. NMR data of amathamine B in CD_3OD

Position	Amathamine B		
	δ_C	δ_H (*J*, Hz)	HMBC (from ^{1}H)
1-N	-	-	-
2	-	-	-
3	106.2	-	-
3a	127.3	-	-
4	120.3	7.33, s	7a, 6, 5, 3a, 3
5	119.6	-	-
6	126.0	7.40, s	7a, 7
7	108.5	-	-
7a	138.3	-	-
8	66.8	4.76	-
9	159.3	-	-
10	93.4	5.24	9,8
11-N	49.9	-	-
12	41.62	2.97	10,13
13	42.1	3.12, m/2.55, m	-

The *N*-methyl protons H-12 showed a strong correlation to C-10 and C-13. Downfield methine proton H-10 showed correlations to carbons C-13, C-9 and C-8. The other downfield methine proton H-8 showed correlations to an indole carbon C-3. The establishment of the two methine and a methylene were reinforced by HSQC (Appendix D.2) and DEPT-135 (appendix D.3) experiments. These data indicated the presence of an *N*-methyl pyrrole, with an electron withdrawing substitute at the double bond. The COSY NMR spectrum (Appendix D.4) indicated that the methylene H-13 were vicinal to the methine H-13. The downfield chemical shift values of the methine C-10 were observed to be reminiscent of an anomeric carbon of cyclic sugars. This and the identification of a molecular formula containing oxygen led to the placement of a hydroxyl at the quaternary carbon C-9.

Formation of the MS^3 fragments *m/z* 300 and 221 from fragment ions *m/z* 412 and 314 require the loss of 41 and 112 mass units respectively. Formation of the MS^2 fragments *m/z* 341 and *m/z* 412 from the parent ion require the loss of 57 and 128 mass units respectively. Both MS^2 fragment losses differ from the MS^3 fragment losses by 16 mass units (57-16 = 41, 128-16 = 112). Losses of 41 and 57 are uneven mass losses, indicating the loss of one nitrogen, as in the similar loss of 43 in the tandem MS of compound 514, these two fragmentations occur in MS^2 and MS^3 analyses, in two different fragmentation pathways, indicating that they represent fragmentation of the same component. This pattern is reflected in the fragmentation of *mz* 128 and 112 mass units, both having a difference of 16 mass units. The even mass lost, 128 and 112 mass units, indicates two nitrogen losses in both fragmentations, consistent with the proposed molecular formula. Therefore,

two separate moieties must fragment off the indole core to give these fragmentation patterns a component containing one nitrogen having a mass loss of 41/57 mass units and another component containing two nitrogen atoms fragmenting to give the loss of 128/112 mass units. Upon determination of the 5,7 dibromo-indole and *N*-methyl hydroxy-pyrrole moieties, the mass loss of 57 was speculated to be the fragmentation of the *N*-methyl hydroxy-pyrrole moiety.

Further NMR spectroscopic analysis was unsuccessful, the final structure for amathamide B **(134)** is proposed from speculation of the presence of an imidazole moiety as present in amathamine B, however as a methyl hydroxy-imidazole moiety. This is proposed due to the presence of a suspected methyl signal, $\delta_{C/H} = 2.84/24.39$. To satisfy the proposed molecular formula an additional hydroxyl was positioned upon the imidazole moiety. These positions were chosen due to the absence of an imidazole proton signal, expected at $\delta_H \sim 7.8$.

3.4 Tandem mass spectrometric analysis of two further brominated compounds from *A. citrina*

3.4.1 Introduction

During the isolation of amathamine A and B semi-purified samples of amathamine C and D were generated. Tandem mass spectrometry analysis indicated these compounds are likely to be brominated indole alkaloids. Amathamine C is proposed to be a tetra-bromoinated variant of amathamine A. Additionally, amathamine D is proposed be a variant of amathamine B with an additional hydroxyl and carbonyl functionalities. Other brominated compounds from *A. citrina* were deemed insufficiently processed from their semi-crude state for analysis.

3.4.2 Amathamine C

The sum difference of mass between amathamine A and C is 78 g/mol, indication of a simple bromine substitution. Based on the interpretation of the tandem MS data (Appendix F.1), compound 592 **(135)** a tetra-bromo variant of compound 514 was proposed. Amathamine C has a longer retention time in reversed-phase chromatography, indicating amathamine C is more non-polar than amathamine A, consistent with the proposed structure.
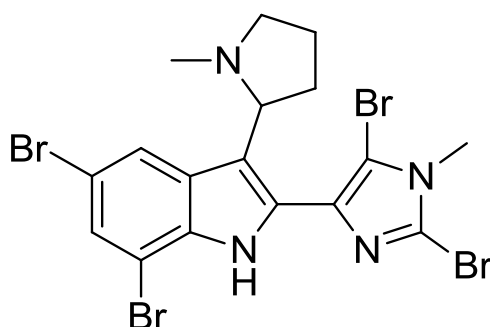
(135)

As in the tandem MS analysis of amathamine A (Table 3.6) the fragmentation of the *N*-methyl pyrrolidine moiety is observed. A loss of 43 mass units in a MS^2 analysis confirmed the presence of an *N*-methyl pyrrolidine moiety, suggesting substitution of bromine likely occurs upon the imidazole ring. A tri-bromo indole core was disregarded because both partially purified compounds amathamine A **(133)** and B **(134)** contain the same 5,7-dibromo indole core. This was further supported by the formation of ion *m/z* 340.9. A mass loss of 252 mass units is required, and is proposed as a loss of 172+Br. A 172 mass unit loss is witnessed in the complete fragmentation of the imidazole moiety in tandem MS analysis of amathamine A.

Table 3.6. Tandem MS analysis of amathamine C

Parent Ion (*m/z*) [M+H⁺]	MS² Fragments Observed (*m/z*)	Mass loss to form MS² fragment (mass units)	MS³ Fragments Observed (*m/z*)	Mass loss to form MS³ fragment (mass units)
593	550	43	470	80
			390	160
			332	218
			239	311
	340.9	252		

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3.4.3 Amathamine D

Based on the interpretation of the tandem MS data (Appendix F.2), the di-bromoinated compound, with the monoisotopic mass 498 g/mol, tentatively called Amathamine D (**136**) was proposed to be a di-brominated variant of amathamine A with additional hydroxyl and carbonyl moieties.
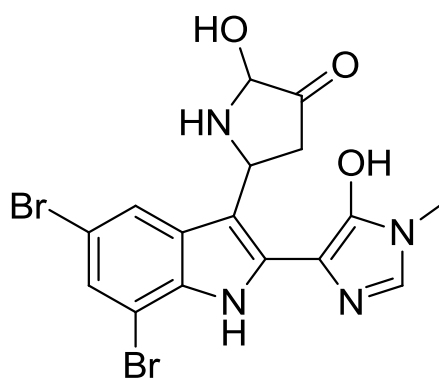
(136)

A mass loss of 75 mass units witnessed in the MS^2 analysis of amathamine D was proposed to be the fragmentation of a pyrrolidine moiety with additional hydroxyl and carbonyl moieties. Data generated from tandem MS analyses was insufficient to establish other structural characteristics of amathamine D. However, as amathamine D was proposed as a di-brominated member of the amathamine family, amathamine D has a isotope pattern consistent with di-bromination, a substitution of the indole bromine with an additional hydroxyl was proposed. Amathamine D has a shorter retention time in reversed-phase chromatography,

indicating amathamine C is more polar than amathamine A, consistent with the

proposed structure.

Table 3.7. Tandem MS analysis of amathamine D

Parent Ion (*m/z*) [M+H⁺]	MS² Fragments Observed (*m/z*)	Mass loss to form MS² fragment (mass units)	MS³ Fragments Observed (*m/z*)	Mass loss to form MS³ fragment (mass units)
469	424	75	381	43
			365	59
			345	79
			312	112
	341	158		

3.5 Future Work

The partial characterisation of amathamines A-D was putatively characterised according to information gathered. The full characterisation of amathamine A, sufficient for publication, was hindered by the potential very long-range HMBC connectivities and no establishment of the absolute configuration. The acquisition of an x-ray diffraction crystallographic structure should solve these two concerns. Additionally, the application of preparative HPLC is potentially advantageous in purification of the sample of amathamine A isolated in this thesis.

The characterisation of amathamine B was hindered by insufficient time invested in isolation and NMR spectroscopic experimentation. Additionally, acquisition of an x-ray diffraction crystallographic structure should provide valuable structural information as well as an absolute configuration determination. Further purification of amathamines A-D is vital for further characterisation.

Chapter Four

Experimental

4.1   Commonly used Solvents and Solutions

The solvents and the compositions of solutions used during this research are listed in Tables 4.1 and 4.2 respectively. MeOH and DCM used for extraction, bench columns and sample preparation were distilled from drum-grade before use. MilliQ water was distilled on an E-Pure still (Barnstead) to an approximate resistance of 17.9-18MΩ.

Table 4.1. Solvents used within this research.

Solvent	Source
Drum-grade dichloromethane (DCM)	Merck
Drum-grade methanol (MeOH)	Merck
HPLC-grade acetonitrile (ACN)	Honeywell International
Methanol-d$_4$ (CD$_3$OD)	Sigma Aldrich
Trifluoroacetic acid (TFA)	Across Organics

Table 4.2. Compositions of solutions used within this research.

Solution	Composition
Extraction Solvent	MeOH:DCM (3:1)
LCMS Solvent A	H$_2$O + 0.01% TFA
LCMS Solvent B	ACN + 0.01% TFA
LCMS Solvent C	ACN:H$_2$O (3:2)
LCMS Solvent D	ACN:H$_2$O (1:1)

4.2   General Experimental Methods

Column fractions greater than 10mL were collected in conical flasks, while fractions of 10mL and under were collected in scintillation vials. Solvent was removed from fractions using a rotary evaporator (Büchi) at 35˚C. Samples were resuspended in MeOH, transferred to scintillation vials, then dried in a heating block (Lab-Line Multi-Block) at 35˚C under a stream of nitrogen gas. Sample masses were determined to four decimal places by weighing on a four figure balance (Mettler, AE-160). Care was taken to ensure vials were clean before all weighings and free of markings and fingerprints by cleaning every vials exterior with MeOH. Lyophilisation was conducted using a FreeZone6 freeze-drier (Labconco).

4.3   Commonly used Fractionation Methods

4.3.1 Reversed-Phase Flash Column Chromatography

Reversed-Phase flash column chromatography was carried out on C_{18} stationary phase (C_{18} YMC Gel ODS-A (120 Å) I-230/70 mesh). The glass column (34 x 370mm) was packed as a slurry in MeOH, and equilibrated to H_2O using successive column volumes of MeOH:H_2O (1:1) then H_2O. Samples were lyophilised and pulverised into a powder, by crushing with a spatula, or made up in minimal amounts of DCM for loading, liquid samples were transferred to the head of the column with a pipette while solid samples were tipped from their vials. Reversed-phase flash columns were run utilising a steep stepped gradient, from

H$_2$O to MeOH, DCM, and, sequentially, back to H$_2$O. A typical solvent system gradient is outlined in Table 4.3

Table 4.3. Typical reversed-phase solvent system gradient.

Sollvent solution	Volume (mL)
H$_2$O	150
H$_2$O:MeOH (1:1)	150
H$_2$O:MeOH (3:7)	150
H$_2$O:MeOH (1:9)	150
MeOH (1:1)	150
DCM:MeOH (1:1)	150
DCM	150
DCM:MeOH (1:1)	150
MeOH	150
H$_2$O:MeOH (1:1)	150
H$_2$O	150

4.3.2 Size Exclusion Chromatography

Size Exclusion Chromatography was carried out on LH-20 stationary phase (Sephadex LH-20, Pharmacia Fine Chemicals) on 35g and 150g sized columns (37 x 440 mm, 55 x 440 mm) packed in MeOH. Samples were dissolved in minimal amounts of MeOH for loading. Size Exclusion columns were run isocratically with MeOH.

4.4   Detection and Characterisation Techniques utilised

4.4.1 Liquid Chromatography Tandem Mass Spectrometry

Samples were fractionated by HPLC (UltiMate 3000; Dionex) coupled to an electrospray ionisation (ESI)-ion trap mass spectrometer (AmaZon X; Bruker Daltonics) for detection. The HPLC and mass spectrometer software, Chromelon (Dionex) and Trap Control respectively (Bruker Daltonics) were controlled by Hystar (Bruker Daltonics). Injections of 20 µL were utilised with a reversed phase Phenomenex, Luna 5µ $C_{18(2)}$ 100 Å, 150 x 4.60 mm column for separation. Solvent gradient utilised (Table 4.4) was a multi-step gradient between two solvents, LCMS solvent A and LCMS solvent B. Each run used a flow rate of 0.2mL/min with a column temperature of 25˚C. Samples were dissolved in MeOH, then were filtered (0.22µm LabServ Millipore filters) prior to analysis.

Table 4.4 Solvent gradient for LCMS experiments

Time (min)	Solvent A (% of total volume)	Solvent B (% of total volume)
0	90	10
2	90	10
27	0	100
39	0	100
45	90	10
50	90	10

Tandem mass spectrometry was acquired using the liquid chromatography parameters above but with modified mass spectrometry methodology. The *m/z* of the protonated ions of interest were selected for, with a slit-width of 2 *m/z*, and the

resulting fragments were recorded. All mass chromatograms, UV chromatograms and mass spectra resulting were analysed in DataAnalysis (Bruker Daltonics).

4.4.2 High Resolution Mass Spectrometry

Accurate masses and molecular formulae were established using a HRESIMS (MicrOTOF; Bruker Daltonics). Samples were made up in distilled MeOH to a concentration of approximately 2 mg/mL and introduced by direct infusion. The instrument was calibrated before each use, within the mass range of interest, using sodium formate (0.2 mg/mL) as the calibrant.

4.4.3 Nuclear Magnetic Resonance Spectroscopy

All NMR spectra were recorded on a 400 MHz spectrometer (AVIII-400; Bruker Daltonics). Chemical shifts were recorded at 300˚K and reported relative to the solvent signal (CD$_3$OD; ^{1}H: δ 3.31 /^{13}C: δ 49.15). Proton spectra were acquired with presaturation of the signals at δ 3.31 and δ 4.78. All heteronuclear and homonuclear one-dimensional and two-dimensional experiments used standard pulse sequences. HMBC spectroscopy utilised an optimised parameter set for ^{2-3}J ^{1}H-^{13}C correlations.

4.5 Work Described in Chapter Three

4.5.1 Isolation of target compounds

Lyophilised *A. citrina* (583.4 g) was collected from Mumbles Pier, Swansea, Wales, by Dr Joanne Porter and Michael Winsa of Herrot-Watt University,

Edinburgh, and exhaustively extracted with MeOH:DCM (3:1). The sample was submerged in approximately 3.25L of solvent and was masticated, via blending, then filtered by vacuum filtration. The residue was resuspended in three more successive aliquots of solvent and re-masticated until the sample was completely extracted, affording a total of thirteen litres of supernatant.

In MeOH fractions of crude extract from reversed-phase (C18, 75g) flash column chromatography the unknown compound identified to be tribrominated with a nominal mass of 514 g/mol eluted, tentatively called amathamide A. Guided my LCMS analysis, these crude MeOH fractions were combined and subjected to an eleven step isolation process of repeated reversed-phase and size exclusion columns to afford a relatively pure sample of amathamide A for analysis. Detailed separation trees are recorded in Appendix G.1 and 2.

From MeOH:H_2O (7:3) fractions of crude extract from reversed-phase (C18, 75g) flash columns, the unknown compound identified to be dibrominated with a nominal mass of 468 g/mol eluted. From multiple identical crude columns equivalent MeOH:H_2O (7:3) fractions, containing compound 468, tentatively called amathamide B, were combined and processed again by reversed-phase flash column chromatography. Fractions with the greatest purities of amathamide B, determined by LCMS analysis, were then treated with size exclusion chromatography (LH-20, 75g), followed by chromatography on a small scale reversed-phase flash column (C_{18}, 10g) to afford a relatively pure fraction of amathamine B for analysis. Detailed separation trees are recorded in Appendix G.1 and.3.

4.5.2 Nuclear Magnetic Resonance Spectroscopic Characterisation of Target Compounds

Samples for analysis were lyophilised to mitigate the contamination of water. Samples were dissolved in CD_3OD (0.5mL, Sigma Aldrich) and transferred into NMR tubes. Experiments were conducted using a Broadband Inverse Probe and the protocol described in Section 4.4.3. Solvent evaporation and potential contamination of water was effectively minimised by sealing the lid of the NMR tube with adhesive tape. Predicted chemical shift values were generated using ChemDraw® Ultra software.[113]

Appendices

Appendix A.1. Collection information of surveyed bryozoan species.

Collection card Number	Species	Collection site
BCC0011	*Bugula flabellata*	Tauranga, New Zealand
BCC0015	*Orthoscuticella*, *Costaticella* (or another *Catenicellidae* species)	White Island, New Zealand
BCC0016	*Orthoscuticella*, *Costaticella* (or another *Catenicellidae* species)	White Island, New Zealand
BCC0017	*Caberea sp.*	White Island, New Zealand
BCC0018	*Hydrozoan / Bryozoan* Mix	White Island, New Zealand
BCC0019	*Hydrozoan / Bryozoan* Mix	White Island, New Zealand
BCC0035	*Orthoscuticella, Costaticella* or *Catenicellidae sp.* (Same as BCC0016)	White Island, New Zealand
BCC0047	*Bugula neritina*	Omokoroa Mooring, New Zealand
-	*Bugula neritina*	Mumbles, Wales
-	*Amathia verticillatum* (Formerly *Zoobotryon*)	Sulphur Point Mariner, Tauranga
-	*Amathia citrina*	Mumbles, Wales
-	*Amathia imbricata*	Mumbles, Wales
-	*Alcyonidium hirsutum*	United Kingdom
-	*Bugula plumosa*	United Kingdom
-	*Schizoporella japonica*	United Kingdom
-	*Bugula fulva*	Scotland
-	*Eucratea loricata*	United Kingdom

Appendix A.2. Chromatograms of bryozoan species surveyed by LCMS.

Appendix A.2.1 *Bugula neritina*.
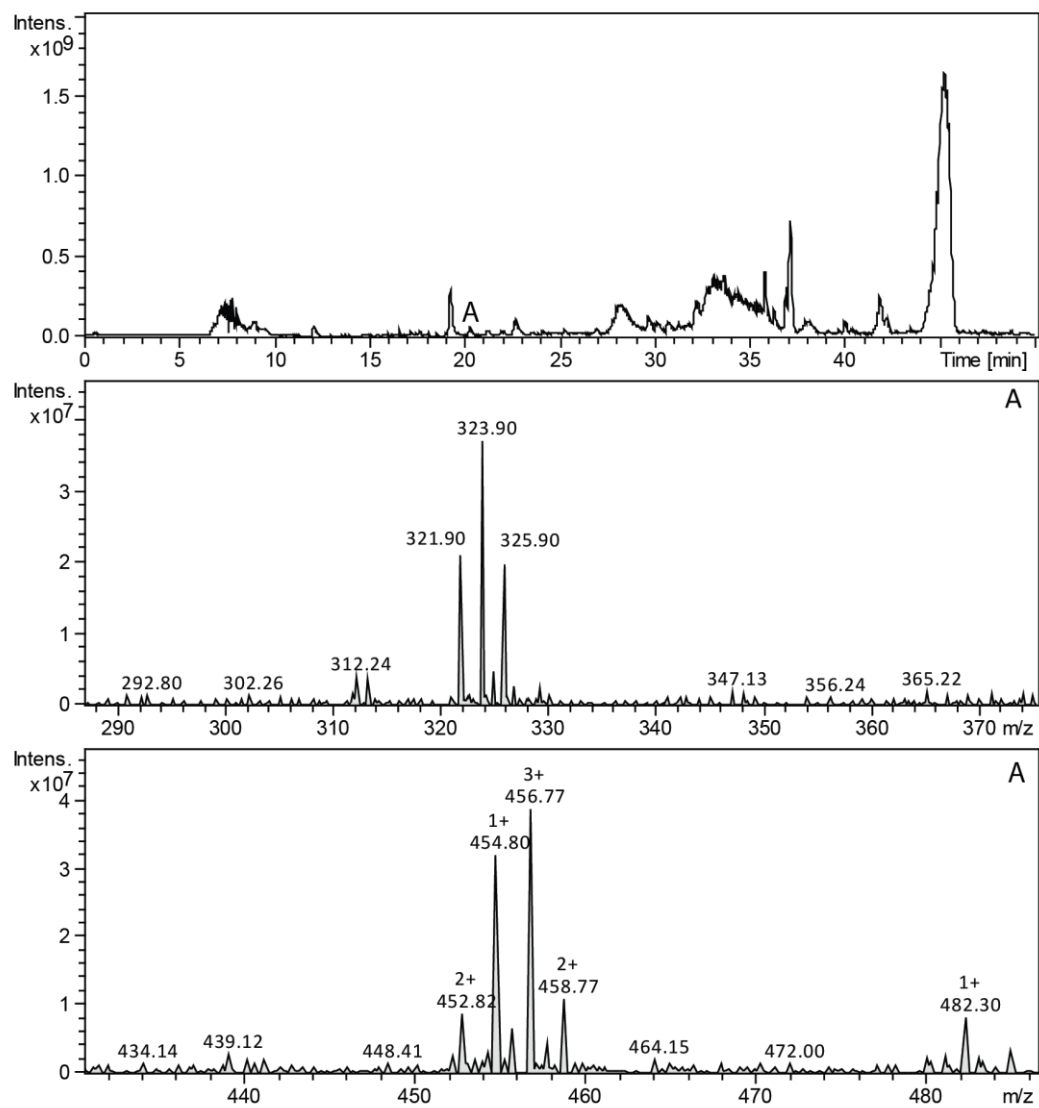
Appendix A.2.2 *Orthoscuticella*, *Costaticella* (or another *Catenicellidae* species)

BCC0016
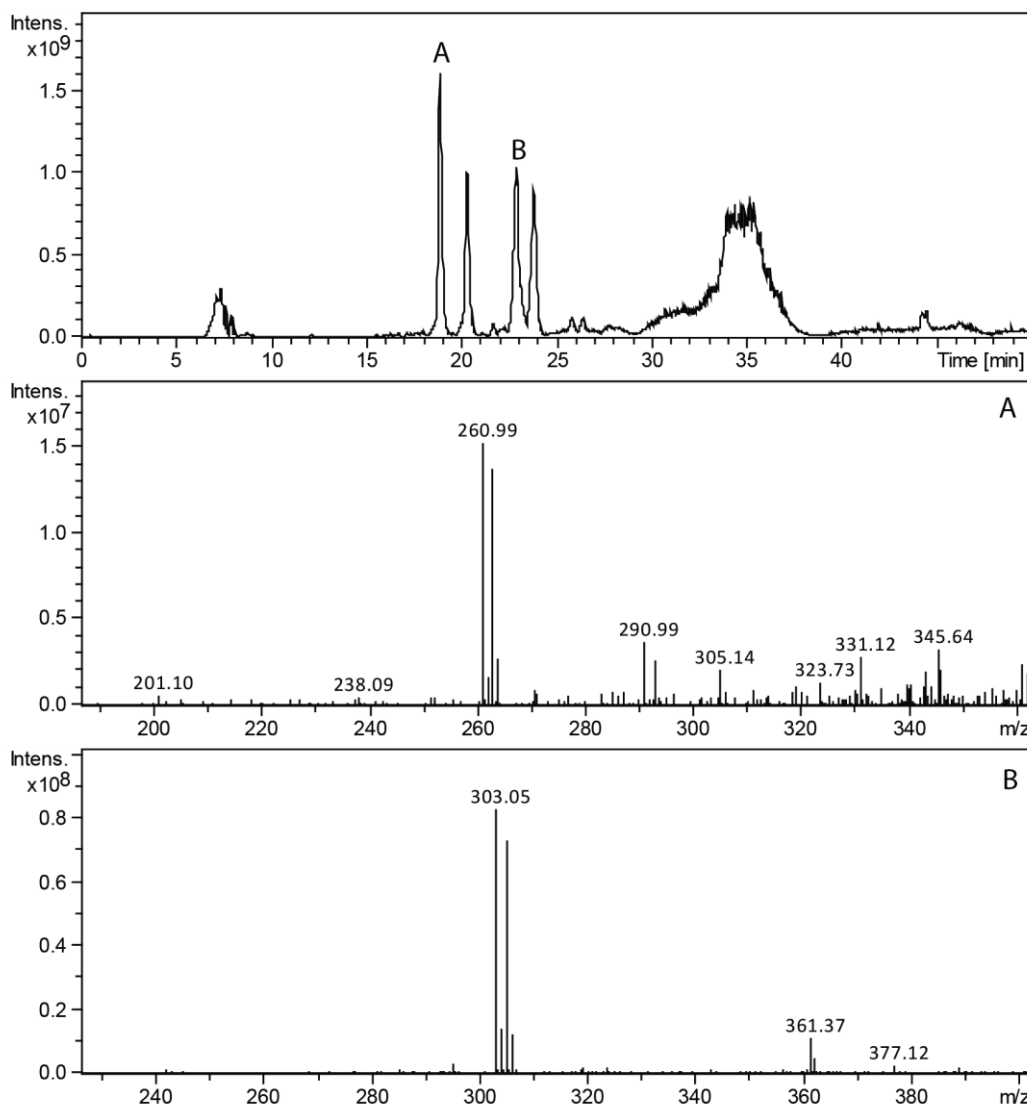
Appendix A.2.3 *Orthoscuticella*, *Costaticella* (or another *Catenicellidae* species)

BCC0015
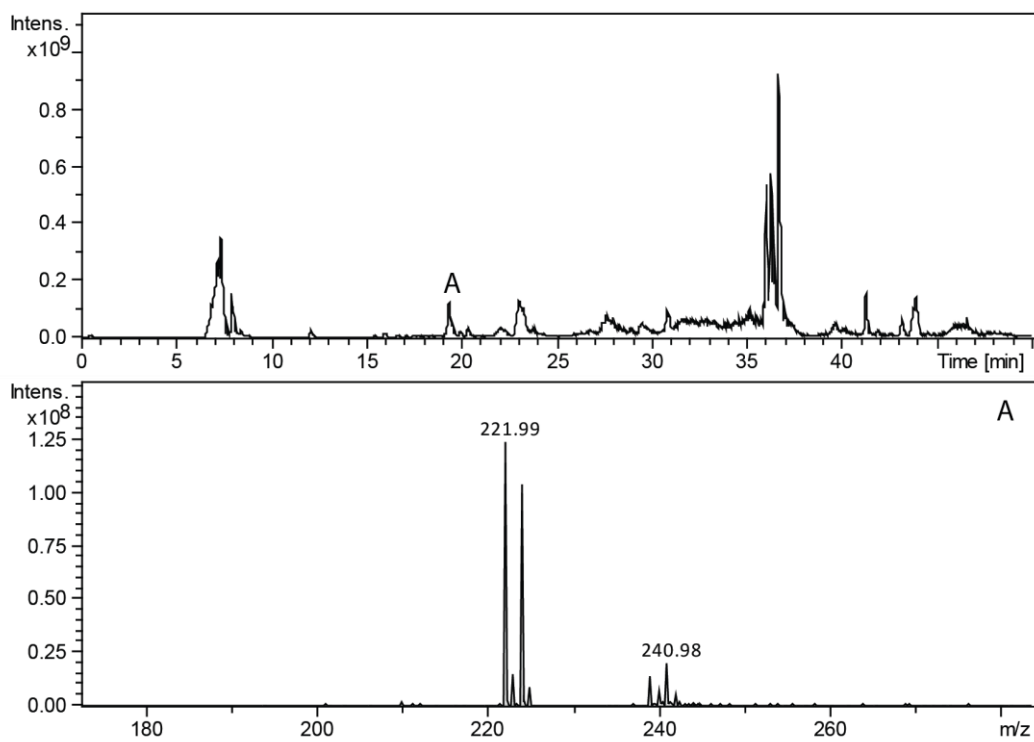
Appendix A.2.4 *Amathia imbricata*
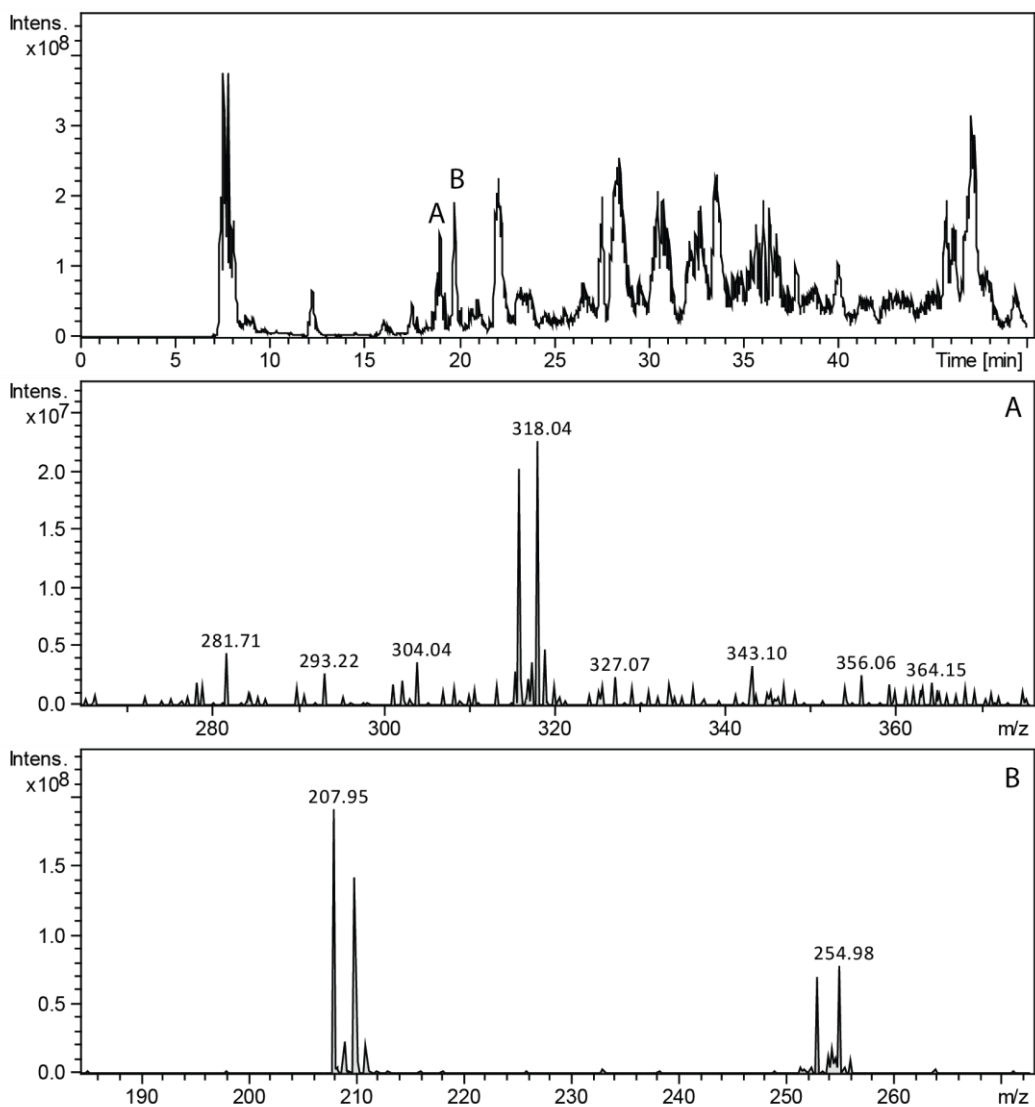
Appendix B.1. HSQC NMR spectrum of amathamine A

CL2.134.1 HSQC135 edited

COSY 134.1 fraction left over

Appendix B.3. HMBC NMR spectrum of amathamine A

CL2.134.1 HMBC Cigar

Appendix B.5. Selective ROESY NMR spectrum of amathamine A, δ_H 7.86 selectively irradiated
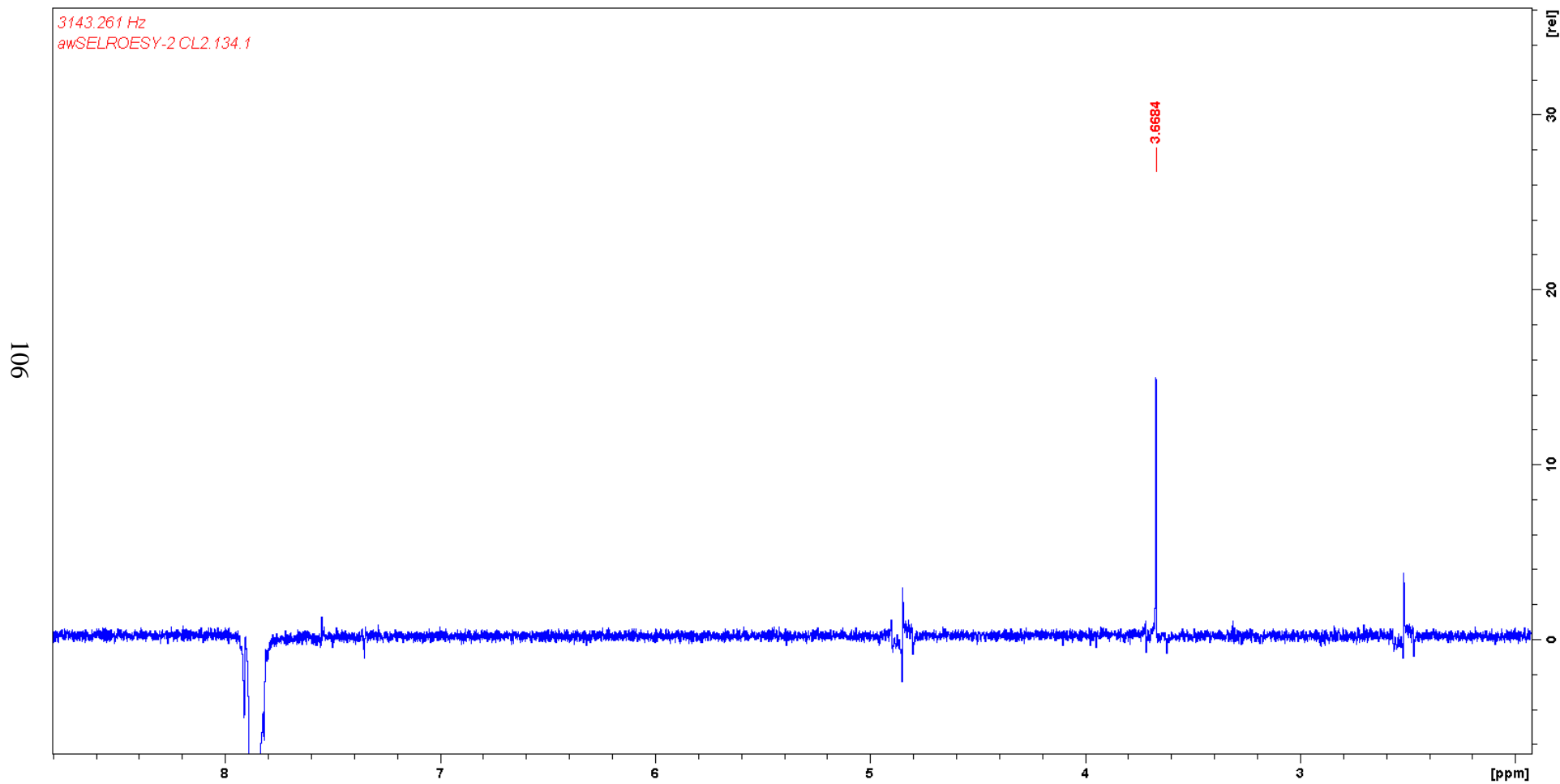
Appendix C. Tandem MS spectra of amathamine A
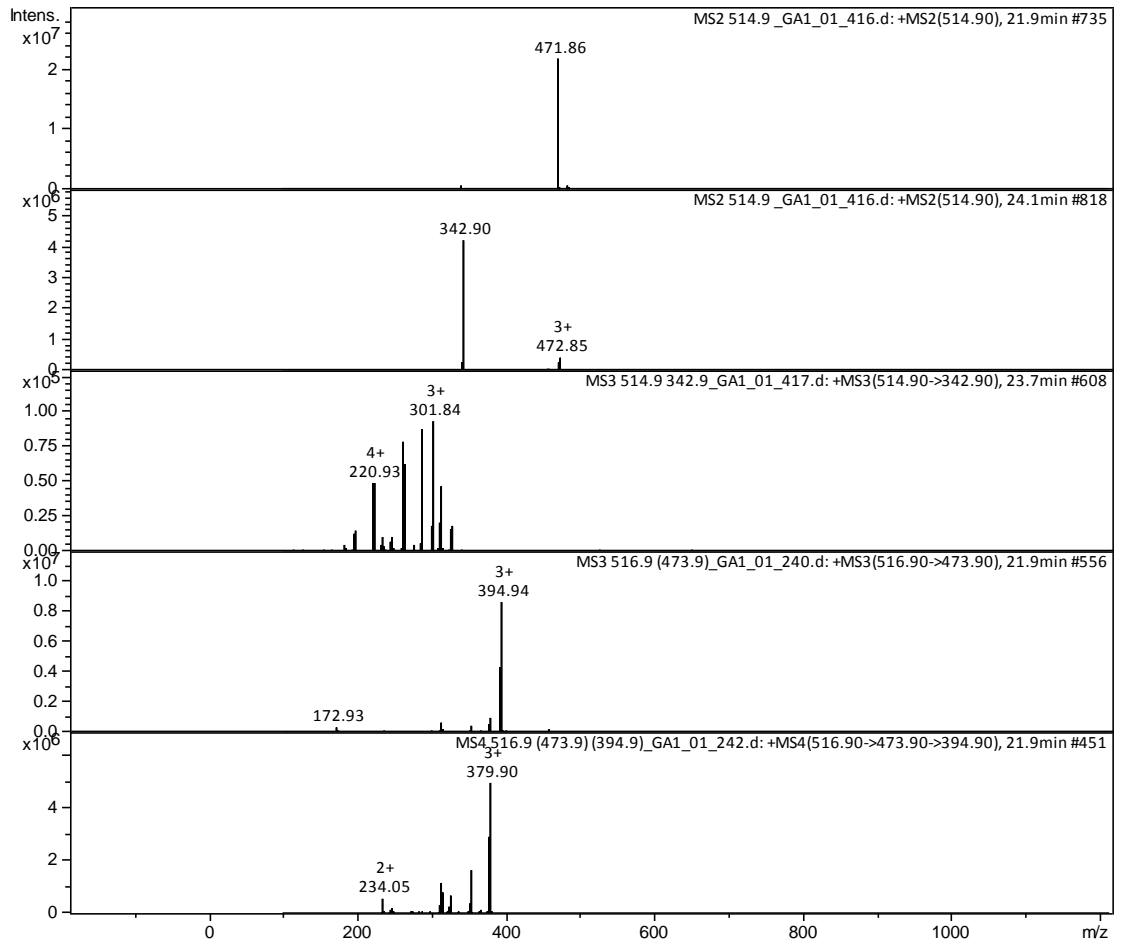
Appendix D.1. HMBC NMR spectrum of amathamine B
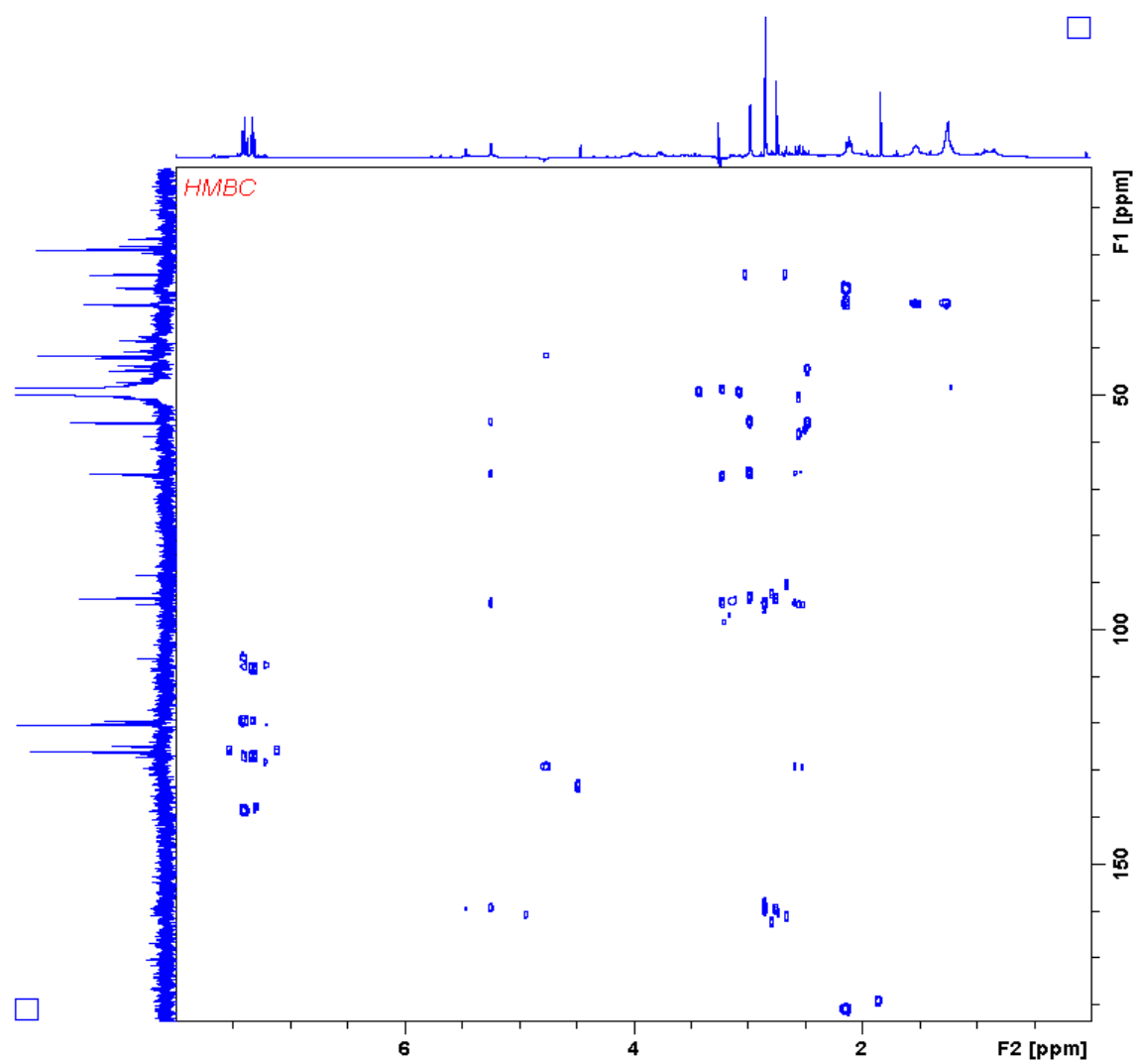
Appendix D.2. HSQC NMR spectrum of amathamine B
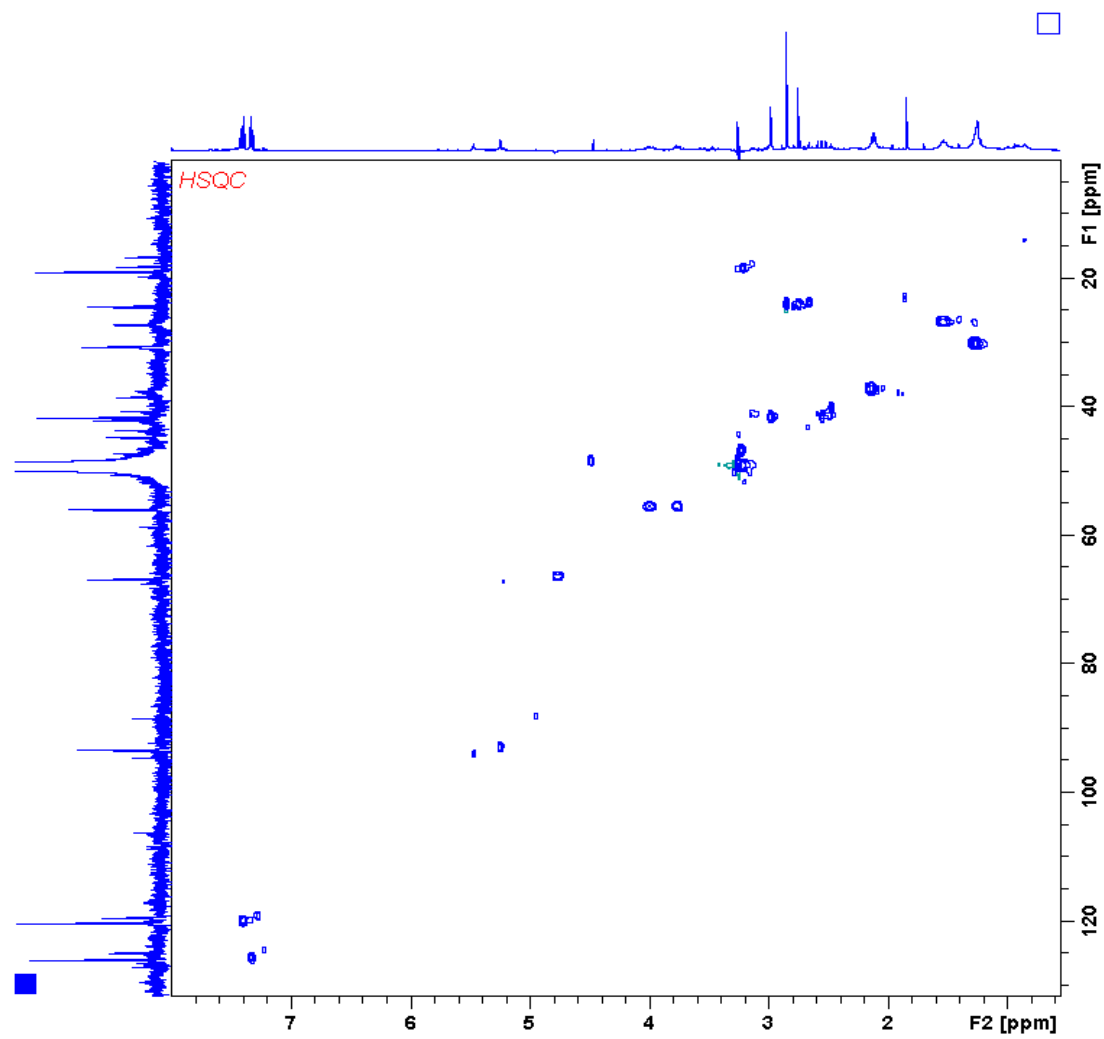
Appendix D.3. DEPT135 NMR spectrum of amathamine B

COSY JS1.79.4
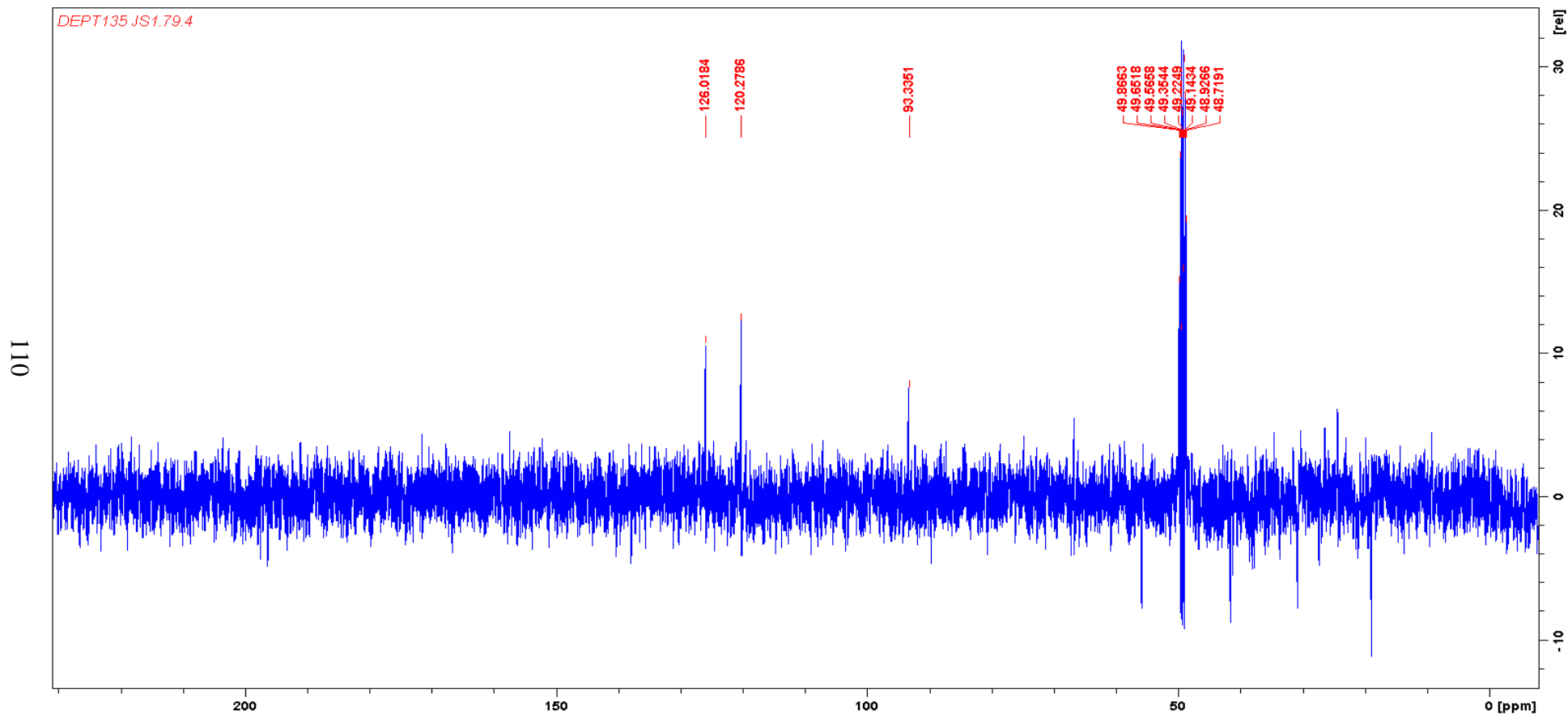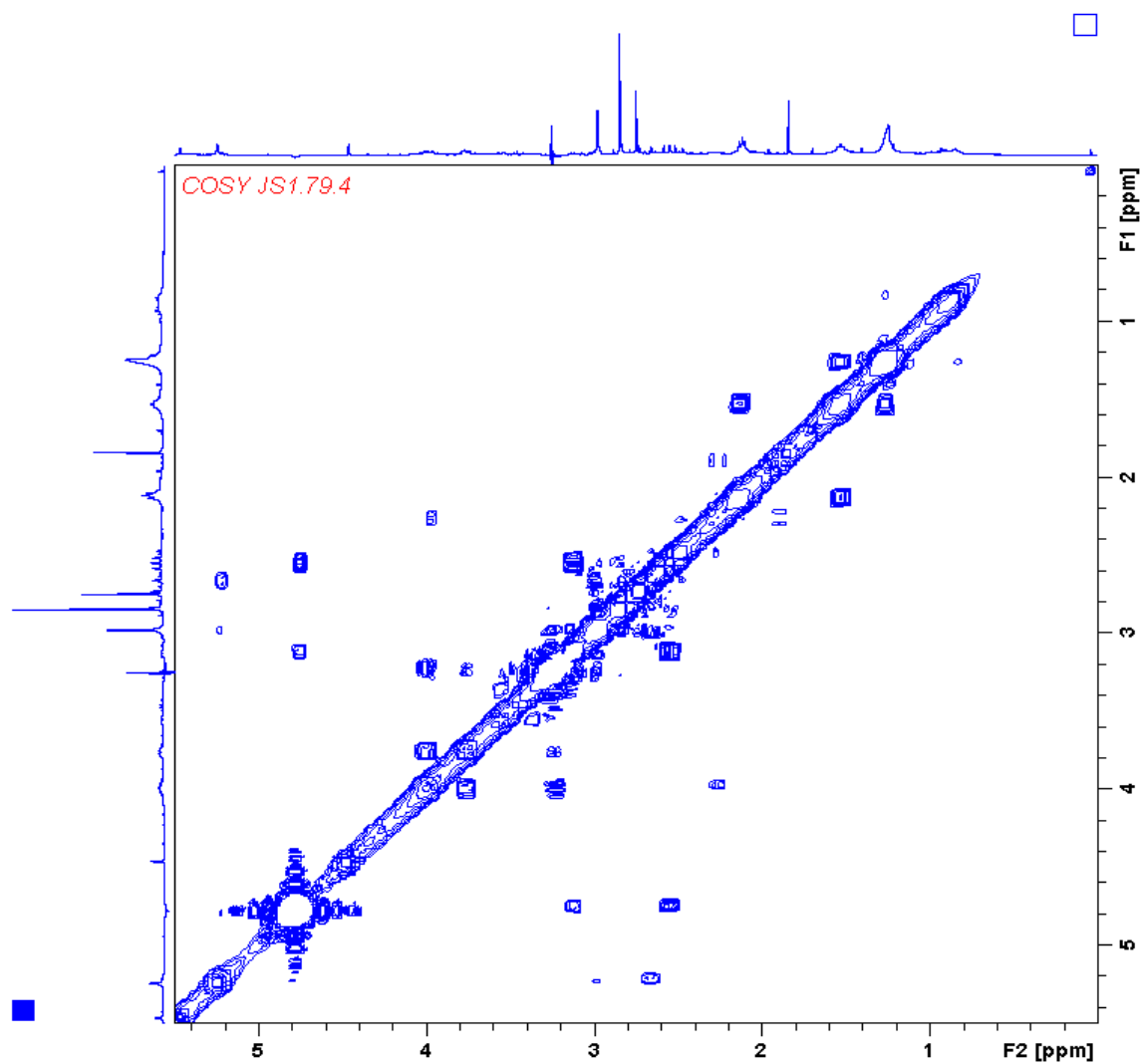
Appendix E.1. Tandem MS spectra of compound 468

Appendix F.1. Tandem MS spectra of amathamine C

Appendix F.2. Tandem MS spectra of amathamine D

Appendix G.1. Separation Trees for the processing of the crude extract

Amathia citrina , freeze dried weight 583g
methanol : dichloromethane (3:1) extract

7.38g   7.07g   7.05g   7.019g   5.02g   7.2g

75g C18 Column (mg)

JS1.17.1	17.2	17.3	17.4	17.5	17.6	17.7	17.8	17.9	17.10	17.11	17.12	17.13	17.14	17.15	17.16	17.17
531.8	820.7	734.3	416.2	191.1	320.5	274.3	472.9	442.4	243.3	897.5	82.2	76.1	82.3	102.4	178.8	304.8

75g C18 Column (mg)

JS1.26.1	26.2	26.3	26.4	26.5	26.6	26.7	26.8	26.9	26.10	26.11	26.12	26.13	26.14	26.15	26.16
468.2	745.0	634.5	441.2	100	250.9	164.6	107.1	530.6	220.3	736.7	258	170.4	210	189.3	285.8

75g C18 Column (mg)

JS1.30.1	30.2	30.3	30.4	30.5	30.6	30.7	30.8	30.9	30.10	30.11	30.12	30.13	30.14	30.15	30.16
–	–	496.3	305.9	175.5	238.7	173.4	339	469.5	179.7	341.3	607.4	82.3	296.1	231.6	

75g C18 Column (mg)

JS1.34.1	34.2	34.3	34.4	34.5	34.6	34.7	34.8	34.9	34.10	34.11	34.12	34.13	34.14	34.15
1234.7	746.8	485.3	421.6	mg	477.1	495.7	407.8	575.9	1066.2	580.6	182.8	116.7	175.6	167.1

75g C18 Column (mg)

JS1.35.1	35.2	35.3	35.4	35.5	35.6	35.7	35.8	35.9	35.10	35.11	35.12	35.13	35.14	35.15	35.16
1987.7	1560.2	675.9	233.5	293.1	224.9	504.5	259.9	446.7	298.2	825.4	320.5	107.7	86.2	33.5	6

75g C18 Column (mg)

JS1.64.1	64.2	64.3	64.4	64.5	64.6	64.7	64.8	64.9	64.10	64.11	64.12	64.13	64.14	64.15	64.16
1110.3	752.3	787.3	315.4	281.5	346.4	339.7	570.1	412.2	245.1	774.9	746.7	169.5	256.8	411.2	300.1

Appendix G.2. Separation Trees for the isolation of compound 514.

Amathia citrina , freeze dried weight 583g

methanol : dichloromethane (3:1) extract

C18 Columns (75g)	C18 Column (75g)	C18 Column (75g)	C18 Column (75g)
7.07 and 7.05g	7.019g	7.019g	5.02g
JS1.34.5-7 and JS1.35.5-7	JS1.17.7-17.9	JS1.26.7	JS1.30.7
2.48g	0.944g	164.6mg	173.4mg

75g C18 Column (mg)

JS1.19.1	19.2	19.3	19.4	19.5	19.6	19.7	19.8	19.9	19.10	19.11	19.12	19.13	19.14	19.15	19.16	19.17	19.18
-	-	63	72.4	319.7	39.4	44.9	32.3	25.7	29.4	69.3	131.7	51.2	94.3	44.1	10.2	2.9	1

35g LH-20 Column (mg)

JS1.22.1	22.2	22.3	22.4	22.5	22.6	22.7	22.8	22.9	22.10	22.11	22.12	22.13	22.14	22.15	22.16	22.17	22.18
-	1.5	2.7	2.9	2.7	-	3	-	-	-	2.9	6.9	0.9	18.9	8.7	6	1.3	-

22.25	22.24	22.23	22.22	22.21	22.20	22.19
1.5	0.1	0.2	0.1	0.2	0.8	0.6

75g LH-20 Column (mg)

JS1.28.1	28.2	28.3	28.4	28.5	28.6	28.7	28.8	28.9	28.10	28.11	28.12	28.13	28.14	28.15	28.16	28.17
-	10.1	63.6	19.6	5.8	3.8	1.7	0.8	1.2	3.2	2.6	8.7	0.8	0	0	0.4	0.2

LH-20 Column(150g)

JS1.32.1	32.2	32.3	32.4	32.5	32.6	32.7	32.8	32.9	32.10	32.11	32.12	32.13
2.3	93.5	18.7	4.2	1.6	2.3	2.4	6.1	3.5	9	2	-	-

75g C18 Column (mg)

JS1.39.1	39.2	39.3	39.4	39.5	39.6	39.7	39.8	39.9	39.10	39.11	39.12	39.13	39.14	39.15
-	-	29	57	89.5	58.1	34.8	27.2	56.7	19.7	56.7	47.9	51.9	3.9	-

75g LH-20 Column (mg)

JS1.40.1	40.2	40.3	40.4	40.5	40.6	40.7	40.8	40.9	40.10	40.11	40.12	40.13	40.14
0.6	47.1	12.9	3.2	2.6	0.6	0.4	1.5	1.9	1.3	-	-	-	-

Combined samples

JS1.22.13. 28.6-8. 32.5. 32.6. 40.9 and 40.10

14.5mg

35g LH-20 Column (mg)

JS1.46.1	46.2	46.3	46.4	46.5	46.6	46.7	46.8	46.9	46.10	46.11	46.12	46.13	46.14	46.15	46.16	46.17	46.18	46.19	46.20
0.6	6	1.3	1.3	1.3	1.2	1.7	1.1	0	0	0	0.6	0	1.2	0	0	-	-	-	-

Combined samples

JS1.17.10, 26.8-10, 30.8-10, 35.8-10

3.094g

75g C18 Column (mg)

JS1.51.1	51.2	51.3	51.4	51.5	51.6	51.7	51.8	51.9	51.10	51.11	51.12	51.13	51.14	51.15	51.16	51.17	51.18	51.19
170	203.2	305.3	62.6	0	34.7	25.6	60.5	58.8	44	37.1	36.9	100	424.7	127.7	305.9	36.2	10.7	-

35g LH-20 Column (mg)

JS1.53.1	53.2	53.3	53.4	53.5	53.6	53.7	53.8	53.9	53.10
1.2	20.4	4.6	4.6	4	1.4	1.4	1.3	3.2	1.5

35g LH-20 Column (mg)

JS1.54.1	54.2	54.3	54.4	54.5	54.6	54.7	54.8	54.9	54.10
2	23.5	6.1	3.7	2.4	1.6	1.9	2.5	4.4	0.9

Appendix G.3. Separation Trees for the isolation of compound 468.

Amathia citrina, freeze dried weight 583g

methanol : dichloromethane (3:1) extract

combined fractions
JS1.17.4, 26.4, 30.4, 34.4
1.50g

combined fractions
JS1.17.3, 26.3, 30.3, 34.3, 35.3, 64.3, 64.4
3.49g

75g C18 Column (mg)

JS1.58.1	58.2	58.3	58.4	58.5	58.6	58.7	58.8	58.9	58.10	58.11	58.12	58.13	58.14a	58.14b	58.15	58.16	58.17
321	303.5	28.8	18.8	8	10.1	9.7	19	9.8	81.2	6.9	4.4	24.1	8.4	2.3	9.1	36.2	0.3

75g C18 Column (mg)

JS1.65.1	65.2	65.3	65.4	65.5	65.6	65.7	65.8	65.9	65.10	65.11	65.12a	65.12b	65.13	65.14	65.15
1,323.90	743.2	80.4	126.5	17.5	10.9	15.8	20	12.1	89.1	17.4	8.3	13.2	3.7	4.3	127.7

150g LH-20 Column (mg)

JS1.76.1	76.2	76.3	76.4	76.5	76.6	76.7
1.5	18	10.4	22.6	17.5	6	12.8

10g C18 Column (mg)

JS1.79.1	79.2	79.3	79.4	79.5	79.6	79.7	79.8	79.9	79.10
-	1.3	1.9	2.4	3.3	4.5	2	1.7	1.3	-

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