New Halogenated Secondary Metabolites from Red Algae of the South Pacific

by
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Abstract

An NMR- and MS-directed study led to the isolation and structure elucidation of several halogenated secondary metabolites from a New Zealand and a Tongan red alga. An extensive investigation was carried out on the New Zealand red alga *Rhodophyllis membranacea* following mass spectrometric evidence for an unusual tetrahalogenated indole with the exceptionally rare inclusion of bromine, chlorine and iodine within a fraction of a semi-purified extract. Due to the difficulty associated with the structure elucidation of proton deficient molecules, a strategic isolation and structure elucidation of several polyhalogenated indoles was employed in order to unequivocally assign the halogen positions on the indolic core. This resulted in the isolation and characterisation of 11 new tetrahalogenated indoles (**123–133**), four of which contain bromine, chlorine and iodine (**124** and **129–131**) and represent the first isolation of such compounds. Additionally, four new pentahalogenated indoles (**134–137**) and an uncharacterised tribromo-trichloroindole were isolated. The synthetically known compound 4-chloroisatin (**138**) was isolated as a new marine natural product, while 4-chloro-3-hydroxyl-3-(2-oxopropyl)-2-oxindole (**139**) was established to be an artefact of isolation. Several compounds were found to exhibit antifungal properties against *Saccharomyces cerevisiae*.

A detailed examination of the Tongan alga *Callophycus serratus* led to the isolation of six new meroditerpenoids: callophycol C (**227**), iodocallophycols E (**228**) and F (**229**), iodocallophycoic acid B (**230**), deiiodocallophycoic B (**231**) and callophycoic acid I (**232**). The relative configurations in compounds **228–231** are proposed to differ from closely related compounds in the literature. Iodocallophycol E (**228**) exhibited moderate cytotoxicity against the HL-60 cell line with an IC₅₀ value of 6.0 μ M.

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Glossary

δ	Chemical shift (ppm)
^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{15}N NMR	Nitrogen-15 nuclear magnetic resonance
^1H NMR	Proton nuclear magnetic resonance
J	Scalar coupling constant (Hz)
T_1	Spin-lattice relaxation
m/z	Mass-to-charge ratio
B-16	Murine melanoma cell line
BACE-1	Beta-site amyloid precursor protein cleaving enzyme
br	Broad
C_{18}	Octadecyl derivatised silica
C_8	Octyl derivatised silica
CIGAR	Constant time inverse-detected gradient accordion rescaled
COSY	Correlation spectroscopy (^1H to ^1H shown as)
d	Doublet
DESI	Desorption electrospray ionisation
DFT	Density functional theory
DIOL	2,3-dihydroxypropoxy-propyl-derivatised silica
EC_{50}	Effective concentration that gives 50% of the maximal response
GI_{50}	Concentration that inhibits growth by 50%
HepG2	Human liver carcinoma cell line
HILIC	Hydrophilic interaction liquid chromatography
HL-60	Human promyelocytic cell line
HMBC	Heteronuclear multiple-bond correlation (^1H to ^{13}C shown as)
HP20	PSDVB stationary support
HP20ss	PSDVB stationary support
HPLC	High-performance liquid chromatography
HRESIMS	High-resolution electrospray ionisation mass spectrometry
HSQC	Heteronuclear single-bond correlation
HT-29	Human colorectal adenocarcinoma cell line
IC_{100}	Inhibitory concentration in 100% test subjects
IC_{50}	Inhibitory concentration in 50% test subjects
LC	Liquid chromatography
LH20	Cross-linked dextran-based size-exclusion chromatographic medium
m	Multiplet
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MS	Mass spectrometry
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NMR	Nuclear magnetic resonance

NOE	Nuclear Overhauser enhancement (^1H to ^1H shown as)
P388	Murine leukaemia cell line
Pos.	Position
ppm	Parts per million
PSDVB	Poly(styrene-divinylbenzene)
q	Quartet
quin	Quintet
R_f	Retention factor
s	Singlet
SAM	<i>S</i> -adenosyl-L-methionine
SAR	Structure-activity relationship
SCUBA	Self contained underwater breathing apparatus
t	Triplet
TLC	Thin layer chromatography
TOCSY	Total correlated spectroscopy (^1H to ^1H shown as)
V-BrPO	Vanadium-dependent bromoperoxidase
V-ClPO	Vanadium-dependent chloroperoxidase
V-HPO	Vanadium-dependent haloperoxidase
V-IPO	Vanadium-dependent iodoperoxidase

Chapter 1

Introduction

The field of marine natural products chemistry has developed considerably since the discovery of sponge-derived nucleosides in the early 1950s.^{1,2} The literature contains a significant number of chemically intriguing, biologically active compounds sourced from a variety of marine organisms, including red algae.^{3–7}

1.1 Marine Red Algae

Fossil evidence shows algae to be an ancient group of organisms that have existed since the Cambrian period.⁸ Throughout this time, they have flourished, becoming an abundant and considerably diverse group of organisms. The presence of photosynthetic pigments give the characteristic green, golden, red and brown hues observed in many algae, and it is the colours exhibited by them that have influenced the names by which they are commonly known—the green, golden, red and brown algae.⁹ The largest of the three groups is the red algae.¹⁰

Red algae (phylum Rhodophyta) consist of over 7000 described species.¹¹ Their global distribution is widespread with speciation abundant in tropical and subtropical regions.^{10,12} While they can be found growing on coastal rocks alongside green and brown algae, their populations tend to be denser at greater depths than those of the other two algal types owing to the presence of accessory pigments (*e.g.* phycoerythrin) that can capture the shorter wavelengths of light penetrating deeper waters.^{12,13} Of the macroalgae, red algae are the most prolific producers of secondary metabolites—molecules that are not involved in the normal metabolic processes required for life, but whose biochemical function contributes to the overall survival of the producing organism.¹² Thus, secondary metabolites may inhibit the growth of nearby algae thereby reducing competition for space, or prevent the

overgrowth of fouling organisms, or provide chemical defence against grazing animals and pathogens.¹² The levels and compositions of algal secondary metabolites are influenced by temporal and spatial variations and environmental factors such as light levels, temperature, nutrients, salinity and biotic interactions.¹⁴

Red algae were among the first organisms to be studied by marine natural product chemists due to their ease of accessibility before the arrival of SCUBA.^{12,13} Since then, the natural products literature has become rife with publications of biologically active compounds derived from red algae, many of which are halogenated. Examples include maharone (**1**) and 5-bromomaharone (**2**),¹⁵ the monoterpene plocamenone (**3**),¹⁶ the diterpenes **4–7**¹⁷ and the omaezallenes (**8–10**).¹⁸ Maharone (**1**) and 5-bromomaharone (**2**) were isolated from *Asparagopsis taxiformis*;¹⁵ an alga that has also been found by various research groups to produce more than 100 simple halogenated compounds, many of which are volatile.^{19–22} It has been suggested that volatile halocarbons such as those produced by *A. taxiformis* could be the reason for the smell of the ocean.²³ Both **1** and **2** exhibited activity in an assay against the bioluminescent bacteria *Vibrio fischeri* (EC₅₀ 0.16 μ M observed for both compounds).¹⁵ Plocamenone (**3**), originally isolated in 1979, was the subject of several structural revisions before the structure depicted below was reported in 2012. Potent cytotoxicity was observed for **3** against the P388 (murine leukemia) cell line (IC₅₀ 0.16 μ g/mL).¹⁶ The diterpenes **4–7** were isolated from *Sphaerococcus coronopifolius* and displayed varying levels of antiproliferative activity in HepG2 (human liver carcinoma) cells (IC₅₀ 42.9–279.9 μ M). The highest activity was comparable to that shown by the positive control.¹⁷ Compounds **8–10** displayed antifouling activity against the cypris larvae of the barnacle *Amphibalanus amphitrite* with EC₅₀ values of 0.22, 0.30 and 1.50 μ g/mL, respectively.¹⁸ Chemically intriguing, biologically active halogenated metabolites such as **1–10** are the reason that red algae continue to captivate marine natural product researchers.

1.2 Halogenated Natural Products

The halogens are in group 17 of the period table[†] and comprise fluorine, chlorine, bromine, iodine and astatine.²⁴ The first four are reactive non-metals that form a variety of compounds including diatomic, organic and inorganic molecules.²⁵ Some of their physicochemical properties are presented in Table 1.1.²⁶ Astatine is a highly unstable, radioactive element; its most stable isotope has a half-life of only eight hours.²⁴ Compared with the other halogens, little is known about astatine.

Halogenated natural products were once considered to be artefacts of isolation or chemical accidents of nature but they are now recognised as authentic natural products.²³ In 1968 there were fewer than 30 such known natural products from both the terrestrial and marine environments.²⁸ Today, that number has significantly increased to more than 5000²⁸ with the largest proportion being represented by brominated molecules followed by chlorinated, iodinated and fluorinated compounds.

[†]Also in group 17, is the recently discovered tennessine, which is a highly radioactive element that is predicted to be metallic.²⁴

Table 1.1. Some Physicochemical Properties of the Common Halogens.

	Bond dissociation energy ²⁶	Bond length ²⁷	Dipole moment ²⁶	Hydration energy ²⁶	Standard electrode potential, ²⁷ $E^\ominus$	
	CH_3X	$\text{C}-\text{X}$	CH_3X	X^-	$\text{X}_2 + 2\text{e}^- \rightleftharpoons 2\text{X}^-$	Electronegativity ²⁷
Halogen	(kJ mol^{-1})	($\text{\AA}$)	(Debye)	(kJ mol^{-1})	(V) [‡]	(Pauling scale)

[‡]The reverse half-cell reaction depicts oxidation and the corresponding magnitude of $E^\ominus$ is equal but opposite in sign.

The chemical and structural diversity of halogenated natural products varies considerably from simple compounds containing one carbon (*e.g.* haloform) to complex molecules comprised of more than 50 carbons.²⁹ Equally diverse are the terrestrial and marine organisms that biosynthesise these extraordinary molecules, such as fungi, plants, sponges, algae, corals, tunicates, bacteria, cyanobacteria and even humans.²⁸ In humans, iodine is an essential element required in the diet for the production of the metabolism-regulating hormone thyroxine (**11**).^{30,31} An imbalance in iodine can disrupt thyroxine (**11**) biosynthesis, which can lead to hypo- or hyperthyroidism.³¹ The vast majority of halogenated natural products have been covered in several reviews published by Gribble^{23,28–30,32–38} while Wang *et al.* have provided the most recent review of the naturally occurring organoiodides.³⁹

11

As with non-halogenated secondary metabolites, those that are halogenated are believed to facilitate advantages in the survival of an organism.³⁴ In some compounds, the presence of halogen substituents show increased biological activity when compared to their non-halogenated counterparts. For example, rebeccamycin (**12**) exhibits strong cytotoxicity against the murine B16 (melanoma) and P388 cell lines, but the semi-synthetic dechlororebeccamycin (**13**) shows a significant decrease in activity.⁴⁰ The antibiotic vancomycin (**14**) is another example. It

disrupts peptidoglycan cross-linking in susceptible bacteria by preferentially binding to intermediates terminating in specific amino acids. Upon the removal of both chlorine substituents the molecule has increased flexibility, which leads to a decreased specificity of the binding site and consequently reduced activity.⁴¹ Compounds **12** and **14** were originally isolated from terrestrial-derived bacteria,^{42,43} however, the largest proportion of the total number of halogenated natural products have originated from marine organisms.³⁴ This is unsurprising considering the halogen-rich environment that is sea water (Table 1.2).³⁷

Table 1.2. Concentration of Common Halides in the Oceans and Sedimentary Rock.³⁷

---

12 R = Cl

13 R = H

14

1.2.1 Brominated and Chlorinated Marine Natural Products

The concentration of chloride ions in the oceans seemingly dwarfs that of bromide, fluoride and iodide by being *ca.* 600, 7000 and 1 million times more abundant, respectively (Table 1.2).³⁷ Despite its great abundance, the relative proportion of reported chlorinated to either brominated or iodinated marine natural products is small; bromine and iodine-containing natural products occur in significantly higher numbers than their relative abundances would suggest. This is due to the mechanisms by which halogens are incorporated. Biohalogenation is typically an enzymatic oxidation process (see Section 1.2.4) and the oxidation potential increases in the order fluoride<chloride<bromide<iodide (see Table 1.1). Thus, the last two halogens are more readily susceptible to oxidation and this leads to a higher occurrence of brominated and iodinated natural products than is to be expected on the basis of their relative concentrations in seawater.⁴⁴

Perhaps the earliest known bromine-containing metabolite is 6,6'-dibromoindigo (**15**), more famously known as Tyrian purple.³⁶ The intensely purple coloured compound has been used as a dye for centuries but it was only in 1909 that it was isolated from the mollusc *Murex brandaris* and characterised.⁴⁵ Since then, the number of brominated metabolites derived from the marine environment has increased significantly.⁴⁴

A considerable number of marine-derived halogenated natural products carry both bromine and chlorine.⁴⁶ Approximately 40% are substituted by at least one chlorine atom and 71% by one bromine atom.⁴⁴ Red algae have been a major source of such compounds with numerous examples found in terpenes, such as **16–18**^{45,47,48} and C₁₅ acetogenins, such as **19–21**.^{49,50} Between these compounds moderate antifungal,

cytotoxic, and antibacterial activities was observed.

Halogenated marine natural products substituted by either bromine or chlorine have frequently been isolated from sponges. Unlike algal metabolites, the presence of both bromine and chlorine atoms in sponge-derived metabolites is relatively rare. Mollenynes A (**22**)⁵¹ and B (**23**)⁵² from *Spirastrella mollis*, are examples of sponge-derived bromochloro- substituted natural products. Compounds **22** and **23** differ by an interchange of the halogens and the configurations at positions C-8 and C-9. It is suggested that this could occur *via* a type I dyotropic rearrangement in **22**, whereby a 1,2-shift of the halogens in a concerted manner leads to the formation of **23**.⁵² Investigations of the sponge *Dysidea herbacea* have resulted in the isolation of polychlorinated compounds such as dysidamide (**24**)⁵³ and dysideathiazole (**25**).⁵⁴ Dysideathiazole (**25**) and closely related derivatives isolated in the same study were deduced as strongly deterrent in fish feeding experiments.⁵⁴ A cyanobacterium symbiont is believed to be the true source of chlorinated compounds found in *Dysidea* sponges.⁵⁵

Other examples of marine-derived halogenated natural products have been isolated from ascidians (*e.g.* **26**),⁵⁶ seahares (*e.g.* **27** and **28**),^{57,58} gorgonians (*e.g.* **29**),⁵⁹

fungi (*e.g.* **30**),⁶⁰ soft corals (*e.g.* **31**),⁶¹ bacteria (*e.g.* **32**)⁶² and molluscs (*e.g.* **33**).⁶³ Compound **26** exhibited potent cytotoxicity against the P388 cell line (IC₅₀ 1 ng/mL, respectively).⁵⁶ Compound **28** was ichthyotoxic[†] to *Gambusia affinis* (IC₁₀₀ 3 mg/L over 24 h),⁵⁸ while polycyclic **29** was noted to exhibit antifouling activity against *A. amphitrite* (EC₅₀ 4.1 µg/mL).⁵⁹ The polychlorinated sulfolipid **33**, isolated from the digestive glands of the bivalve mollusc *Mytilus galloprovincialis*, has a highly functionalised side-chain containing 11 chlorine atoms. While **33** was inactive in an antiproliferative bioassay, chlorolipids isolated from *Chrysophyte* species have been found to show antibiotic activity.⁶³

[†]Toxic to fish.

1.2.2 Iodinated Marine Natural Products

The 1811 discovery of iodine was by the French chemist Bernard Courtois. Upon adding excess sulfuric acid to seaweed ash, molecular iodine and its concomitant violet vapour subsequently formed, which condensed to a lustrous black crystalline solid.⁶⁴ Almost a century later, 3,5-diiodotyrosine (**34**) was discovered from the coral *Gorgonia cavolinii* as the first iodinated natural product.³⁹

Between 1896 and 2014 there were 182 naturally occurring organoiodides reported in the literature. More than two-thirds of iodine-containing natural products originated from the marine environment and close to half of those were sourced from marine algae.³⁹ Within the halogenated marine natural products literature, 3% contain

iodinated compounds. This is a significant percentage considering the concentration of iodide in sea water relative to chloride and bromide ions.⁴⁴

Of the isolated iodinated metabolites, several also contain either bromine or chlorine. Most of these are found in small molecules isolated from red algal species of the genera *Asparagopsis*, *Delisea* and *Falkenbergia* and include halogenated derivatives of compounds such as propanones, acetic and propanoic acids, acetimides and butenols.³⁹ Other examples of iodinated marine natural products include oxylipins (*e.g.* **35** and **36** from a brown alga),⁶⁵ acetylenes (*e.g.* **37–39** from a sponge),^{66,67} furanones (*e.g.* **40–42** from a red alga),⁶⁸ prostaglandins (*e.g.* **43** from a soft coral),⁶⁹ terpenes (*e.g.* **44** and **45** from a red alga and soft coral, respectively),^{70,71} depsipeptides (*e.g.* **46** and **47** from a sponge)⁷² and a trisulfated sterol (*e.g.* **48** from a sponge).⁷³ Naturally occurring compounds substituted with chlorine, bromine and iodine are extremely rare with only three examples reported in the literature (see Section 3.5.5).^{19–21,74} A range of biological activities have been reported for iodinated natural products including cytotoxic, antiinflammatory, antibacterial, antifungal, anticonvulsant, antihistaminic, antifouling and enzyme-inhibiting.³⁹

39

40 R = H

41 R = OH

42 R = OAc

43

44

45

46 R = Me

47 R = H

48

1.2.3 Fluorinated Marine Natural Products

Fluorine-containing metabolites are rarely encountered. A number of factors contribute to the low occurrence of naturally occurring organofluorides. Despite fluorine being the thirteenth most common element present in the Earth's crust, most is contained within insoluble minerals severely limiting bioavailability. Fluoride has the lowest oxidation potential of the halides, which prevents biohalogenation by the same mechanisms used for chlorine, bromine and iodine. Additionally, the high

hydration energy of the fluoride ion renders it a poor nucleophile in water (Table 1.1).^{26,75}

From the marine environment only fluoroacetate (**49**) and five 5-fluorouracil analogues (**50–54**) have been isolated from a *Streptomyces* sp. bacterium and the sponge *Phakellia fusca*, respectively.^{76,77} Fluoroacetate (**49**), originally isolated in 1943 as the toxic component of the terrestrial plant *Dichapetalum cymosum*, is the most common organofluoride found from natural sources.²⁶ Interestingly, compound **50** is an anticancer drug used in the treatment of head and neck, colorectal, bladder, and breast cancers,⁷⁸ and surprisingly, it has been suggested that *de novo* biosynthesis of **50–54** is not carried out by *P. fusca* but instead **50** is accumulated by the sponge from industrial effluent.⁷⁶

1.2.4 Vanadium-dependent Haloperoxidases

The insertion of halogens into biological substrates typically occurs *via* enzymatic oxidation. The incorporation of fluorine and in some instances chlorine, is an exception as the enzyme involved facilitates a nucleophilic substitution reaction. Several families of enzymes that catalyse biohalogenation have been identified, such as the vanadium-dependent and haem iron-dependent haloperoxidases and the non-haem iron-dependent, flavin-dependent and *S*-adenosyl-L-methionine (SAM)-dependent halogenases.⁷⁹ Vanadium-dependent haloperoxidases (V-HPOs) are the predominant halogenating enzymes found in algae and have been studied extensively by Butler and co-workers.^{79–84}

The first V-HPO was discovered in 1983 from the brown alga *Ascophyllum nodosum* and more have since been found in other macroalgae,^{84,85} fungi⁸⁶ and bacteria.^{79,87} V-HPOs catalyse halide oxidation by hydrogen peroxide to form a two-electron oxidised species (*e.g.* hypohalous acid, hypohalite or an enzyme-bound halonium-type species), which can then proceed to react *via* electrophilic halogenation with the organic substrate or with a second equivalent of hydrogen peroxide when a suitable organic substrate is lacking (Scheme 1.1).⁷⁹ The oxidation state of the vanadium centre remains constant throughout the process and thus, the V-HPO functions as a Lewis acid catalyst rather than a redox catalyst.⁷⁹

Scheme 1.1. Scheme of the halide and peroxide reaction with a vanadium haloperoxidase.⁸⁰

V-HPOs are categorised according to the enzyme's ability to catalyse the oxidation of the halide with the lowest oxidation potential (for oxidation potentials see Table 1.1). Thus, a chloroperoxidase (V-ClPO) is capable of catalysing the oxidation of chloride, bromide and iodide ions; bromoperoxidase (V-BrPO) can catalyse the oxidation of bromide and iodide, and iodoperoxidase (V-IPO) can catalyse the oxidation of the iodide ion only.⁸⁰ Thermodynamic studies indicate that it is impossible for the oxidation of fluoride ions to occur by the reduction of hydrogen peroxide (Scheme 1.2) implying that no such fluoroperoxidase exists.²⁶ The V-BrPOs appear to be the most prevalent, particularly in algae, and research has been carried out on their catalytic oxidative properties. Two such studies have shown that the enzyme, in the presence of aqueous bromide and hydrogen peroxide, catalyses the regiospecific oxidation of an indole derivative⁸² and the stereoselective bromination and subsequent cyclisation of a terpene.⁸¹ Each reaction is thought to proceed through a bromonium ion intermediate. The observed regiospecificity and stereoselectivity suggests that the organic substrate is oriented in a specific way

within the active site of V-BrPO.^{81,82} Organic substrate selectivity is also known for the V-BrPOs,⁸¹ although the factors governing this selectivity are yet to be determined.⁷⁹ It is also intriguing that similar V-HPOs can exist in two related algae, yet halogenated natural products can be predominant in one but not the other.⁷⁹

Scheme 1.2. Standard electrode potential and redox equilibria of fluoride and hydrogen peroxide.²⁷

1.3 Proposed Research

The research described in this thesis was aimed at identifying and characterising new secondary metabolites from marine sponges and red algae, particularly those that are halogenated. An NMR and MS-guided isolation methodology was employed to facilitate these efforts. In particular, it was anticipated that using MS to guide the isolation process would be extremely powerful due to the isotopic nature of bromine and chlorine. Where possible, compounds isolated in this study were submitted to the School of Biological Sciences for biological activity assessment.

Chapter 2

Screening of Marine Organisms

Once an organism has been selected for investigation, it is extracted with organic solvent. A typical crude extract contains a vast array of compounds ranging in polarities from hydro- to lipophilic. In the scope of polarities present, compounds at the outer extremes are largely primary metabolites (compounds that are essential to normal metabolic processes within an organism) that constitute a large proportion of the total number of compounds present within a crude extract. By comparison, secondary metabolites represent a much smaller amount of the crude extract mass and generally possess amphiphilic properties with a combination of polar and non-polar functional groups.⁸⁸ Within a biological system, this amphiphilic nature enables partial solubility in water and the ability to traverse across a cell-membrane to interact with the substrate.⁸⁹ It is partly for this reason that secondary metabolites entice the interest of natural product chemists.

2.1 Targeting Secondary Metabolites

The large chemical diversity and range of polarities within a crude extract often results in solubility issues, making analyses at this early stage very problematic. A preliminary purification step performed on the crude extract either substantially minimises or completely circumvents these issues. The hypothetical diagram illustrated in Figure 2.1 can be used as a chromatographic guideline for the preliminary purification of a crude extract so that further analyses can be carried out with ease. The majority of the mass is anticipated to elute from reversed-phase chromatographic media in the early (polar compounds such as salts and sugars) and late stages (non-polar compounds such as lipids), leaving the central region or ‘mass-window’ to contain compounds of intermediate polarity.⁹⁰ Therefore, the aim

would be to target this region during the purification of a crude extract, as it should be comprised of mostly secondary metabolites.

Figure 2.1. Mass distribution of a biological extract and the elution profile from a reversed-phase chromatographic medium. Adapted from Ryan.⁹¹

2.1.1 PSDVB and Cyclic Loading

Our research group utilises the ‘mass-window’ concept (Figure 2.1) during the early purification of a crude extract. Before it can be employed, the extract must first be loaded onto a chromatographic medium for purification—cyclic loading serves this purpose. The technique ‘cyclic loading’ uses reversed-phase poly(styrene-divinylbenzene) (PSDVB) beads and was developed by Northcote and West at VUW for the preliminary purification of a crude sponge extract.⁹⁰ While cyclic loading can be employed using any chromatographic adsorbent medium, PSDVB is particularly attractive as it is inexpensive, chemically inert and stable throughout the pH range. Respectively, the latter two properties mean that PSDVB can be recycled repeatedly without concern and the adsorption and retention of compounds can be facilitated with the use of ionic buffers. In addition to sponges,^{89,91–93} the technique has since been used successfully by VUW natural products research groups for the initial fractionation of algal,¹³ sea cucumber,^{94,95} nudibranch⁹² and extremophilic microbial⁹⁶ extracts, thus becoming an integral method in our laboratories.

Conceptually, cyclic loading is the reverse of chromatography—instead of sequentially eluting compounds of interest from the chromatographic medium, the

technique sequentially adsorbs them.⁸⁹ The general cyclic-loading procedure is as follows: an organism of interest is extracted with organic solvent and the crude extract is passed through a column containing PSDVB where most of the non-polar compounds adsorb to the stationary phase. The eluent is subsequently diluted with an equal volume of H₂O (100% v/v) and recycled through the column. The dilution and recycling steps are repeated until all compounds of interest are adsorbed to the PSDVB; usually a four-fold dilution of the extract is sufficient. Adding H₂O incrementally leads to a gradual increase in eluent polarity. This effectively minimises any risk of precipitation and also increases the affinity of compounds, still in solution, to the stationary phase, thus ensuring their adsorption. Once cyclic loading is complete, the column is washed with H₂O to remove salts, then successively eluted with H₂O/organic modifier mixtures of decreasing polarity. Cyclic loading is pictorially presented in Figure 2.2.

The resulting fractions obtained from the elution of a PSDVB column are subsequently back-loaded (a modified cyclic loading technique) onto another PSDVB column. This is to effect a solvent change due to the tendency of some H₂O/organic modifier mixtures frothing vigorously under reduced pressure. The fraction is diluted with H₂O (100% v/v) and recycled (back-loaded) onto PSDVB. The eluent is collected and the process repeated once more—at this stage most of the compounds should have re-adsorbed onto the PSDVB column. The column is then blown dry to minimise the amount of H₂O present after which point elution is carried out using a pure organic solvent that can be concentrated without difficulty to provide samples that are ready for screening.

2.2 Natural Products Screening

Screening a partially purified organic extract of a given organism serves to determine whether the organism is worthy of a more detailed scientific investigation before considerable time and resources have been invested. Two main screening techniques

exist: bioassay-guided screening and spectroscopy-guided screening. The former technique tends to be more commonly used among natural product researchers as a primary method.

Figure 2.2. Schematic of the cyclic loading procedure. Adapted from West.⁹⁰

2.2.1 Bioassay-guided Screening

Bioassay-guided fractionation has been used extensively to screen and guide the isolation of natural products.^{97,98} A source organism is selected following a positive result of the extract against a specific biological assay. The extract is then purified and the fractions reassessed for biological activity in the same assay. Those producing a positive result undergo repeated purification/testing processes until the biologically active compound is isolated. As a result, the method ultimately yields a compound of potential pharmaceutical interest and forms the reason (validly) for the preference of this method among researchers in the field. However, the

complex mixtures of compounds (even after one purification step) is problematic because the biological activity of major components could mask minor, potentially novel active compounds that may pass through the assay undetected. Additionally, assay results can be confounded by interference compounds (those causing false positives) or synergistic effects between two or more components both of which can be misleading.⁹⁸

2.2.2 Spectroscopy-guided Screening

Spectroscopy-based screening focuses on the chemical make-up of compounds within a crude extract. No information on biological activity is offered but there is perception is that structural novelty frequently leads to interesting biological activity.⁸⁹

Mass-spectrometry coupled with liquid chromatography (LC-MS) is an example of a widely-used spectroscopic screening tool. High sensitivity and mass accuracy in addition to the wealth of characteristic information provided, such as chromatographic retention time, molecular mass and molecular formula, make it a particularly powerful screening tool.⁹⁹ Further advantages are offered by the use of tandem MS which can provide structural information in the form of fragment ions generated from the parent molecular ion. The main downfall in the technique is in the difficulty of optimising ionisation conditions to suit all compounds present within the matrix.¹⁰⁰

Nuclear magnetic resonance (NMR) spectroscopy is an alternative spectroscopic screening tool. NMR has the capability of providing elaborate structural information with regards to chemical motifs and substructures of molecules within the often complex mixture of a partially purified extract. Gaining this information early makes it a useful dereplication tool when used in conjunction with structural library databases, offering a greater chance for the isolation of novel compounds. Knowledge of chemical functionalities, such as acidic or basic moieties, is particularly valuable for guiding decisions in successive chromatographic techniques, the purification of

which may benefit from the use of ion-exchange media or pH buffers. However, when compared with MS- and bioassay-guided screening, the technique is comparatively insensitive. Although, sensitivity has significantly improved with technological advancements in NMR field strength, micro-coils and cryogenic systems, coupled with the development of capillary and micro-probes. These developments have facilitated the structure elucidation of sub-milligram quantities of natural product compounds, including that of hemi-phorboxazole A (**55**), the structure of which was elucidated from a mere total mass of 16.5 μg .^{101–104}

2.2.3 NMR-guided Screening, VUW

NMR screening of partially purified extracts is the method employed in our research group. The extracts of a selected organism are cyclic loaded onto PSDVB (Section 2.1.1) and the column is sequentially eluted using solutions of 1) 30% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ 2) 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ and then 3) Me_2CO . The Me_2CO fraction can be immediately concentrated under reduced pressure but the 30% and 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ fractions require back-loading onto PSDVB for the reason outlined in Section 2.1.1. These three fractions consecutively represent the polar, intermediate, and non-polar regions of the diagram provided in Figure 2.1 with the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ fraction thereby constituting the ‘mass-window’ and therefore, theoretically, contains most of the compounds of interest. Once concentrated, the fractions can be analysed using NMR spectroscopy.

The NMR screening methods of our research laboratory have developed over

time. Naturally, the first screening method began with the simple 1D ^1H NMR experiment.⁹⁰ The success of this screening tool was observed through the isolation of several novel compounds.^{90,105–107} However, signals attributed to primary metabolites obscured those belonging to potentially interesting secondary metabolites, especially in the region δ_{H} 0.5–5.5, and structural information was limited to olefinics and aromatics.^{89,90} Further developments of the NMR screen led to the generation of a paper mask compiled from the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ COSY and HSQC screens⁸⁹ and later a digital HSQC mask.^{13,91,108}

The paper mask was generated from the collation of several COSY or HSQC screen spectra with correlations occurring regularly at any given position being traced onto paper. A screen spectrum was subsequently overlaid on the appropriate mask to identify unique correlations within the screen, and was assigned a rating depending on the position, number and signal intensity of unique correlations present, and the availability of a given organism.⁸⁹ Though the COSY is significantly more sensitive, preference was given to the HSQC mask due to the additional information obtained from the carbon dimension enabling the ability to determine distinct chemical functionalities. The paper mask was further developed into a computer-based adaptation—the digital HSQC mask. A selected HSQC screen applied to the digital mask (compilation and superimposition of the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ HSQC screen spectra) was evaluated by comparing common (correlations appearing frequently at a given position) and uncommon correlations (those appearing less frequently at a given position) between the mask and the screen. As a result, common correlations appear strong and easily discernible to those that are uncommon, which are weak by comparison.^{13,91,108}

While the HSQC screen successfully served its purpose, leading to the isolation of a multitude of novel compounds, the capacity to only detect protonated carbon centres presented a limiting factor and indicated room for improvement. A greater level of information is provided by the HMBC experiment and accordingly, developments towards an HMBC screen were instigated.⁹³ Information obtained about non-

protonated functional groups and chemical motifs and the ability to elucidate substructures are huge advantages offered by the use of this experiment as a screening tool. Families of compounds can be identified at the earliest stage of the purification process providing a powerful tool to aid in the decision of whether of an organism is worthy of further investigation.⁹³ While the HMBC is theoretically less sensitive than the HSQC experiment, its simple pulse sequence and the absence of carbon decoupling make it more robust and almost as sensitive in practise. A generic HMBC spectrum generated from a 75% Me₂CO/H₂O screen fraction is provided in Figure 2.3.

Figure 2.3. A generic HMBC spectrum (600 MHz, CD₃OD) of a 75% Me₂CO/H₂O screen fraction showing a multitude of correlations throughout the spectrum.

Research conducted within our laboratory has mainly focused on sponges and algae, owing to the interesting, often novel secondary metabolites that originate from these organisms. In this study, the screening approach (see Appendix A for the screening and NMR protocol) initially involved both of these taxonomic groups but quickly progressed towards and remained focused on red algae of either Tongan or

New Zealand origin. Using an NMR-based spectroscopic screening methodology, six marine organisms were screened, of which two red algae were investigated extensively.

2.3 Marine Sponges

Sponges produce a diverse array of interesting, biologically active compounds. Owing to their soft-bodies and sessile nature, these organisms tend to produce toxic secondary metabolites as chemical defences to facilitate their survival by deterring predators, preventing competitor encroachment, parasitic infection or overgrowth of fouling organisms.¹⁰⁹ For example, the strong odorous exudate of *Latrunculia magnifica* (now re-classified as *Negombata magnifica*)¹¹⁰ causes the immediate fleeing of fish when released into the sea and has been found to induce rapid death in aquarium fish.¹¹¹ The major metabolite of the sponge is the cytotoxin latrunculin A (**56**).¹¹²

Between 20–30% of all marine natural products reported in recent years have been from sponges alone.^{3–7} Many of these exhibit cytotoxic activity in tumour-based cell line assays (*e.g.* monanchomycalin A and B, **57** and **58**),¹¹³ but sponge-derived metabolites have also been found to display other biological activities including antifungal (*e.g.* ceratinadin A and B, **59** and **60**),¹¹⁴ antimalarial (*e.g.* tsitsikammamine C, **61**),¹¹⁵ antiinflammatory (*e.g.* tedanol, **62**)¹¹⁶ and antiviral (*e.g.* polybrominated diphenyl ethers **63** and **64**)¹¹⁷ activity. With such a broad range of biological activities and a large diversity of chemically intriguing molecules, it is not difficult to see why sponges are popular organisms to study in marine natural products laboratories.

In this research, two Tongan sponge specimens were selected for screening. A brief investigation of each ensued, the results of which are detailed below.

2.3.1 PTN4_08B

The unidentified sponge, PTN4_08B, was collected from Vava'u, Tonga, in 2009 (Figure 2.4). The sponge was soft, springy and irregularly shaped with a smooth pale-brown surface. The ^1H NMR spectrum of the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction (Figure 2.5) consisted of an almost pure compound. Further analysis of the 2D data revealed the presence of an atypical steroid as indicated by the three deshielded oxymethines: a triplet of doublets resonating at δ_{H} 4.16, δ_{C} 78.7 and two broad singlets at δ_{H} 4.74, δ_{C} 75.5 and δ_{H} 4.80, δ_{C} 75.5. In contrast, the ^1H NMR spectrum of the 100% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction (Figure 2.6) displayed at least two steroidal compounds, each containing the typical A-ring CH-3 oxymethine proton resonance

(*dddd*, δ_{H} 3.52, δ_{C} 71.8 and *dddd*, δ_{H} 3.59, δ_{C} 71.3). While the regular steroid failed to attract further attention, the unusual steroid confined to the 75% Me₂CO/H₂O fraction was intriguing and warranted additional purification and analyses.

Figure 2.4. Photo of PTN4.08B.
Image courtesy of Rob Keyzers, 2009.

The 75% Me₂CO/H₂O fraction was partitioned on a second PSDVB column, then a DIOL column and then reversed-phase HPLC (C₁₈). This final purification step resulted in the isolation of a 1:1 mixture of the trisulfated steroids, halistanol sulfate (**65**) and topsentinol K trisulfate (**66**). Compound **65** was originally isolated from a Japanese collection of *Halicondria* cf. *moorei* as the active component in an undescribed antimicrobial assay.¹¹⁸ An Indonesian *Topsentia* sp. sponge was reported as the source of **66**, which was subsequently discovered to inhibit the aspartic protease BACE-1.¹¹⁹ A multitude of compounds have been reported within this class of steroids and as such further analyses were discontinued.

Figure 2.5. ^1H NMR spectrum of the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction from PTN4_08B (600 MHz, CD_3OD).

Figure 2.6. ^1H NMR spectrum of the 100% Me_2CO screen fraction from PTN4_08B (600 MHz, CDCl_3).

2.3.2 PTN3_19C

PTN3_19C is a sponge that is easily recognised because of its striking blood-red appearance (Figure 2.7). Collected from ‘Eua, Tonga, during a 2008 expedition, the sponge was originally screened by Singh⁹³ and was tentatively identified as *Plakortis quasiampfiaster* based on the morphological and chemotaxonomic similarity. The 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction displayed a simple ^1H NMR spectrum with one or

two major compounds, and fractionation of the sample by Singh led to the isolation and identification of two known pyrroloacridines, plakinidine A (**67**) and plakinidine B (**68**).⁹³ The isolation of these compounds concluded Singh's investigation of the sponge and the 30% Me₂CO/H₂O and 100% Me₂CO fractions remained unexplored, providing an opportunity for a re-examination of the sponge.

Figure 2.7. Underwater photo of PTN3_19C.
Image courtesy of Dan Crossett, 2016.

67 R = H

68 R = Me

During this study, PTN3_19C was extracted with MeOH and the extract subjected to the preliminary purification procedures outlined in Section 2.1.1. This resulted in three strongly pigmented screen fractions: 1) 30% Me₂CO/H₂O (intense red) 2) 75% Me₂CO/H₂O (intense red) and 3) 100% Me₂CO (vibrant purple). The ¹H NMR spectra of both the 30% (Figure 2.8) and 75% Me₂CO/H₂O screen fractions (Figure 2.9) showed each to be almost pure with clear resonances attributable to plakinidines. Conversely, the 100% Me₂CO fraction (Figure 2.10) lacked any plakinidine-related signals but consisted mainly of two compounds. Further purification of the 100% Me₂CO fraction on silica gel afforded the major compounds, which were determined to be the new α -exomethylene- γ -methyl- γ -tetradecyl- γ -

butyrolactone (**69**) (2.7 mg) and α -exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**) (13.4 mg) (Scheme 2.1).

69 R = H

70 R = OH

Figure 2.8. ^1H NMR spectrum of the 30% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction from PTN3_19C (600 MHz, CDCl_3).

Figure 2.9. ^1H NMR spectrum of the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction from PTN3_19C (600 MHz, CD_3OD).

Figure 2.10. ^1H NMR spectrum of the 100% Me_2CO screen fraction from PTN3_19C (600 MHz, CDCl_3).

Scheme 2.1. Isolation scheme of γ -butyrolactone compounds **69** and **70** from PTN3_19C.

α -Exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**)

A protonated molecule was detected using HRESIMS at m/z 309.2778, consistent with the molecular formula $\text{C}_{20}\text{H}_{36}\text{O}_2$, which required three double bond equivalents. All protons and carbons were observed in their respective ^1H and ^{13}C NMR spectra.

The protonated centres were attributed to an exomethylene (methylidene), five distinct methylenes, a saturated alkyl chain and two methyls on the basis of the 1D and multiplicity-edited HSQC NMR spectra. Three non-protonated carbons were identified as an ester carbonyl (δ_C 170.2), olefinic carbon (δ_C 136.1) and a quaternary oxygenated carbon (δ_C 84.1).

Beginning with the methylidene protons of CH₂-19 (δ_H 6.22, 5.60; δ_C 122.0), HMBC correlations were observed to C-1 (δ_C 170.2), C-2 (δ_C 136.1) and C-3 (δ_H 2.79, 2.67; δ_C 39.6) establishing the attachment point as C-2. Allylic (long-range) COSY correlations between the protons of H₂-19 and H₂-3, and HMBC correlations from H₂-3 to C-1, C-2 and C-19 confirmed this connectivity. This segment was further extended to include the oxygenated quaternary carbon C-4 (δ_C 84.1), the methylenes CH₂-5 (δ_H 1.64; δ_C 41.5) and CH₂-6 (δ_H 1.34; δ_C 23.9), and the methyl CH₃-20 (δ_H 1.38; δ_C 26.7) through the following correlations: in the HMBC experiment, H₂-3 correlated to C-4, C-5 and C-20, H₃-20 correlated to C-3, C-4 and C-5 and in the COSY experiment, a correlation was observed between H₂-5 and H₂-6. The C-1 carbonyl and $\Delta^{2,19}$ accounted for two double bond equivalents and with no other candidates remaining to account for the third, a ring was implied leading to the establishment of a γ -lactone (Figure 2.11). This was supported by three observations: 1) the C-1 δ_C chemical shift was indicative of an ester carbonyl 2) the C-4 δ_C chemical shift suggested oxygenation at this centre and 3) the molecular formula required two oxygens. Compounds bearing this α -exomethylene- γ -lactone moiety are not without precedence in the literature.^{120,121}

The α -exomethylene- γ -lactone motif of **69** (Figure 2.11) satisfied all double bond equivalents as required by the molecular formula, indicating the remainder of the structure to be acyclic and fully saturated, which was supported by the chemical shifts of the unassigned carbons. The presence of a lone methyl triplet (CH₃-18: δ_H 0.88; δ_C 14.3), a pair of methylenes (CH₂-17: δ_H 1.25–1.35; δ_C 22.9 and CH₂-16: δ_H 1.25–1.35; δ_C 32.1) and a cluster of methylenes (δ_H 1.25–1.35; δ_C 29.5–29.9) accounting for 18 protons and comprising the remaining nine carbon resonances,

Figure 2.11. COSY and HMBC correlations establishing the cyclic substructure of the α -exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**).

implied the rest of the structure to be a saturated C_{12} linear alkyl chain extending from the methylene CH_2 -6. A selective 1D TOCSY experiment (200 ms spin-lock mixing time) irradiating the H_3 -18 proton resonance displayed coherence transfer through the alkyl chain to H_2 -5 (Figure 2.12), validating the methyl connection to the structure and providing the planar structure of **69**. NMR data is presented in Table 2.1.

Figure 2.12. 1D TOCSY coherence transfer from H_3 -18 to H_2 -5 in α -exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**).

α -Exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**)

For compound **70**, a protonated molecule was detected at m/z 325.2729 using HRESIMS. This was found to be consistent with the molecular formula $C_{20}H_{36}O_3$, which required three double bond equivalents and differed only by one additional oxygen with respect to **69**. All 20 carbons were observed in the ^{13}C NMR spectrum and the 1D and 2D NMR spectra revealed the compound to be closely related to **69**. The multiplicity-edited HSQC experiment revealed an exomethylene, four distinct

Table 2.1. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for α -Exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**) (CDCl_3).

69

Pos.	^{13}C			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}^\dagger$ (Hz)	δ (ppm)	mult	J (Hz)		
1	170.2	C						
2	136.1	C						
3a	39.6	CH ₂	135	2.79	dt	16.9, 2.6	3b, 19a, 19b	1, 2, 4, 5, 19, 20
3b			134	2.67	dt	16.9, 2.6	3a, 19a, 19b	1, 2, 4, 5, 19, 20
4	84.1	C						
5	41.5	CH ₂	123	1.64	dd	10.0, 7.3	6	3, 4, 6, 7, 20
6	23.9	CH ₂		1.34	m		5	4, 5, 7
7–15	29.5–29.9	CH ₂		1.25–1.35	m			
16	32.1	CH ₂		1.25–1.35	m			
17	22.9	CH ₂		1.25–1.35	m			
18	14.3	CH ₃	127	0.88	t	7.0	17	16, 17
19a	122.0	CH ₂	163	6.22	t	2.8	3a, 3b, 19b	1, 2, 3
19b			161	5.60	t	2.5	3a, 3b, 19a	1, 3
20	26.7	CH ₃	122	1.38	s			3, 4, 5

† Measured from the unsuppressed $^1J_{\text{CH}}$ in the HMBC experiment

methylenes, a saturated alkyl chain, an oxymethine and two methyls. Similarly to **69**, three non-protonated carbons were identified as an ester carbonyl (δ_{C} 168.2), olefinic carbon (δ_{C} 139.6) and a quaternary oxygenated carbon (δ_{C} 87.7).

As with **69**, analysis of the 1D and 2D NMR spectra of **70** began at the exomethylene. The methylenes of CH₂-19 (δ_{H} 6.31, 5.91; δ_{C} 125.2) correlated to C-1 (δ_{C} 168.2), C-2 (δ_{C} 139.6), and to the oxymethine CH-3 (δ_{H} 4.56; δ_{C} 74.5) in the HMBC spectrum and to H-3 in the COSY spectrum. HMBC correlations from H-3 to C-1, C-2 and C-19 validated the connectivity, with additional correlations to the quaternary oxygenated carbon C-4 (δ_{C} 87.7), the methylene CH₂-5 (δ_{H} 1.67; δ_{C} 39.7) and to the methyl CH₃-20 (δ_{H} 1.32; δ_{C} 19.2) extending the substructure. The methyl protons H₃-20 displayed HMBC correlations to C-3, C-4 and C-5, supporting the established C-3–C-5 connectivity (Figure 2.13).

TOCSY experiments (200 ms spin-lock mixing time) showed the remainder of the structure to be analogous to **69** with irradiation of the methyl H₃-18 (δ_{H} 0.87; δ_{C} 14.1) exhibiting transfer to H₂-5 and vice versa for the irradiation of H₂-6, thus establishing

the planar structure of **70**. NMR data is provided in Table 2.2.

Figure 2.13. COSY and HMBC correlations establishing the cyclic substructure in α -exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**).

Table 2.2. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for α -Exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**) (CDCl_3).

Pos.	^{13}C			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}^\dagger$ (Hz)	δ (ppm)	mult	J (Hz)		
1	168.2	C						
2	139.6	C						
3	74.5	CH	147	4.56	t	2.3	19a,19b	1,2,4,5,19,20
4	87.7	C						
5	39.7	CH_2	128	1.67	dd	9.3,7.4	6	3,4,6,7,20
6	23.9	CH_2		1.39	m		5	4,5,7
7–15	29.5–29.9	CH_2		1.25–1.35	m			
16	31.9	CH_2		1.25–1.35	m			
17	22.7	CH_2		1.25–1.35	m			
18	14.1	CH_3	126	0.87	t	7.0	17	16,17
19a	125.2	CH_2	164	6.31	d	2.5	3,19b	1,2,3
19b			162	5.91	d	2.2	3,19a	1,3
20	19.2	CH_3	127	1.32	s			3,4,5

† Measured from the unsuppressed $^1J_{\text{CH}}$ in the HMBC experiment

Compounds **69** and **70** were submitted to the School of Biological Sciences for assessment of cytotoxicity in the HL-60 (human promyelocytic leukaemia) cell line bioassay. Each exhibited moderate activity with IC_{50} values of 1.13 and 2.70 μM for **69** and **70**, respectively.

Natural products containing the β -hydroxyl- γ -lactone moiety are not without precedence in our laboratory. A compound labelled AJS12.36A (**71**) featuring a triunsaturated alkyl chain was isolated by Singh from an undescribed sponge also collected from 'Eua, Tonga.⁹³ Other structurally related compounds to **69** and **70** in the chemical literature were isolated from sponges of the genus *Plakortis*. An investigation of a Canary Islands *Plakortis* sp. sponge resulted in the isolation of plakolide A (**72**), which demonstrated moderate to weak cytotoxicity, ranging from 1.1–5.0 $\mu\text{g/mL}$ in several mammalian cell line bioassays.¹²⁰ *Plakortis quasiampfiaster*, collected from Vanuatu, was the source of 17 closely related compounds named the amphiasterins (**73–89**). Compounds **73–89** are γ -lactones that vary in the functionality at the α -position and in the length and degree of unsaturation of the alkyl side chain.¹²¹

At this time, the configurational assignments of **69** and **70** remain undetermined due to the degradation of both compounds. Although, procurement of either compound was completed with ease, the focus of this research had switched towards red algal specimens and as such, further analyses on PTN3.19C were discontinued.

71

72

- 73** $m = 31$ $n = 9$
74 $m = 35$ $n = 9$
75 $m = 31$ $n = 9$, Δ saturated
76 $m = 35$ $n = 9$, Δ saturated

- 77** $m = 10$
78 $m = 12$
79 $m = 16$
80 $m = 10$, Δ saturated
81 $m = 12$, Δ saturated

2.4 Marine Red Algae

A variety of compounds have been isolated from red algae, with terpenoids such as the diterpenes **90–92**,¹²² and bromophenols such as **93–95**¹²³ amongst the most prevalent.^{3–5} Additionally, red algal metabolites typically contain a high level of halogen substitution and a large number of novel terpenoid carbon skeletons.¹²⁴ These structural characteristics make red algae a particularly attractive source of interesting, potentially bioactive secondary metabolites.

A total of four red algal specimens were selected for screening using our spectroscopic-screening protocol. Where possible, algae were identified by Dr Joe Zuccarello at the School of Biological Sciences, VUW. Only two were chosen for further study.

2.4.1 PTN4_34B

PTN4_34B (Figure 2.14) is an unidentified red alga collected from ‘Eua, Tonga in June 2016. The 75% Me₂CO/H₂O screen fraction displayed resonances in the 1D and 2D NMR spectra attributed to a sugar moiety with the anomeric proton doublet clearly visible at δ_{H} 4.76 in the ¹H NMR spectrum (Figure 2.15). Aromatic singlet resonances above δ_{H} 8.50 (Figure 2.16) attached to highly shielded carbons resonating between δ_{C} 92–108 were observed in the 1D and 2D NMR spectra of the 100% Me₂CO fraction, which revealed the presence of a class of molecules containing a porphyrin core. Other interesting olefinic and aromatic resonances were observed between δ_{H} 5.50–8.50, however, further investigation of both screens was discontinued in favour of other red algal specimens.

Figure 2.14. Underwater photo of PTN4_34B.
Image courtesy of Dan Crossett, 2016.

Figure 2.15. ^1H NMR spectrum of the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction from PTN4_34B (600 MHz, CD_3OD).

Figure 2.16. ^1H NMR spectrum of the 100% Me_2CO screen fraction from PTN4_34B (600 MHz, CDCl_3).

2.4.2 PTN4_35B

The unidentified red alga, PTN4_35B (Figure 2.17) was collected from ‘Eua, Tonga, in June 2016 and was selected for screening. Despite obvious morphological differences between PTN4_34B and PTN4_35B, the NMR profile observed for the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction of PTN4_35B (Figure 2.18) was very similar to

that of PTN4_35B (Figure 2.15). Notably, resonances pertaining to a similar sugar moiety to that found in PTN4_34B were observed. As such, this alga was discounted from further investigation.

Figure 2.17. Photo of PTN4_35B.
Image courtesy of Dan Crossett, 2016.

Figure 2.18. ^1H NMR spectrum of the 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ screen fraction from PTN4_35B (600 MHz, CD_3OD).

2.4.3 PTN4_20A

The red alga PTN4_20A (Figure 3.1) was collected in January 2012 and 2014 from Wellington, New Zealand, and was identified as *Rhodophyllis membranacea*.

An extensive natural product investigation ensued following mass spectrometric evidence for the presence of a multitude of polyhalogenated indoles. Further details of this investigation are described in Chapter 3.

2.4.4 PTN4_34F

PTN4_34F (Figure 4.1) was collected in June 2016 from ‘Eua, Tonga, and was identified as *Callophycus serratus*. Signals in the aromatic region, in addition to a deshielded *gem*-dimethyl, were identified in the screen 1D and 2D NMR spectra. This result prompted a more detailed study of this alga, the results of which are outlined in Chapter 4.

Chapter 3

Polyhalogenated Indoles from the New Zealand Red Alga *Rhodophyllis membranacea*

*The research described in this chapter includes and expands on work that was published in the Journal of Natural Products.*¹²⁵

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3.1 Secondary Metabolites of New Zealand Red Algae

New Zealand red algal metabolites have predominantly been reported by the Blunt and Munro group at the University of Canterbury. Over the period between 1978 and 1988, Blunt and Munro contributed a total of 24 new red algae compounds to the marine natural products literature.^{126–133} Algae of the genera *Laurencia*, *Plocamium* and *Delisea* were among those studied and examples of halogenated compounds include the brominated polyether thyriferol (**96**),¹²⁸ the isomeric acetylenic cyclic ethers **97** and **98**,¹³² the sesquiterpene isolaurenisol (**99**)¹³⁰ and a series of 2(5*H*)-furanones (**100–104**)¹³³ were isolated. Thyriferol (**96**) was found to be inactive against several strains of bacteria,¹²⁸ but was subsequently shown to possess potent cytotoxicity against the P388 cell line (IC₅₀ 16 nM).¹³⁴ Compounds **97–104** were not assessed in any biological assay.^{128,130,132}

In addition to the research of the Blunt and Munro group, three other independent

groups have worked on New Zealand red algal specimens and reported halogenated compounds. König and Wright isolated several compounds from a *Laurencia* sp., including four new C₁₅ acetogenins (**105–108**) and a structural isomer of isolaurenisol (**109**).¹³⁵ Compounds **105–109** were not assessed for biological activity, but the subsequent re-isolation of **109** by a separate research group¹³⁶ showed that it lacked cytotoxicity (IC₅₀ > 88.6 μ M) against several mammalian cell lines. Popplewell and Northcote reported the isolation of the nitrogenous bromophenol, colensolide A (**110**), from Northland specimens of *Osmundaria colensoi*.¹³⁷ While colensolide A (**110**) lacked activity against the HL-60 cell line and the MC²155 strain of *Mycobacterium smegmatis*, several other known compounds isolated alongside **110** demonstrated moderate cytotoxicity and antibacterial activity, respectively. The third research group reported polyhalogenated indoles isolated from *Rhodophyllis membranacea*.¹³⁸ Their work is discussed in Section 3.2.

105 $R_1 = \text{OAc}$ $R_2 = \text{Cl}$ $R_3 = \text{OAc}$

106 $R_1 = \text{OH}$ $R_2 = \text{OAc}$ $R_3 = \text{OAc}$

107 $R_1 = \text{OAc}$ $R_2 = \text{OH}$ $R_3 = \text{OAc}$

108

109

110

3.2 *Rhodophyllis membranacea*

The red alga *R. membranacea* (Figure 3.1) is one of 49 accepted species within the genus *Rhodophyllis* and it is distributed throughout Australia and New Zealand.¹¹ A specimen collected off the Kaikoura Coast of New Zealand was investigated by Brennan and Erickson in 1978 following a positive hit of the crude extract in an antifungal bioassay.¹³⁸ Their report is amongst the earliest record of natural products derived from New Zealand marine organisms with Blunt and Munro acknowledged for facilitating algal collection and performing the biological assays. Several polyhalogenated indoles were reported, including 2,3,4- and 2,3,7-trihalogenated indoles (**111–115**) and a number of 2,3,4,7-tetrahalogenated indoles (**116–122**).¹³⁸ While the structures of **112–115** were verified *via* synthesis,¹³⁹ the others were not and the ¹H NMR data of many of the tetrahalogenated indoles could not be unambiguously assigned.¹³⁸ As such, there was obvious scope for a re-examination of *R. membranacea*.

R. membranacea has been found by our research group to grow abundantly in the subtidal region along the shoreline of Moa point, Wellington, New Zealand—while searching for a morphologically similar red alga for the undergraduate

Figure 3.1. Photo of *Rhodophyllis membranacea*.

111	$R_1 = \text{Cl}$	$R_2 = \text{Cl}$	$R_3 = \text{Cl}$	$R_4 = \text{H}$
112	$R_1 = \text{Cl}$	$R_2 = \text{Cl}$	$R_3 = \text{H}$	$R_4 = \text{Cl}$
113	$R_1 = \text{Cl}$	$R_2 = \text{Cl}$	$R_3 = \text{H}$	$R_4 = \text{Br}$
114	$R_1 = \text{Br}$	$R_2 = \text{Cl}$	$R_3 = \text{H}$	$R_4 = \text{Br}$
115	$R_1 = \text{Br}$	$R_2 = \text{Br}$	$R_3 = \text{H}$	$R_4 = \text{Br}$
116	$R_1 = \text{Br}$	$R_2 = \text{Br}$	$R_3 = \text{Br}$	$R_4 = \text{Br}$
117	$R_1 = \text{Cl}$	$R_2 = \text{Br}$	$R_3 = \text{Br}$	$R_4 = \text{Br}$
118	$R_1 = \text{Br}$	$R_2 = \text{Cl}$	$R_3 = \text{Br}$	$R_4 = \text{Br}$
119	$R_1 = \text{Cl}$	$R_2 = \text{Cl}$	$R_3 = \text{Br}$	$R_4 = \text{Br}$
120	$R_1 = \text{Cl}$	$R_2 = \text{Cl}$	$R_3 = \text{Br}$	$R_4 = \text{Cl}$
121	$R_1 = \text{Cl}$	$R_2 = \text{Cl}$	$R_3 = \text{Cl}$	$R_4 = \text{Br}$
122	$R_1 = \text{Cl}$	$R_2 = \text{Cl}$	$R_3 = \text{Cl}$	$R_4 = \text{Cl}$

teaching laboratory, *R. membranacea* was inadvertently and repeatedly collected over several years. Initially, the alga was disregarded as a prospective organism for research purposes and instead was screened by Cori Jones (a visiting student from the University of Kansas) for its potential applicability in the teaching laboratory. During that enquiry, however, the alga became interesting from a research perspective following mass spectrometric evidence for the presence of more than 20 tri-, tetra-, penta- and hexahalogenated indoles. Consequently, *R. membranacea* became the immediate focus of this study in the search to isolate and

characterise any unusual polyhalogenated species.

A spectroscopic-guided isolation methodology led to the isolation of 21 compounds. Three are known tetrahalogenated indoles (**116**, **119** and **121**) previously reported by Brennan and Erickson¹³⁸ but herein presented with full assignment of ¹H and ¹³C NMR data, and 11 are new tetrahalogenated indoles (**123–133**). In addition, four new pentahalogenated (**134–137**), one hexahalogenated indole identified only by HRESIMS data as a tribromo-trichloroindole, and the synthetically known, albeit new marine natural products, 4-chloroisatin (**138**) and the aldol product of **138**, 4-chloro-3-hydroxyl-3-(2-oxopropyl)-2-oxindole (**139**) (see Section 3.8), were isolated.

3.3 Purification and Isolation of Polyhalogenated Indoles

Specimens of *R. membranacea* were collected from Moa Point, Wellington, New Zealand, in January 2012 and 2014. The alga was extracted with MeOH and the extracts were cyclic loaded onto PSDVB (as described in Chapter 2.1.1) then eluted with 30% Me₂CO/H₂O, 75% Me₂CO/H₂O and Me₂CO. The 30% Me₂CO/H₂O fraction appeared to contain one major component and purification of the sample by flash column chromatography on Diol yielded 4-chloro-3-hydroxyl-3-(2-oxopropyl)-2-oxindole (**139**) (0.70 mg). The Me₂CO fraction was further purified on silica, followed by size-exclusion chromatography using Sephadex LH20, then C₁₈ HPLC to yield 4,7-dibromo-2,3-dichloroindole (**119**) (1.3 mg) and 7-bromo-2,3-dichloro-6-iodoindole (**129**) (0.70 mg) (Scheme 3.1).

Additional extractions of the alga were treated in a similar fashion. Fractions eluting from the Sephadex LH20 column, that contained the compounds of interest (determined using LC-MS), were dissolved in 75% MeOH/MeCN, centrifuged,[†] and the supernatant was separated and combined as one sample. A portion (280 mg) was partitioned on C₁₈ HPLC to provide six fractions (Scheme 3.1)—under these HPLC conditions (85% MeOH/H₂O), the elution profile displayed distinct separation of the halogenated indoles according to the number of halogens present on the indolic core (Figure 3.2). These were identified using HRESIMS as tri-, tetra-, penta-, and hexahalogenated indoles.

3.3.1 Purification of Tetrahalogenated Indoles

Further purification of the HPLC fraction containing the tetrahalogenated indoles was carried out on C₁₈ HPLC. This provided more of **119** (11.7 mg) and **129** (5.6 mg), in addition to 5-bromo-2,3,4-trichloroindole (**133**) (1.3 mg) and 14 other

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[†]Attempts to re-dissolve these fractions using a variety of solvent mixtures persistently left some undissolved material.

Scheme 3.1. Isolation scheme of compounds **119**, **129**, **138**, **139** and fractions containing tri-, tetra-, penta- and hexahalogenated indoles from *Rhodophyllis membranacea*.

Figure 3.2. The reversed-phase C_{18} HPLC elution profile (diode array trace) of a fraction containing a mixture of polyhalogenated indoles. UV (y-axis) was detected between 210 and 400 nm and the overall chromatographic runtime (x-axis) was 25 min. The intensity of UV absorption (qualitative) is shown based on colour. Elution with 85% MeOH/ H_2O led to a distinct separation between polyhalogenated indole types as displayed.

fractions. C_8 HPLC was used to purify one of these fractions, which yielded 7-bromo-2,3,4-trichloroindole (**121**) as the major compound (1.5 mg). Other fractions were purified either directly from Diol, or Diol then C_{18} HPLC to provide 2,3,4,7-tetrabromoindole (**116**) (1.0 mg), 2,3,4-trichloro-7-iodoindole (**123**) (1.3 mg), 2-bromo-3,4-dichloro-7-iodoindole (**124**) (0.13 mg), 2,3,6,7-tetrabromoindole (**125**) (1.2 mg), 3,6,7-tribromo-2-chloroindole (**126**) (1.3 mg), 2,6,7-tribromo-3-chloroindole (**127**) (3.7 mg), 6,7-dibromo-2,3-dichloroindole (**128**) (7.6 mg), 2,7-dibromo-3-chloro-6-iodoindole (**130**) (0.70 mg), 3,7-dibromo-2-chloro-6-iodoindole (**131**) (0.08 mg) and 2,3,7-trichloro-6-iodoindole (**132**) (0.06 mg) (Scheme 3.2).

3.3.2 Purification of Pentahalogenated Indoles

Excellent separation of the fraction containing the pentahalogenated indoles was achieved using C_{18} HPLC eluting with 55% MeCN/ H_2O (Figure 3.3). The first fraction was collected at $t_R = 52$ min and the final fraction of interest was collected at $t_R = 84$ min. A series of later eluting peaks (present in low levels) were collected together. However, each chromatographic runtime was 138 min with the fractions of interest concentrated within a relatively short 32 min window in the middle of the analysis. To shorten the overall HPLC runtime, the pentahalogenated indoles were separated from the late-eluting compounds using C_{18} HPLC eluting from 85-100% MeOH/ H_2O , then the fraction containing the pentahalogenated indoles

Scheme 3.2. Isolation scheme of tetrahalogenated indoles from *Rhodophyllis membranacea*.

was further purified using C₁₈ HPLC (55% MeCN/H₂O) as a single continuous chromatographic run with sample injected at 45 min intervals (Figure 3.4). Further purification using the same column and conditions led to the purification of 5,7-dibromo-2,3,4-trichloroindole (**137**) (1.2 mg). Other fractions were purified using DIOL and C₁₈ HPLC to yield pure 2,3,4,5,7-pentabromoindole (**134**) (1.6 mg), 2,3,4,6,7-pentabromoindole (**135**) (0.30 mg) and 4,5,7-tribromo-2,3-dichloroindole (**136**) (1.7 mg). The isolation scheme of the pentahalogenated indoles is presented in Scheme 3.3.

Figure 3.3. Reversed-phase HPLC elution profile of pentahalogenated indoles from 55% MeCN/H₂O.

Figure 3.4. Reversed-phase HPLC elution profile of pentahalogenated indoles from 55% MeCN/H₂O as a single continuous run with sample injected at 45 min intervals.

Scheme 3.3. Isolation scheme of pentahalogenated indoles from *Rhodophyllis membranacea*.

3.3.3 Purification of Hexahalogenated Indole

Purification of the fraction containing the hexahalogenated indoles was carried out in a similar manner to that used for the pentahalogenated indoles. The sample was first fractionated using C₁₈ HPLC to remove late-eluting non-hexahalogenated indoles, before a single continuous chromatographic run was performed using C₁₈ HPLC with sample being injected at *ca.* 35 min intervals. Five fractions were collected (Scheme 3.4) and based on the ¹H NMR spectrum (using the NH proton resonance as a guide) and the HRESIMS data, the third fraction appeared most pure.

3.3.4 Isolation of 4-Chloroisatin

A separate methanolic extract of *R. membranacea* was fractionated on a PSDVB column, eluting with mixtures of MeOH and H₂O. Further purification using Diol and C₁₈ HPLC led to the isolation of 4-chloroisatin (**138**) (1.3 mg) in the presence of its inseparable tautomer. The isolation of **138** is detailed in Scheme 3.1.

Scheme 3.4. Isolation scheme of hexahalogenated indoles from *Rhodophyllis membranacea*.

3.4 Structure Elucidation

Evidence for the presence of the indolic core (**140**) was found using UV spectrophotometry. The number of bromines and chlorines for each molecule was confirmed from the characteristic molecular ion cluster in the mass spectrum. The presence of iodine was evidenced by the significantly increased mass and match to the high mass accuracy data.

140

Given the low H:C,N,X ratio, the difficulty in elucidation lay in where the protons and the halogens were located around the aromatic core. The structure elucidation began with the tetrahalogenated indoles since these presented the least challenging structures of the polyhalogenated indoles isolated in this study. Once the tetrahalogenated indoles were unequivocally assigned, the structures of the pentahalogenated indoles were elucidated.

3.5 Tetrahalogenated Indoles

The combination of NMR spectroscopy and HRESIMS was instrumental in guiding the isolation and differentiation between the different tetrahalogenated indoles. In

many cases, the ^1H NMR spectrum of two closely related indoles differing only by the substitution of one halogen for another appeared almost indistinguishable (Figure 3.5). The aromatic protons of all isolated tetrahalogenated indoles appeared as doublets with *ca.* 8 Hz coupling constants, diagnostic of *ortho*-substitution on the benzenoid ring; *vicinally*-coupled protons on pyrrole rings exhibit a much smaller coupling constant (2–3 Hz).¹⁴⁰ In contrast, but equally diagnostic, the one-bond proton-carbon coupling ($^1J_{\text{CH}}$) constants observed for an indole standard were found to be significantly larger for those located around the pyrrole ring than the benzenoid ring (Tables 3.1 and 3.2), consistent with those reported for benzene¹⁴¹ and pyrrole.¹⁴² Since the magnitude of the $^1J_{\text{CH}}$ value increases with increasing *s*-character of the C-H bond, angular distortion and electronegative substituents,¹⁴⁰ this result is expected. The $^1J_{\text{CH}}$ values were measured for tetrahalogenated indoles **116**, **119**, **121** **123–133** and were not consistent with proton substitution on the pyrrole ring of the indole. Consequently, the *ortho*-protons were limited to three possible regioisomers providing either a 2,3,4,5-, 2,3,4,7-, or 2,3,6,7-tetrahalogenated indole.

Figure 3.5. Stacked ^1H NMR spectra (600 MHz, CDCl_3) of (a) **123** (blue) and **124** (black), (b) **125** (blue) and **126** (black); (c) **127** (blue) and **128** (black); and (d) **129** (blue) and **130** (black).

Table 3.1. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Indole (**140**) (CDCl_3).

140

Pos.	^{13}C or $^{15}\text{N}^\dagger$			^1H			COSY	HMBC (^1H to ^{13}C or $^{15}\text{N}^\dagger$)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-256	NH		8.09	br s			
2	124.3	CH	184	7.21	t	2.7	3	1,3,3a,7a
3	102.7	CH	175	6.60	ddd	3.0,2.1,0.9	2	1,2,3a(w),7a
3a	127.9	C						
4	120.8	CH	159	7.70	d	8.0	5	3,3a(w),6,7a
5	119.9	CH	159	7.17	td	7.5,1.0	4,6	3a,7
6	122.1	CH	159	7.24	td	7.6,1.0	5,7	4,7(w),7a
7	111.1	CH	159	7.41	d	8.1	6	1(w),3a,5
7a	135.8	C						

† Determined from a ^{15}N CIGAR experiment

(w)Weak

Table 3.2. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Indole (**140**) ($\text{DMSO}-d_6$).

Pos.	^{13}C or $^{15}\text{N}^\dagger$			^1H			COSY	HMBC (^1H to ^{13}C or $^{15}\text{N}^\dagger$)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-246	NH		11.06	br s			2(w),3,3a,7a(w)
2	125.2	CH	184	7.33	t	2.7	3	1,3,3a,7a
3	101.0	CH	174	6.41	ddd	2.9,2.0,0.9	2	1,2,3a,7a
3a	127.6	C						
4	120.0	CH	159	7.53	dd	7.8,0.7	5	3,6,7a
5	118.8	CH	159	6.98	td	7.5,1.0	4,6	3a,7
6	120.9	CH	159	7.07	td	7.5,1.1	5,7	4,7a
7	111.4	CH	159	7.39	dd	8.1,0.7	6	1,3a,5
7a	135.8	C						

† Determined from a ^{15}N CIGAR experiment

(w)Weak

In order to differentiate between the regioisomers and determine the proton positions, the conspicuous carbon chemical shifts of C-3, C-7 and C-7a (indole, **140**: δ_{C} 102.7, 111.1, 135.8 (CDCl_3), respectively, see Tables 3.1 and 3.2 for full NMR data) coupled with the incidence of three-bond correlations in the HMBC spectrum appearing stronger than two-bond correlations, were used as diagnostic tools. A close examination of how many strong three-bond correlations from the protons to these carbons was particularly valuable. To illustrate this point, strong three-bond correlations to C-3, C-7 and C-7a would be observed from protons situated on C-

4 and C-5 (Figure 3.6a), protons positioned on C-5 and C-6 would show strong correlations to C-7 and C-7a (Figure 3.6b), and protons on C-6 and C-7 would display a strong correlation to C-7a only (Figure 3.6c). Additionally, of the three possibilities, the 2,3,4,5 tetrahalogenated indole was expected to be the only one that would contain a proton connected to a carbon resonating near δ_C 111 (CH-7) similarly to that found for indole (Tables 3.1 and 3.2).

Figure 3.6. Three-bond HMBC correlations to C-3, C-7 and C-7a in (a) 2,3,6,7-, (b) 2,3,4,7- and (c) 2,3,4,5-tetrasubstituted indole.

The next challenge was to determine where the halogens were situated. While iodinated carbon resonances are very distinct from that of brominated and chlorinated carbons,[†] similarities often observed in the carbon chemical shifts of the latter two can complicate the assignment of these centres. NMR methods can be employed to facilitate with the differentiation between brominated and chlorinated carbons. Measurements of the ^{13}C spin-lattice relaxation (T_1) times and integration of the halogenated carbon centres in question have been used to distinguish between the two, which works on the basis of brominated carbons generally having shorter T_1 times than chlorinated carbons.¹⁴⁵ Other methods involve the acquisition of high-resolution NMR data to observe the $^{35}\text{Cl}/^{37}\text{Cl}$ isotope shift in a 1D ^{13}C NMR spectrum,¹⁴⁶ and in 2D band-selective HSQC^{52,147} and HSQMBC experiments.¹⁴⁸

In this study, a strategy was employed that involved the isolation and full NMR and mass spectrometric characterisation of a tetrahalogenated indole containing one halogen type. From there, the successive isolation of tetrahalogenated indoles constituting the same substitution pattern, but where one halogen was replaced with another, were targeted and systematically characterised accordingly. Two isomeric

[†]This is well known phenomenon described by a variety of explanations and is referred to as either the heavy halogen, heavy atom or spin-orbit coupling effect.^{143,144}

tetrabromindoles were isolated, one exhibiting a 2,3,4,7- substitution pattern (**116**) and the other a 2,3,6,7-tetrabromindole (**125**). With the exception of **133**, all of the tetrahalogenated indoles isolated displayed either of these two substitution patterns. Where possible, high-resolution ^{13}C NMR data was acquired by using a 6.0 s acquisition time so that the $^{35}\text{Cl}/^{37}\text{Cl}$ isotope shift could be observed to give further support towards the proposed assignment of the chlorinated carbons. While some of the tetrahalogenated indoles isolated in this study were successfully crystallised, their quality was insufficient for single-crystal X-ray diffraction.

3.5.1 2,3,6,7-Tetrabromindole

Negative-ion mode HRESIMS analysis of **125** provided evidence for the molecular formula $\text{C}_8\text{H}_3\text{Br}_4\text{N}$ (m/z 427.6928, $[\text{M} - \text{H}]^-$) (Figure 3.7), which required six degrees of unsaturation, fully accounted for by the indolic core. All protons and carbons were identified in the ^1H and ^{13}C NMR spectra. Two were assigned as aromatic methine doublets (CH 4: δ_{H} 7.31; δ_{C} 119.1 and CH 5: δ_{H} 7.42; δ_{C} 126.2) in a multiplicity-edited HSQC experiment, thus indicating one exchangeable proton (δ_{H} 8.45) and six non-protonated sp^2 hybridised carbons (δ_{C} 135.4, 127.2, 119.2, 111.5, 106.8, 95.6) were present.

Figure 3.7. Experimental and calculated mass spectra for 2,3,6,7-tetrabromindole (**125**).

The methine protons correlated to each other in a COSY spectrum, as was the case for all the tetrahalogenated indoles isolated in this study. Analysis of the HMBC spectrum indicated a 2,3,6,7-tetrahalogenated indole through three-bond

correlations to the three distinctive carbons C-3, C-7 and C-7a, as illustrated in Figure 3.6a. HMBC correlations were observed from H-4 to C-3 (δ_{C} 95.6), C-6 (δ_{C} 119.2) and C-7a (δ_{C} 135.4), and from H-5 to C-3a (δ_{C} 127.2), C-7 (δ_{C} 106.8) and weakly to C-6. With one carbon left to assign, this could therefore be attributed to C-2 (δ_{C} 111.5). The chemical shifts of C-3a and C-7a were comparatively similar to indole (**140**; Tables 3.1 and 3.2). In contrast, the chemical shifts of C-2, C-3, C-6, and C-7 were more shielded than the corresponding carbons of indole (**140**), and as required by the molecular formula must therefore contain bromine at these sites to provide the structure of **125** as 2,3,6,7-tetrabromoindole. NMR data is provided in Tables 3.3 and 3.4.

Table 3.3. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 2,3,6,7-Tetrabromoindole (**125**) (CDCl_3).

Pos.	^{13}C or $^{15}\text{N}^\dagger$			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-245	NH		8.45	br s			
2	111.5	C						
3	95.6	C						
3a	127.2	C						
4	119.1	CH	168	7.31	d	8.4	5	3,6,7a
5	126.2	CH	168	7.42	d	8.4	4	3a,6,7
6	119.2	C						
7	106.8	C						
7a	135.4	C						

† Determined from a ^{15}N CIGAR experiment

Table 3.4. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 2,3,6,7-Tetrabromoindole (**125**) ($\text{DMSO}-d_6$).

Pos.	^{13}C			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-	NH		12.74	br s			
2	-	C						
3	93.0 [†]	C						
3a	126.8	C						
4	118.5	CH	168	7.33	d	8.5	5	3,6,7a
5	125.2	CH	168	7.46	d	8.5	4	3a,4,7
6	118.0 [†]	C						
7	106.7	C						
7a	135.5 [†]	C						

-Not observed

[†]Determined from an HMBC experiment

3.5.2 3,6,7-Tribromo-2-chloroindole

The molecular formula of **126** was determined to be $\text{C}_8\text{H}_3\text{Br}_3\text{ClN}$ from the HRESIMS data (m/z 383.7438, $[\text{M} - \text{H}]^-$) (Figure 3.8). Inspection of the 1D and 2D NMR data revealed close similarities to **125** with the exception of the appearance of a carbon resonance at δ_{C} 124.0 in place of δ_{C} 111.5. HMBC correlations were observed from H-4 (δ_{H} 7.31) to C-3 (δ_{C} 91.5), C-6 (δ_{C} 119.2) and C-7a (δ_{C} 133.8), and from H-5 (δ_{H} 7.43) to C-3a (δ_{C} 126.8) C-6 and C-7 (δ_{C} 106.9). The similarity in chemical shifts of C-3, C-6 and C-7 to those of **125** clearly pointed to bromination at those positions. Consequently, C-2 (δ_{C} 124.0) was assigned as the chlorinated centre based on its higher chemical shift compared to that of **125**. Thus, the structure **126** was deduced as 3,6,7-tribromo-2-chloroindole. NMR data is provided in Tables 3.5 and 3.6.

Figure 3.8. Experimental and calculated mass spectra for 3,6,7-tribromo-2-chloroindole (**126**).

Table 3.5. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 3,6,7-Tribromo-2-chloroindole (**126**) (CDCl_3).

126

Pos.	^{13}C or $^{15}\text{N}^\dagger$			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-248	NH		8.43	br s			
2	124.0	C						
3	91.5	C						
3a	126.8	C						
4	119.2 ‡	CH	168	7.31	d	8.4	5	3,6,7a
5	126.3	CH	169	7.43	d	8.4	4	3a,6,7
6	119.2 ‡	C						
7	106.9	C						
7a	133.8	C						

† Determined from a ^{15}N CIGAR experiment

‡ Interchangeable

Table 3.6. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 3,6,7-Tribromo-2-chloroindole (**126**) ($\text{DMSO}-d_6$).

Pos.	^{13}C			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-	NH		12.82	br s			
2	-	C						
3	88.5 †	C						
3a	126.4	C						
4	118.3	CH	168	7.33	d	8.4	5	3,5,6,7a
5	124.9	CH	169	7.47	d	8.4	4	3a,4,7
6	117.8 †	C						
7	106.5 †	C						
7a	133.9 †	C						

-Not observed

† Determined from an HMBC experiment

3.5.3 2,6,7-Tribromo-3-chloroindole

Compound **127** provided an identical molecular formula to **126** ($\text{C}_8\text{H}_3\text{Br}_3\text{ClN}$; m/z 383.7435, $[\text{M} - \text{H}]^-$), but differences in the ^1H NMR spectrum suggested that it was a regioisomer. Through comparison of HMBC data, it was established that the structural alteration was an exchange of bromine and chlorine at C-2 (δ_{C} 108.8) and C-3 (δ_{C} 109.0) yielding the structure 2,6,7-tribromo-3-chloroindole (**127**). NMR data is presented in Tables 3.7 and 3.8.

Table 3.7. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 2,6,7-Tribromo-3-chloroindole (**127**) (CDCl_3).

Pos.	^{13}C or $^{15}\text{N}^\dagger$			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-248	NH		8.33	br s			
2	108.8 ‡	C						
3	109.0 ‡	C						
3a	125.5	C						
4	118.1	CH	168	7.37	d	8.4	5	3,6,7(w),7a
5	126.1	CH	168	7.41	d	8.4	4	3a,6,7
6	119.2	C						
7	107.1	C						
7a	134.7	C						

† Determined from a ^{15}N CIGAR experiment

‡ Interchangeable

(w)Weak

Table 3.8. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 2,6,7-Tribromo-3-chloroindole (**127**) ($\text{DMSO}-d_6$).

Pos.	^{13}C			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-	NH		12.67	br s			
2	111.5	C						
3	105.5	C						
3a	125.0	C						
4	117.5	CH	169	7.40	d	8.4	5	3,6,7a
5	125.1	CH	169	7.45	d	8.4	4	3a,6,7
6	118.0	C						
7	107.0	C						
7a	134.8	C						

-Not observed

3.5.4 6,7-Dibromo-2,3-dichloroindole

A molecular formula of $\text{C}_8\text{H}_3\text{Br}_2\text{Cl}_2\text{N}$ was indicated for **128** through the HRESIMS data (m/z 339.7936, $[\text{M} - \text{H}]^-$) (Figure 3.9). The analogous ^1H and HMBC NMR data to **127** inferred identical halogen substitution at positions C-3, C-6 and C-7. This was supported through the close comparison of the carbon chemical shifts of C-3 (δ_{C} 105.2) with that of compound **127** and of C-6 (δ_{C} 119.2) and C-7 (δ_{C} 107.1) with those of compounds **125–127**, establishing the substitution of C-3

with chlorine and both C-6 and C-7 with bromine. This indicated that the remaining chlorine was positioned at C-2 (δ_{C} 121.8). In the high-resolution ^{13}C NMR spectrum, the $^{35}\text{Cl}/^{37}\text{Cl}$ isotope shift was visualised—two peaks were observed resonating at δ_{C} 105.202 and δ_{C} 105.198 with 0.6 Hz difference between them, supporting the assignment of chlorine at C-3 (Figure 3.10). The effect was less pronounced in the C-2 resonance, which instead was visualised as a broadened peak. Although the reason for this is uncertain, it is perhaps attributed to a combination of factors. Despite the clear observation of the $^{35}\text{Cl}/^{37}\text{Cl}$ isotope shift for C-3, non-protonated sp^2 hybridised carbons show the isotope shift to a significantly smaller extent than protonated sp^2 hybridised carbons.¹⁴⁸ For the C-2 resonance, this is probably further exacerbated by unresolved scalar coupling of the quadrupolar ^{14}N ($I=1$) of the pyrrole which causes broader linewidths.¹⁴⁹ Accordingly, the structure of **128** was assigned as 6,7 dibromo-2,3-dichloroindole with full NMR data presented in Tables 3.9 and 3.10.

Figure 3.9. Experimental and calculated mass spectra for 6,7-dibromo-2,3-dichloroindole (**128**).

Figure 3.10. High-resolution (*top*; at = 6.0 s) and default (*bottom*; at = 1.5 s) ^{13}C NMR spectra of 6,7-dibromo-2,3-dichloroindole (**128**) showing the $^{35}\text{Cl}/^{37}\text{Cl}$ isotope shift (150 MHz, CDCl_3).

Table 3.9. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 6,7-Dibromo-2,3-dichloroindole (**128**) (CDCl_3).

Pos.	^{13}C or $^{15}\text{N}^\dagger$			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-251	NH		8.47	br s			
2	121.8	C						
3	105.2	C						
3a	125.2	C						
4	118.2	CH	168	7.35	d	8.4	5	3,6,7a
5	126.1	CH	168	7.42	d	8.4	4	3a,6,7
6	119.2	C						
7	107.1	C						
7a	133.0	C						

† Determined from a ^{15}N CIGAR experiment

Table 3.10. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 6,7-Dibromo-2,3-dichloroindole (**128**) ($\text{DMSO}-d_6$).

Pos.	^{13}C or $^{15}\text{N}^\dagger$			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-239	NH		12.76	br s			3,3a
2	122.9	C						
3	102.1	C						
3a	124.5	C						
4	117.7	CH	168	7.40	d	8.4	5	3,6,7a
5	125.3	CH	169	7.47	d	8.4	4	3a,7
6	118.2	C						
7	107.0	C						
7a	133.1	C						

† Determined from ^{15}N CIGAR experiment

3.5.5 7-Bromo-2,3-dichloro-6-iodoindole

The molecular formula of **129** was identified as $\text{C}_8\text{H}_3\text{BrCl}_2\text{IN}$ from the HRESIMS spectrum (m/z 387.7799, $[\text{M} - \text{H}]^-$) (Figure 3.11). All proton and carbon resonances were observed in their corresponding 1D NMR spectrum. Strong HMBC correlations were displayed from H-4 (δ_{H} 7.25) to C-3 (δ_{C} 105.2), C-6 (δ_{C} 94.3) and C-7a (δ_{C} 132.4) and from H-5 (δ_{H} 7.63) to C-3a (δ_{C} 125.9) and C-7 (δ_{C} 111.9) with an additional weak correlation observed to C-6. Without the weak two-bond correlation from H-5 to C-6, the chemical shift at δ_{C} 94.3 would suggest

bromination at C-3. From the previously identified tetrahalogenated indoles **125**–**128**, it was evident that the chemical shift of C-6 was not in accordance with bromine substitution. On the other hand, the carbon chemical shifts of C-5 and C-6 were consistent with iodine substitution, given that iodine causes significant shielding of the *ipso*- (δ_{C} 94.5, CDCl_3) and deshielding of the *ortho*- carbons (δ_{C} 137.5, CDCl_3) in iodobenzene.[†] With this in mind, C-7 was assigned to be substituted with bromine and was relatively more deshielded when compared with compounds **125**–**128** due to the neighbouring iodine. The remaining two chlorines were therefore positioned on carbons C-2 (δ_{C} 121.7) and C-3 (δ_{C} 105.2) with their respective chemical shifts corresponding to those observed in **128** supporting this assignment. Additionally, the high-resolution ^{13}C NMR spectrum showed a similar result to that observed for **128** (Figure 3.12). As such, the structure of **129** was provided as 7-bromo-2,3-dichloro-6-iodoindole. This completed the full structural characterisation of a naturally occurring bromochloroiodo- compound for the first time. Full NMR data is provided in Tables 3.11 and 3.12.

Natural products containing three different halogens are extremely rare, with bromochloriodomethane (**141**),^{19,20} 1-bromo-1-chloro-3-iodoisopropanol (**142**)²¹ and a bromochloriodoketone (**143**)⁷⁴ comprising the only literature examples. However, **141** and **142** were never physically isolated but were rather detected from a mixture of compounds using GC-MS analysis,^{19–21} and although **143** was claimed as being isolated, the report lacks evidence pertaining to this compound; it does not describe the isolation procedure utilised to obtain the compound, no details were provided as to how its structure was determined and no experimental data was reported.⁷⁴

[†]Determined from NMR data acquired on iodobenzene

Figure 3.11. Experimental and calculated mass spectra for 7-bromo-2,3-dichloro-6-iodoindole (**129**).

Figure 3.12. High-resolution (*top*; at = 6.0 s) and default (*bottom*; at = 1.5 s) ^{13}C NMR spectra of 7-bromo-2,3-dichloro-6-iodoindole (**129**) showing the $^{35}\text{Cl}/^{37}\text{Cl}$ isotope shift (150 MHz, CDCl_3).

Table 3.11. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 7-Bromo-2,3-dichloro-6-iodoindole (**129**) (CDCl_3).

129

Pos.	^{13}C or $^{15}\text{N}^\dagger$			^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1	-250	NH		8.33	br s			
2	121.7	C						
3	105.2	C						
3a	125.9	C						
4	118.6	CH	169	7.25	d	8.4	5	3,6,7(w),7a
5	132.2	CH	169	7.63	d	8.4	4	3a,6,7
6	94.3	C						
7	111.9	C						
7a	132.4	C						

† Determined from a ^{15}N CIGAR experiment
(w)Weak

Table 3.12. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for 7-Bromo-2,3-dichloro-6-iodoindole (**129**) ($\text{DMSO}-d_6$).

Pos.	^{13}C or $^{15}\text{N}^\dagger$		$^1J_{\text{CH}}$ (Hz)	^1H			COSY	HMBC (^1H to ^{13}C)
	δ (ppm)	mult		δ (ppm)	mult	J (Hz)		
1	-239	NH		12.67	s			2,3,3a,7a
2	122.7	C						
3	102.1	C						
3a	125.1	C						
4	117.9	CH	169	7.26	d	8.3	5	3,3a(w),6,7(w),7a
5	131.3	CH	169	7.64	d	8.3	4	3a,7,7a(w)
6	95.5	C						
7	111.9	C						
7a	132.6	C						

† Determined from ^{15}N CIGAR experiment
(w)Weak

3.5.6 2,7-Dibromo-3-chloro-6-iodoindole

HRESIMS analysis determined the molecular formula of **130** as $\text{C}_8\text{H}_3\text{Br}_2\text{ClIN}$ (m/z 431.7283, $[\text{M} - \text{H}]^-$). The proton and carbon chemical shifts of the aromatic methines closely resembled those of compound **129** suggesting that the halogen substitutions at C-3 and C-6 were identical. Analogous ^{13}C and HMBC NMR data to **129** verified the substitution of C-3 (δ_{C} 109.0) and C-6 (δ_{C} 94.4) with chlorine and iodine, respectively, and further indicated substitution of C-7 (δ_{C} 112.0) with bromine. The remaining bromine was therefore positioned on C-2 (δ_{C} 108.9) and the structure of **130** was assigned as 2,7 dibromo-3-chloro-6-iodoindole. The ^1H and ^{13}C NMR data for **130** and tetrahalogenated indoles henceforth (**116**, **119**, **121**, **123**, **124**, **131–133**) are tabulated together in Tables 3.13–3.16.

3.5.7 3,7-Dibromo-2-chloro-6-iodoindole

The molecular formula of **131** was determined as $\text{C}_8\text{H}_3\text{Br}_2\text{ClIN}$ (m/z 431.7280, $[\text{M} - \text{H}]^-$), regioisomeric with **130** as indicated by dissimilarities in the proton chemical shifts. This was confirmed following an analysis of the HMBC data, and the structure was established as 3,7-dibromo-2-chloro-6-iodoindole (**131**).

3.5.8 2,3,7-Trichloro-6-iodoindole

Compound **132** revealed a molecular formula of $\text{C}_8\text{H}_3\text{Cl}_3\text{IN}$ from the HRESIMS spectrum (m/z 343.8302, $[\text{M} - \text{H}]^-$) (Figure 3.13). The placement of iodine was established at C-6 (δ_{C} 91.1) through its similar carbon chemical shift to those observed in **129–131**, and the strong three-bond HMBC correlation from H-4 (δ_{H} 7.20). As such, the chlorines were situated at C-2,[†] C-3 (δ_{C} 105.2) and C-7 (δ_{C} 120.9) and the structure of **132** was deduced as 2,3,7-trichloro-6-iodoindole.

Figure 3.13. Experimental and calculated mass spectra for 2,3,7-trichloro-6-iodoindole (**132**).

3.5.9 2,3,4,7-Tetrabromoindole

A deprotonated molecule observed using HRESIMS indicated a molecular formula of $\text{C}_8\text{H}_3\text{Br}_4\text{N}$ for **116** (m/z 427.6920, $[\text{M} - \text{H}]^-$). The ^1H NMR spectrum was evidently dissimilar to tetrabromoindole **125**, indicating this to be a regioisomer. All protons and carbons were accounted for in the ^1H and ^{13}C NMR spectra, two of which were identified as protonated aromatic methines (CH-5: δ_{H} 7.24; δ_{C} 127.1 and CH-6: δ_{H} 7.19; δ_{C} 126.3) in a multiplicity-edited HSQC experiment. The presence of a 2,3,4,7-tetrahalogenated indole was indicated through HMBC correlations to C-7 and C-7a, as illustrated in Figure 3.6b. Strong three-bond correlations were observed in the HMBC spectrum from H-5 to C-3a (δ_{C} 125.0) and C-7 (δ_{C} 103.6), and from H-6 to C-4 (δ_{C} 112.6) and C-7a (δ_{C} 135.0). Additionally, weak two-bond correlations from H-5 to C-4 and H-6 to C-7 were observed. The final two brominated

[†]Due to paucity of mass, this resonance was not observed in the ^{13}C NMR spectrum.

carbons could therefore be assigned to C-2 (δ_{C} 113.8) and C-3 (δ_{C} 95.1) and their congruent chemical shifts with those of **125** supported this assignment. Thus, the structure of **116** was established as 2,3,4,7-tetrabromoindole.

3.5.10 2-Bromo-3,4-dichloro-7-iodoindole

The molecular formula of **124** was determined as $\text{C}_8\text{H}_3\text{BrCl}_2\text{IN}$ using negative-ion mode HRESIMS (m/z 387.7804, $[\text{M} - \text{H}]^-$). The near indistinguishable chemical shifts of CH-5 (δ_{H} 6.93; δ_{C} 124.0) and CH-6 (δ_{H} 7.44; δ_{C} 132.2) with those of **123** (See Tables 3.13–3.16), coupled with similarities in the HMBC data, indicated an identical halogen substitution pattern at C-4 (chlorinated; δ_{C} 126.0) and C-7 (iodinated; δ_{C} 74.0). The remaining two carbons, C-2 (δ_{C} 110.3) and C-3 (δ_{C} 108.6), exhibited chemical shifts analogous to those found in compounds **127** and **130**, indicating these positions to be substituted by bromine and chlorine, respectively. Thus, the structure was determined to be 2-bromo-3,4 dichloro-7-iodoindole (**124**).

The structures of **119**, **121** and **123** were deduced in a similar fashion to compounds **116** and **124**.

3.5.11 5-Bromo-2,3,4-trichloroindole

HRESIMS analysis identified the molecular formula $\text{C}_8\text{H}_3\text{BrCl}_3\text{N}$ for compound **133** (m/z 295.8444, $[\text{M} - \text{H}]^-$) (Figure 3.14). All protons and carbons were observed in the ^1H and ^{13}C NMR spectra. A 2,3,4,5-tetrasubstituted indole was suggested from the shielded carbon chemical shift of the protonated carbon C-7 (δ_{C} 110.8) and by the observation of an HMBC correlation to C-7a in accordance with Figure 3.6c. The HMBC experiment displayed strong correlations from H-6 (δ_{H} 7.43) to C-4 (δ_{C} 125.2) and C-7a (δ_{C} 133.0) and a weak correlation to C-5 (δ_{C} 116.1), whereas H-7 (δ_{H} 7.08) strongly correlated to C-5 and C-3a (δ_{C} 123.3) and weakly to C-4. The chemical shift of C-4 was similar to that observed in compounds **121**, **123**

and **124**, indicating chlorine substitution at that position. The shielded carbon chemical shift of C-5, relative to indole (**140**), was in agreement with bromine substitution. The final two carbons, C-2 and C-3 (δ_C 121.0 and 104.0, respectively), must therefore be substituted with chlorine, yielding the structure of **133** as 5-bromo-2,3,4-trichloroindole.

Figure 3.14. Experimental and calculated mass spectra for 5-bromo-2,3,4-trichloroindole (**133**).

Table 3.13. ^1H (600 MHz) NMR Data for Tetrahalogenated Indoles **116**, **119**, **121**, **123**, **124** and **130–133** (CDCl_3).

Pos.	Compound								
	116	119	121	123	124	130	131	132	133
1	8.59	8.44	8.40	8.30	8.30	8.34	8.41	8.38	8.39
2									
3									
3a									
4						7.25	7.19	7.20	
5	7.24	7.23	7.03	6.94	6.93	7.62	7.64	7.62	
6	7.19	7.21	7.27	7.45	7.44				7.43
7									7.08
7a									

Table 3.14. ^{15}N (60 MHz)[†] and ^{13}C (150 MHz) NMR Data for Tetrahalogenated Indoles **116**, **119**, **121**, **123**, **124** and **130–133** (CDCl_3).

Pos.	Compound								
	116	119	121	123	124	130	131	132	133
1	-245	-250	-251	-246	-242	-247	-	-	-253
2	113.8	123.3	123.4	121.8	110.3	108.9	124.0	-	121.0
3	95.1	105.5	104.7	105.0	108.6	109.0	91.5	105.2	104.0
3a	125.0	123.7	122.6	122.8	122.1	126.2	127.6	126.5	123.3
4	112.6	112.0	124.8	126.1	126.0	118.5	119.6	118.0	125.2
5	127.1	126.9	123.5	124.1	124.0	132.1	132.3	132.1	116.1
6	126.3	126.4	126.0	132.2	132.2	94.4	94.3	91.1	128.0
7	103.6	103.7	102.8	74.0	74.0	112.0	111.8	120.9	110.8
7a	135.0	132.8	132.8	136.0	137.7	134.2	133.3	130.8	133.0

–Not observed

†Determined from a ^{15}N CIGAR experiment

Table 3.15. ^1H (600 MHz) NMR Data for Tetrahalogenated Indoles **116**, **119**, **121**, **123**, **124** and **130–132** ($\text{DMSO}-d_6$).

Pos.	Compound							
	116	119	121	123	124	130	131	132
1	12.97	13.00	13.00	12.67	12.58	12.58	12.74	12.85
2								
3								
3a								
4						7.27	7.17	7.22
5	7.26	7.27	7.11	6.98	6.96	7.63	7.63	7.62
6	7.32	7.35	7.41	7.57	7.55			
7								
7a								

Table 3.16. ^{15}N (60 MHz)[†] and ^{13}C (150 MHz) NMR Data for Tetrahalogenated Indoles **116**, **119**, **121**, **123**, **124** and **130–132** ($\text{DMSO}-d_6$).

Pos.	Compound							
	116	119	121	123	124	130	131	132
1	-233	-239	-239	-235	-	-237	-	-
2	116.4	124.6	124.4	124.1	-	111.1	124.9	-
3	92.2	101.9	101.3	101.2	-	105.7	89.1	102.4 [‡]
3a	124.2	122.6	121.6	120.6	121.3 [‡]	125.5	126.8 [‡]	126.1
4	110.9	110.2	122.6	123.4	126.0 [‡]	117.8	118.9	117.4
5	126.2	126.2	122.9	123.3	123.1	132.1	131.4	132.1
6	126.3	126.5	126.2	132.5	132.4	95.5	95.4	92.3 [‡]
7	103.9	104.1	103.4	76.0	76.3 [‡]	111.8	111.6	120.8 [‡]
7a	135.2	133.0	133.1	136.0	138.7 [‡]	134.2	133.5	131.2

–Not observed

†Determined from a ^{15}N CIGAR experiment

‡Determined from an HMBC experiment

3.5.12 ^{15}N NMR

Further verification towards the proposed structure of tetrahalogenated indoles **116**, **119**, **121** and **123–132** was found using the ^{15}N CIGAR NMR experiment. The CIGAR (Constant Time Inverse-detection Gradient Accordion Rescaled) is an HMBC experiment that is optimised for multiple $^nJ_{\text{XH}}$ ($\text{X} = \text{C}, \text{N}$) values¹⁵⁰ making it particularly suited for detecting long-range ^1H - ^{15}N correlations due to the large variation in $^nJ_{\text{NH}}$ coupling constants.^{151,152} Using indole (**140**) as a guide, correlations were expected to be observed from H-2, H-3 and H-7 (weakly) to N-1 (Tables 3.1 and 3.2). No correlations were observed in the ^{15}N CIGAR experiments for **116**, **119**, **121** and **123–132**. This observation provided further evidence that neither C-2, C-3 or C-7 were protonated. With regard to 5-bromo-2,3,4-trichloroindole (**133**), the expected correlation from H-7 to N-1 is lacking, but this is likely due to paucity of material.

When the tetrahalogenated indoles were grouped and the nitrogen chemical shift[†] analysed, it became apparent that the nitrogen chemical shift, in conjunction with other experimental data, could also be used as a diagnostic tool. Relative to indole, all exhibited shielded nitrogen chemical shifts, and this appeared to be dependent on the halogen substituted at C-2, C-3 and C-7 (or lack of halogen at C-7 in the case of **133**). For example, 9 or 10 ppm differences were observed between tetrahalogenated indoles with bromine substituted at these three sites and that with bromine at C-7 and chlorine at both C-2 and C-3 (Figure 3.15). Interestingly, the $\text{DMSO}-d_6$ ^{15}N data appeared more shielded than the CDCl_3 ^{15}N data by *ca.* 10 ppm.

3.6 Pentahalogenated Indoles

With one less proton to work from, when compared with the tetrahalogenated indoles, the structure elucidation of the pentahalogenated indoles using NMR

[†] CH_3NO_2 as the reference standard

Figure 3.15. ^{15}N chemical shifts of halogenated indoles varying in substitution at C-2, C-3 and C-7.

spectroscopy was far more challenging because all six positions needed to be considered; the lack of $^3J_{\text{HH}}$ coupling constants meant that the aromatic proton could be positioned on either the benzenoid or pyrrole ring. However, the magnitude of the $^1J_{\text{CH}}$ could be used as a diagnostic tool to determine which ring the proton was located on. Of the six possibilities, the most readily assigned compounds would be presented by 2,4,5,6,7- or 2,3,4,5,6-pentahalogenated indole due to the shielded nature of the protonated carbon (*i.e.* CH-3 and CH-7, respectively) of each indole substitution type. The others, however, were less obvious and a similar logic to that used for the tetrahalogenated indoles was used to reason the substitution pattern of the remaining four possible pentahalogenated indoles. Thus, a proton situated on C-4 would display strong correlations to C-3 and C-7a (Figure 3.16a); a proton positioned on C-5 would correlate strongly to C-7 (Figure 3.16b); and a proton on either C-2 or C-6 would exhibit a strong correlation to C-7a (Figure 3.16c and d, respectively). To distinguish between a protonated C-2 and C-6, the ^{15}N CIGAR experiment would be expected to show a correlation from H-2 to N-1 if the proton was positioned at C-2.

Once the position of the proton and corresponding substitution pattern was ascertained, the halogen positions needed to be determined. The unequivocal assignment of the tetrahalogenated indoles isolated in this study provided a gauge by which the ^{13}C and ^{15}N NMR data of the pentahalogenated indoles could be compared to in order to ascertain their structures. Although far fewer compounds were isolated, a similar strategy to that used for the elucidation of

the tetrahalogenated indoles was employed, which began with pentabromoindole, of which two regioisomers (**134** and **135**) were isolated. The NMR data for the pentahalogenated indoles (**134–137**) is presented in Tables 3.17 and 3.18.

Figure 3.16. Three-bond HMBC correlations to C-3, C-7 and C-7a in 2,3,5,6,7-, 2,3,4,6,7-, 2,3,4,5,7-, and 3,4,5,6,7-pentasubstituted indole.

3.6.1 2,3,4,5,7-Pentabromoindole

The HRESIMS data for **134** provided the deprotonated molecular ion m/z 505.6029, which was consistent with the molecular formula $C_8H_2Br_5N$ (Figure 3.17). All carbon resonances (δ_C 133.6, 129.3, 126.2, 119.1, 115.0, 114.9, 103.7, 95.8) were observed in the ^{13}C NMR spectrum. One protonated carbon (CH-6: δ_H 7.63; δ_C 129.3) was observed in the multiplicity-edited HSQC spectrum, indicating the other proton was exchangeable (δ_H 8.64). A ^{15}N CIGAR experiment revealed the chemical shift of N-1 at δ_N -245.

Figure 3.17. Experimental and calculated mass spectra for 2,3,4,5,7-pentabromoindole (**134**).

On the basis of the deshielded carbon chemical shift of the protonated carbon CH-6, 2,4,5,6,7- and 2,3,4,5,6-pentabromoindole were discounted as possible structures.

The $^1J_{\text{CH}}$ value was measured at 174 Hz, which was suggestive of a proton situated on the pyrrole, however, a comparison made with indole (Table 3.1) showed this value to be inconsistent with that of CH-2 and therefore, both of C-2 and C-3 had to be substituted by bromine. In addition, no correlation to N-1 was observed in the ^{15}N CIGAR experiment and an analogous chemical shift of N-1 (δ_{N} -245) to **116** and **125** supported bromination at C-2, C-3 and C-7. This eliminated 3,4,5,6,7-pentabromoindole and left 2,3,5,6,7-, 2,3,4,6,7- and 2,3,4,5,7-pentabromoindole as remaining possibilities.

The HMBC data was used to establish which of the three regioisomers was in hand. Strong three-bond correlations were observed from H-6 to C-4 (δ_{C} 115.0) and C-7a (δ_{C} 133.6). As depicted by Figure 3.16c, the correlation to C-7a is consistent with 2,3,4,5,7-substitution of the indole. Weaker two-bond correlations were also observed from H-6 to C-5 (δ_{C} 119.1) and C-7 (δ_{C} 103.7). The final carbons were assigned to C-2 (δ_{C} 114.9), C-3 (δ_{C} 95.8) and C-3a (δ_{C} 126.2) through a comparison of carbon chemical shifts to **116** and **125**. The assignment of C-2 and C-3 were validated by HMBC correlations from the exchangeable proton NH-1 in DMSO- d_6 acquired data. Thus, the structure of (**134**) was deduced as 2,3,4,5,7-pentabromoindole. Compound **134** was crystallised and submitted to Associate Professor Martyn Coles for analysis using single X-ray crystallography, which confirmed the proposed structure of **134** (Figure 3.18). Although crystals were obtained for the additional pentahalogenated indoles isolated in this study, their quality was not sufficient for analysis by single X-ray crystallography.

3.6.2 2,3,4,6,7-Pentabromoindole

HRESIMS analysis of **135** gave rise to the deprotonated molecular ion m/z 505.6032, which was consistent with the molecular formula $\text{C}_8\text{H}_2\text{Br}_5\text{N}$, but differences in the 1D and 2D NMR data indicated a regioisomer of **134**. Both protons and all carbons (δ_{C} 135.7, 129.8, 123.9, 118.9, 114.2, 112.7, 106.6, 95.4) were observed in the 1D NMR spectrum. The multiplicity-edited HSQC identified the protonated carbon

(CH-5: δ_{H} 7.60; δ_{C} 129.8) and the ^{15}N CIGAR spectrum revealed the N-1 chemical shift as δ_{N} -243.

Figure 3.18. X-ray crystal structure of 2,3,4,5,7-pentabromoindole.

Once again, both 2,4,5,6,7- and 2,3,4,5,6-pentabromoindole were eliminated as potential structures due to the deshielded carbon chemical shift of the protonated carbon (CH-6). The carbon and nitrogen chemical shifts supported bromination at both C-2 (δ_{C} 114.2), C-3 (δ_{C} 95.4) and C-7 (δ_{C} 106.6). These data suggested the compound to be either a 2,3,5,6,7- or 2,3,4,6,7-pentabromoindole. The former possibility was discounted following an analysis of the HMBC data, which did not show the strongest correlations to both C-3 and C-7a. Strong three-bond HMBC correlations from H-5 to C-3a (δ_{C} 123.9) and C-7, and additional weaker HMBC correlations to C-4 (δ_{C} 112.7) and C-6 (δ_{C} 118.9) were observed. The remaining carbon resonance was assigned to C-7a (δ_{C} 135.7) to give rise to 2,3,4,6,7-pentabromoindole (**135**).

3.6.3 4,5,7-Tribromo-2,3-dichloroindole

The molecular formula $\text{C}_8\text{H}_2\text{Br}_3\text{Cl}_2\text{N}$ (m/z 417.7031, $[\text{M} - \text{H}]^-$) for **136** was determined from the HRESIMS data (Figure 3.19). Almost identical chemical shifts to **134** for the protonated carbon (CH-6: δ_{H} 7.64; δ_{C} 129.4) superficially suggested that **136** possessed the same substitution pattern. The HMBC spectrum was also found to resemble that observed for **134**. The strongest correlations were displayed from H-6 to C-4 (δ_{C} 114.5) and C-7a (δ_{C} 131.4) with weaker correlations to C-5 (δ_{C} 119.0) and C-7 (δ_{C} 104.0) also visible. Together, these data inferred substitution of C-4, C-5, and C-7 with bromine, and C-2 (δ_{C} 124.3) and C-3 (δ_{C} 105.9) with chlorine—the chemical shift of N-1 (δ_{N} -251) supported this assignment (Figure 3.15). Furthermore, the $^{35}\text{Cl}/^{37}\text{Cl}$ isotope shift was observed for C-2 and C-3 in a high-resolution ^{13}C NMR spectrum, providing additional evidence for substitution with chlorine these two carbons. Therefore, the structure of **136** is proposed.

Figure 3.19. Experimental and calculated mass spectra for 4,5,7-tribromo-2,3-dichloroindole (**136**).

3.6.4 5,7-Dibromo-2,3,4-trichloroindole

HRESIMS analysis for **137** revealed a deprotonated molecule at m/z 373.7557 consistent with the molecular formula $\text{C}_8\text{H}_2\text{Br}_2\text{Cl}_3\text{N}$ (Figure 3.20). The 1D and 2D NMR data were more similar to that of **136**, suggesting the same substitution pattern and the N-1 chemical shift (δ_{N} -251) indicated chlorine substitution at C-2 (δ_{C} 123.5) and C-3 (δ_{C} 105.2) and bromine substitution at C-7. Furthermore, the

similar proton and carbon chemical shifts of CH-6 (δ_{H} 7.62; δ_{C} 129.4) suggested that the *ortho*- carbons were brominated. This was supported through the observation of strong HMBC correlations to C-4 (δ_{C} 124.9) and C-7a (δ_{C} 131.7) and weak correlations to C-5 (δ_{C} 116.2) and C-7 (δ_{C} 103.1), which also inferred chlorine substitution at C-4. The assignment of chlorine to C-2, C-3 and C-4, and bromine to C-5 agreed favourably with the carbon chemical shifts of **133**. Thus, the structure of **137** is proposed as 5,7-dibromo-2,3,4-trichloroindole.

Figure 3.20. Experimental and calculated mass spectra for 5,7-dibromo-2,3,4-trichloroindole (**137**).

Table 3.17. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Pentahalogenated Indoles **134–137** (CDCl_3).

134	135	136	137					
Compound								
	134		135		136		137	
	δ_{C} or $\delta_{\text{N}}^{\dagger}$	δ_{H}	δ_{C} or $\delta_{\text{N}}^{\dagger}$	δ_{H}	δ_{C} or $\delta_{\text{N}}^{\dagger}$	δ_{H}	δ_{C} or $\delta_{\text{N}}^{\dagger}$	δ_{H}
Pos.	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
1	−245	8.64	−243	8.65	−251	8.50	−251	8.50
2	114.9		114.2		124.3		123.5	
3	95.8		95.4		105.9		105.2	
3a	126.2		123.9		125.0		123.9	
4	115.0		112.7		114.5		124.9	
5	119.1		129.8	7.60	119.0		116.2	
6	129.3	7.63	118.9		129.4	7.64	129.4	7.62
7	103.7		106.6		104.0		103.1	
7a	133.6		135.7		131.4		131.7	

† Determined from ^{15}N CIGAR experiment

Table 3.18. ^{15}N (60 MHz), ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Pentahalogenated Indoles **134**, **136** and **137** ($\text{DMSO}-d_6$).

Pos.	Compound					
	134		136		137	
	δ_{C} or $\delta_{\text{N}}^{\dagger}$ (ppm)	δ_{H} (ppm)	δ_{C} or $\delta_{\text{N}}^{\dagger}$ (ppm)	δ_{H} (ppm)	δ_{C} or $\delta_{\text{N}}^{\dagger}$ (ppm)	δ_{H} (ppm)
1	-232	13.20	-	13.24	-	13.30
2	117.5		124.0 [‡]		125.4	
3	93.9		106.7		101.6	
3a	125.5		124.0 [‡]		122.38 [‡]	
4	113.0		112.4		122.39 [‡]	
5	117.7		117.3		114.6	
6	128.4	7.80	128.4	7.78	128.6	7.81
7	104.5		105.0		104.1	
7a	133.8		131.9		132.1	

-Not observed

[†]Determined from ^{15}N CIGAR experiment

[‡]Interchangeable

3.7 Hexahalogenated Indole

The five fractions collected from the C_{18} HPLC column described in Scheme 3.4, together contained a variety of hexahalogenated indoles ranging from dibromo-tetrachloro- to hexabromoindole. The third fraction appeared by NMR (by visualisation of the NH-1 resonance) and HRESIMS data to consist of mainly one compound identified as $\text{C}_8\text{HBr}_3\text{Cl}_3\text{N}$ (m/z 451.6667, $[\text{M} - \text{H}]^-$) (Figure 3.21). A permutation-combination calculation indicates that there are 20 constitutional isomers of a tribromo-trichloro indole where each halogen is connected to carbon. With only an exchangeable proton present on the molecule, it was clear that NMR spectroscopy would be ineffective for the complete structure elucidation—HMBC correlations from NH-1 in $\text{DMSO}-d_6$ combined with the carbon and nitrogen chemical shifts would have suggested the halogen types present at C-2 and C-3. However, paucity of mass meant that these data were unobtainable. Furthermore, efforts to crystallise the sample for X-ray diffraction were unsuccessful. As such, the structure of this compound remains unsolved.

The hexahalogenated indoles of *R. membranacea* are intriguing since all carbon centres in the molecule, that would otherwise be protonated, are substituted by a halogen. Synthetic hexachlorinated indole has been described in the chemical literature,¹⁵³ but naturally-derived compounds with halogen substitution of every

available carbon is rare. Some examples have been found from the red alga *A. taxiformis* including carbon tetrabromide,¹⁹ perchloroacetone,¹⁹ and a number of trihaloacrylic acids.²²

Figure 3.21. HRESIMS (*left*), and ^1H (600 MHz, *centre*) and ^{13}C (150 MHz, *right*) NMR spectra (CDCl_3) of a tribromo-trichloroindole.

3.8 Isatin Derivatives

A deprotonated molecule detected using HRESIMS analysis provided the molecular formula of 4-chloro-3-hydroxyl-3-(2-oxopropyl)-2-oxindole (**139**) as $\text{C}_{11}\text{H}_9\text{ClNO}_3$ (m/z 238.0276, $[\text{M} - \text{H}]^-$). The structure was elucidated through the examination of the 1D and 2D NMR spectra and confirmed by comparison with the literature.¹⁵⁴ The methyl ketone moiety suggested an artefact of isolation, arising through an aldol condensation of the C-3 ketone of 4-chloroisatin (**138**) with acetone.¹⁵⁴ This was verified upon the re-extraction of and subsequent purification (in the absence of acetone) that led to the isolation of a mixture of 4-chloroisatin and its tautomeric form (**138**, m/z 179.9858, $[\text{M} - \text{H}]^-$) with no evidence for the presence of 4-chloro-3-hydroxyl-3-(2-oxopropyl)-2-oxindole (**139**). Interestingly, isatin (the dechlorinated version of **138**) is believed to be the first recognised tautomeric molecule.¹⁵⁵

3.9 Biological Activity

Several compounds were submitted to the School of Biological Sciences where they were tested for biological activity. Antifungal activities of compounds **119**, **125**, **127–129**, **138** and **139** were assessed against wild-type *Saccharomyces cerevisiae* (baker’s yeast) by monitoring inhibition of yeast growth in the presence of the compounds. These compounds were also tested in a standard 48 h MTT (3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide) cell proliferation assay,¹⁵⁶ against the HL-60 promyelocytic leukaemia cell line and showed similar activity to that observed against *S. cerevisiae* (Table 3.19). The similarities in IC₅₀ values between the two assays is unusual as yeast possess robust xenobiotic[†] defence mechanisms and consequently, significantly higher doses of a compound (five to ten times) are typically required to inhibit yeast growth when compared with mammalian cell-based assays.^{157,158} The fractions containing tri-, penta- and hexahalogenated indoles (fractions D2, D4 and D5, respectively, from Scheme 3.1) were also tested and exhibited antifungal activity with the pentahalogenated indoles showing the greatest level of activity. These results are consistent with Brennan and Erickson’s observation of the extract of *R. membranacea* exhibiting antifungal activity¹³⁸ and suggest that the polyhalogenated indoles may be responsible.

Table 3.19. IC₅₀ values of **119**, **125**, **127–129**, **138** and **139** Against the HL-60 Cell Line and *Saccharomyces cerevisiae*,

Compound	HL-60 (μ M)	<i>S. cerevisiae</i> (μ M)
119	28	23
125	38	69
127	78	>83 [†]
128	61	63
129	49	39
138	70	>61 [†]
139	61	>41 [†]

[†]Highest concentration tested

[†]A chemical substance found within an organism that is not naturally produced by that organism.

3.10 Related Halogenated Compounds in the Literature

The marine natural products literature contains a multitude of indoles and indole alkaloids with the Marinlit database returning 1130 results from an indole substructure search.⁴⁶ These include compounds derived from hydrozoans, bryozoans, sponges, algae, tunicates and microbes.²⁸ An exhaustive review is beyond the scope of this report but a few selected halogenated examples are detailed.

Indole alkaloids range from monomeric (*e.g.* **144–149**)^{159,160} and dimeric indoles (*e.g.* **150–156**)^{160,161} to more complex structures such as the enantiomeric compounds, similisine A (**157**) and B (**158**).¹⁶² Isolated from the red alga *Laurencia similis*, **157** and **158** are trisindole alkaloids with a [5,5]-spirooxindole moiety.¹⁶² Compounds **150** and **151** displayed cytotoxicity against HT-29 (human colorectal adenocarcinoma; **150** only) and P388 cell lines, although, no measured value was reported. Compounds **144–149** and **152–158** either lacked activity in the bioassay used or no biological activity was noted.

144	R ₁ = SMe	R ₂ = SMe	R ₃ = H
145	R ₁ = SMe	R ₂ = SOMe	R ₃ = H
146	R ₁ = SOMe	R ₂ = SMe	R ₃ = H
147	R ₁ = SOMe	R ₂ = SMe	R ₃ = Br
148	R ₁ = Br	R ₂ = SOMe	R ₃ = H
149	R ₁ = SOMe	R ₂ = SOMe	R ₃ = H

150	R ₁ = SMe	R ₂ = SOMe
151	R ₁ = SOMe	R ₂ = SOMe
152	R ₁ = SMe	R ₂ = Br

The chlorinated bisindole pyrroles lynamicins A–E (**159–163**) were isolated from a strain of actinomycetes and several exhibited broad-spectrum antimicrobial activity against a panel of both Gram-positive and Gram-negative bacteria.¹⁶³ A deep-sea derived *Streptomyces* sp. yielded spiroindimicins A–D (**164–167**). Compounds **165–167** demonstrated moderate to weak cytotoxicity against a range of mammalian cell

lines (IC_{50} 4–15 $\mu\text{g/mL}$).¹⁶⁴

- 153** $R_1 = \text{H}$ $R_2 = \text{H}$
154 $R_1 = \text{H}$ $R_2 = \text{Br}$
155 $R_1 = \text{Br}$ $R_2 = \text{H}$
156 $R_1 = \text{Br}$ $R_2 = \text{Br}$

- 157**
158 *ent*

- 159** $R_1 = \text{H}$ $R_2 = \text{OAc}$ $R_3 = \text{H}$ $R_4 = \text{Cl}$ $R_5 = \text{H}$
160 $R_1 = \text{H}$ $R_2 = \text{OAc}$ $R_3 = \text{H}$ $R_4 = \text{Cl}$ $R_5 = \text{Cl}$
161 $R_1 = \text{Cl}$ $R_2 = \text{H}$ $R_3 = \text{H}$ $R_4 = \text{Cl}$ $R_5 = \text{Cl}$
162 $R_1 = \text{H}$ $R_2 = \text{OAc}$ $R_3 = \text{OAc}$ $R_4 = \text{Cl}$ $R_5 = \text{H}$
163 $R_1 = \text{H}$ $R_2 = \text{OAc}$ $R_3 = \text{OAc}$ $R_4 = \text{H}$ $R_5 = \text{H}$

164

- 165** $R_1 = \text{Me}$ $R_2 = \text{H}$
166 $R_1 = \text{H}$ $R_2 = \text{H}$
167 $R_1 = \text{Me}$ $R_2 = \text{COOMe}$

Examples of iodinated indole alkaloids include hicksoanes A–C (**168–170**) from the gorgonian *Subergorgia hicksoni*,¹⁶⁵ breitfussin A (**171**) and B (**172**) from the hydrozoan *Thuiaria breitfussi*,¹⁶⁶ and plakohypaphorines A–F (**173–178**) from the sponge *Plakortis simplex*.^{167,168} Hicksoanes A–C (**168–170**) were found to deter feeding by goldfish at concentrations between 5–100 $\mu\text{g/mL}$ and are thought to provide chemical antifouling defences to the organism.¹⁶⁵ Comprised of a

combination of pyrrole, oxazole and indole moieties, the structures of beifussin A (**171**) and B (**172**) were determined using a combination of NMR spectroscopy, atomic-force microscopy, and computational methods.¹⁶⁶ Neither were evaluated for biological activity. The isolation of plakohypaphorines A–D (**173–176**) constitute the first iodinated indoles and **174–176** were found to demonstrate antihistaminic activity.¹⁶⁸

3.11 Concluding Remarks

In summary, a spectroscopic-guided investigation of the methanolic extract of *R. membranacea* was instigated due to mass spectrometric evidence for the presence of several polyhalogenated indoles. Following each stage of purification, NMR spectroscopy and MS were used to track and differentiate the polyhalogenated indoles

present within each fraction. This led to the isolation of four naturally occurring tetrahalogenated indoles with the rare inclusion of bromine, chlorine and iodine, in addition to several other closely related tetra- and pentahalogenated indoles. The unequivocal full NMR characterisation of the isolated bromochloriodoindoles would have been far more complicated without having isolated and characterised the additional tetrahalogenated indoles described in this study.

The presence of a diverse array of tri- to hexahalogenated indoles within *R. membranacea* is unusual, especially because there appeared to be little else in the form of secondary metabolites—besides fatty acids and sugar derivatives, other compounds also appeared to be indole derivatives. The impetus for the biosynthesis of so many halogenated indoles, including regioisomers, is intriguing and although these biological aspects were not examined in this study they are possibly worth investigating in the future. Due to the metabolic expense incurred by the biosynthesis of secondary metabolites and assuming that biohalogenation occurs *via* a vanadium haloperoxidase, it seems unlikely that these are accidental products. A vanadium bromoperoxidase has been found to show regiospecific oxidation of an indole derivative at the C-2 position—an unsurprising result since the electron-rich nature of the C-2–C-3 double bond in indole makes these centres particularly susceptible to electrophilic attack. The reaction is suggested to proceed *via* a bromonium ion intermediate.⁸² Perhaps, the presence of many polyhalogenated indoles act synergistically to provide improved chemical defences to the alga.

Chapter 4

New Halogenated Meroditerpenoids from *Callophycus serratus*

4.1 The Genus *Callophycus*

The genus *Callophycus* (formerly *Thysanocladia*)^{11,169} encompasses ten species of red algae distributed across Eastern and Southern Africa, the Philippines, the South Pacific and Australia.¹¹ Six of the ten species are found in Australia¹¹ and as such, it is not surprising that the earliest literature record of natural products derived from the genus *Callophycus*, disseminated from this country.¹⁷⁰

There are three literature reports on Australian *Callophycus* natural products. Research conducted on all six species involved an investigation of the cell-wall polysaccharide contents for potential phycocolloids and also to assess their chemotaxonomic value.¹⁷⁰ The main components were pyruvate- and sulfate-rich galactans comprised mostly of galactose and 3,6-anhydrogalactose. Further analyses determined the structure of the predominant repeating disaccharide found in all six species, to be a novel 4',6'-*O*-(1-carboxyethylidene)carrabiose 2-sulfate (**179**).¹⁷⁰ The second publication, by a separate research group, sought the isolation of biologically active metabolites from *C. oppositifolius*. A bioassay-directed isolation procedure yielded callophycin A (**180**), a moderately cytotoxic tetrahydro- β -carboline that displayed activity against a range of human tumour cell-lines (GI₅₀ values between 1.3 and 4.2 μ M).¹⁷¹ The discovery of **180** and its associated biological activity initiated synthetic endeavours towards callophycin A analogues so that additional biological assessments could be made.¹⁰⁸ The final publication, by a third research group, included *C. tridentifer* in a survey comprising 49 Australian red algal species, which aimed to search for and identify specific bromophenols recognised as key components responsible for seafood flavour.¹⁷² All other literature records of

Callophycus natural products describe the isolation of meroditerpenoids.

4.2 Meroditerpenoids from *Callophycus* spp.

The prefix ‘mero’ in meroditerpene, is derived from the Greek word ‘meros’ meaning ‘part’ and relates to the concept that these class of compounds are derived from mixed biosynthetic pathways that include partial terpenoid origin;¹⁷³ the suffix ‘diterpene’ indicates a C₂₀ terpene composed from the combination of four isoprene (C₅H₈) units.¹⁷⁴

Red algae of the genus *Callophycus* have been found by the Kubanek group to be prolific producers of a myriad of structurally diverse meroditerpenoids.^{44,175–181} Several Fijian specimens of this genus (mainly *C. serratus*) were the subject of an extensive study that resulted in discovery of eleven novel carbon skeletons and the isolation of 45 compounds that were new to the marine natural products literature.^{44,175–181} Each compound was found to possess either tri- or tetrasubstitution of the benzene ring and bromination was prevalent. Far fewer examples of iodinated and chlorinated compounds have been reported.^{44,181} A report featuring the first *Callophycus* derived di-, tri- and tetraiodinated meroditerpenes⁴⁴ was published by Kubanek and co-workers simultaneously to the preparation of this dissertation. Even though a high level of structural diversity exists within this set of metabolites, they can be categorised into four simple classes: the *p*-hydroxybenzoate macrolides,^{176–180} callophycoic acids,^{44,181} bromophycoic acids^{44,175} and callophycols.^{44,181}

4.2.1 *p*-Hydroxybenzoate Macrolides

The largest class, which is comprised of 23 compounds isolated from *C. serratus*, are the *p*-hydroxybenzoate (diterpene-benzoate) macrolides—15- or 16-membered hybrid lactones containing a portion of each of the *p*-hydroxybenzoate and diterpenoid moieties. With the exception of five macrolides, a brominated 6-membered ring substituted with either a methyl or exomethylene (methylidene), is contained within the macrocycle. Bromophycolides A and B and debromophycolide A (**181**–**183**) were the first of those isolated in the series.¹⁸⁰ The relative configuration was determined from NMR data and X-ray crystallographic analysis provided the absolute configuration of compounds **181** and **182**. In debromophycolide A (**183**), an analysis of the NOE data established the relative configuration at the epoxide while a comparison of the NMR data to **181** led to an identical configurational assignment at C-15. Ambiguous NOE data, coupled with the broad multiplicity of H-7 resulting in a lack of adequate information otherwise provided from ¹H–¹H scalar couplings, meant that the configuration at C-7 could not be defined in **183**.¹⁸⁰

An additional seven macrolides were reported in the year following that of **181**–**183**. Bromophycolides C–I (**184**–**190**) share a common carbon backbone with either **181** or **182** and relative configurations were deduced based on ¹H–¹H scalar couplings, NOE data and comparisons to **181** and **182**.¹⁷⁹ Synthetic approaches towards **181** and **185** from geranylgeranyl benzoate have since commenced although, no publications have appeared in the literature in recent years.¹⁸² Geranylgeranyl benzoate was proposed as the precursor molecule to *Callophycus* meroditerpenes in the first publication that appeared in the literature,¹⁸⁰ and recent work completed by the Kubanek group resulted in the isolation of 3-geranylgeranyl-4-hydroxybenzoic acid (**191**).⁴⁴

The isolation of bromophycolides J–Q (**192**–**199**) was accompanied by the discovery of a further two novel carbon skeletons, as exemplified by **192** and **193**.¹⁷⁸ Furthermore, this group of newly found bromophycolides revealed an additional subclass containing a tetrahydropyran ring within the macrocycle such as is found

in **198**. Compounds **193** and **194** are examples of rearranged diterpenes and are thought to arise from a biosynthetic 1,2-methyl shift. Compound **192** is unusual in two respects: 1) to date, it is one of only two C_{28} *Callophycus* meroditerpenes with the extra carbon present as an oxymethyl and 2) it contains an unusual bicyclo[3.1.0]hexane—although a cyclopentenyl unit was encountered in **183**, its formation is envisioned through a naturally occurring cyclisation pathway.¹⁸⁰ Perhaps resulting from an artefact of isolation, this unique bicyclic system was suggested as potentially arising by methanolysis and homoallylic substitution of bromine in bromophycolide O (**197**). The relative configurations of **192–199** were established in a similar manner used for previously reported bromophycolides.¹⁷⁸

Completing the family of macrolides are bromophycolides R–U (**200–203**) and callophycolide A (**204**).^{176,177} While compounds **200–203** are structurally similar to previous bromophycolides, the non-brominated callophycolide A (**204**) barely holds a resemblance; the 15-membered macrocycle lacks a 6-membered ring and contains a longer alkyl chain substituted at the C-14 position. The absolute configuration of 14*S*,15*R* was determined using a combination of Mosher's ester data and CD analysis of a dimolybdenum tetraacetate complex (Snatzke's method).¹⁷⁶

4.2.2 Callophycoic acids and Callophycols

The callophycoic acids and callophycols each comprise a relatively smaller group of meroditerpenes when compared with the *p*-hydroxybenzoate macrolides. Callophycoic acids A–H (**205–212**)¹⁸¹ and iodocallophycoic acid A (**213**)⁴⁴ are either di-, tri- or tetracyclic *p*-hydroxybenzoic acid diterpenoids. Callophycols A (**214**) and B (**215**) and iodocallophycols A–D (**216–219**) lack the carboxyl group altogether in favour of halogen substitution at the corresponding aromatic carbon. Compounds **214** and **215** are regioisomeric through an exchange of halogen substitution and represent the only chlorinated *Callophycus* natural products reported in the literature.¹⁸¹ Similarly, **213** and **216–219** constitute the only iodinated *Callophycus* meroditerpenes.⁴⁴ The relative and absolute configurations of **205** were established by NOE and X-ray crystallographic data, respectively.¹⁸¹ NOE data and computational modelling using Spartan established the relative configuration for iodocallophycoic acid A (**213**). The absolute configuration for **213** was established using electronic circular dichroism spectroscopy and time-dependent density functional theory (DFT) calculations.⁴⁴ The known compound 3-geranylgeranyl-4-hydroxybenzoic acid (**191**) was also isolated alongside **213** and **216–219**.⁴⁴

The isolation of **205–215** exclusive of bromophycolides, was originally thought to result from two genetically distinct *C. serratus* chemotypes, even co-existing within a few meters of each other. Environmental factors were also acknowledged as an alternative possibility.¹⁸³ However, it was later reasoned that non-macrocyclic compounds such as **205–215** originate from *C. densus*, while *C. serratus* specimens give rise to the macrocyclic bromophycolides.¹⁷⁵

205 R = A

207 R = B

206 R = A

208 R = B

209 R = C

210

211 R = H

212 R = Br

214 R₁ = Br R₂ = Cl

215 R₁ = Cl R₂ = Br

213 R₁ = COOH R₂ = H

216 R₁ = Br R₂ = Br

217 R₁ = Br R₂ = I

218 R₁ = I R₂ = Br

219 R₁ = I R₂ = I

4.2.3 Bromophycoic acids

The bromophycoic acids A–F (**220–225**) and bromophycoic acid A methyl ester (**226**) represent the final class of meroditerpenes isolated from a *Callophycus* specimen morphologically similar to *C. serratus*.^{44,175} Whilst having a similar carbon backbone to **211** and **212**, bromophycoic acids A–D, F and bromophycoic acid A methyl ester (**220–223**, **225** and **226**) follow a different cyclisation pathway with the isoprene methyl (generates the exomethylene observed in **211** and **212**) being used to form the decalin system of **220–223**, **225** and **226**. Bromophycoic acid E (**224**) is unique among the bromophycoic acids as it possesses four fused ring systems and the carbon-carbon connectivity between the terpene and benzoic acid moieties, appears to be at a different attachment point on the diterpenoid portion.¹⁷⁵ Unlike **192**, the additional methyl (methyl ester) of **226** is considered to be of natural origin since an ethanolic extraction of *Callophycus* did not yield the ethyl ester equivalent.⁴⁴ Compounds **225** and **226** are recent additions to this set of meroditerpenes whose absolute configurational assignments resulted in the revised configurational assignments for **220–224** as depicted below;⁴⁴ the configurations of **220–224** in the original report were relative and inverted to **225** and **226**.¹⁷⁵

4.2.4 Biological Activity

The *Callophycus* meroditerpenes were assessed for biological activity in a variety of assays, which allowed for consideration of a structure-activity relationship (SAR). The large majority were tested for antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), but only **198**, **199**, **203**, **211**, **213** and **220** displayed levels of inhibition (MIC = 0.8, 1.1, 0.5, 1.6, 1.4 and 1.6 $\mu\text{g/mL}$, respectively) comparable to the current vancomycin treatment (MIC = 2 $\mu\text{g/mL}$).^{175,178} Compared with other bromophycolides, the increased hydrophobicity, conformational restriction or both in **198**, **199** and **203** were considered to be contributing factors.¹⁷⁸ These structural features could also explain the significant decrease in antibacterial activity between **220** and **221** (MIC = 25.0 $\mu\text{g/mL}$).¹⁷⁵ The presence of the carboxylic acid appeared to infer activity in iodocallophycic acid A (**213**) as iodocallophycols A–D (**216–219**) were inactive in the same assay.⁴⁴

Most of the bromophycolides displayed modest activity when tested for cytotoxicity against a range of human tumour cell lines (mean IC₅₀ values varied between 2.0 and 42.6 μM). A superficial consideration of the SAR suggested that hydroxyl replacement of the isopropyl bromine (*e.g.* C-15 in **181**) decreases cytotoxicity; a reduction in potency was observed between compounds **181** and **184** (*ca.* six times less active) and between **187** (*ca.* three times less active) and **201**. The replacement of bromine by a hydroxyl group at other sites was less obvious^{177–180} and no inferences could be made into the SAR between the 15- and 16-membered macrolides.¹⁷⁹

When assessed for antimalarial activity against a drug-resistant *Plasmodium falciparum* parasite, the callophycoic acids, bromophycoic acids and callophycols were inactive,^{175,181} while the vast majority of bromophycolides exhibited low micromolar activity. The most potent activity was exhibited by bromophycolides A (**181**), D (**185**), E (**186**), H (**189**), M (**195**) and S (**201**) with IC₅₀ values ranging between 0.3–0.9 μ M.^{177,178} These results suggest that the macrolide is required for activity, and similarly to the SAR observed in the MRSA bioassay, that bromine substitution at C-15 infers activity.¹⁷⁸ Mechanism of action studies revealed that bromophycolide A (**181**) targets haem crystallisation. The release of free haem molecules, caused by the catabolism of haemoglobin by *P. falciparum*, is lethal to the parasite. To overcome this, the organism crystallises haem into the non-toxic haemozin molecule.¹⁸⁴ The discovery of bromophycolide antimalarial activity and this mechanism of action has seen **181–203**, and any other molecules with structurally similar scaffolds to the bromophycolides, the subject of a patent.¹⁸⁵

As a final note pertaining to biological activity, the bromophycolides are believed to provide fungal resistance to *C. serratus*. Using desorption electrospray ionisation mass-spectrometry (DESI-MS), the Kubanek group studied the distribution of the bromophycolides on the surfaces of *C. serratus*. They revealed that the sites of these compounds were localised over algal surfaces where tissue was compromised by mechanical abrasion.^{183,186} In the same study, ten samples containing natural concentrations of either bromophycolides or callophycoic acids and callophycols, displayed >95% growth inhibition against the pathogenic marine fungus *Lindra thalassiae*. The combination of these results suggest that the localisation of such compounds is the result of possible injury and serve to deter fungal infection.^{183,186}

4.3 Investigation of a Tongan Specimen of *Callophycus serratus*

Callophycus serratus (Figure 4.1) is one of ten species included in the genus *Callophycus*. Certain morphological features shared with other species of this genus can cause confusion when taxonomic identification is concerned, although the manifestation of a mid-rib line traversing the main axes of dried *C. serratus* specimens is a distinctive characteristic.¹⁶⁹ The distribution of the red alga is widespread with this species found in the Philippines, Australia, New Zealand, Papua New Guinea, the Solomon Islands, Fiji and Tonga.^{11,169}

Figure 4.1. Underwater photo of *Callophycus serratus*.
Image courtesy of Dan Crossett, 2016.

A specimen of the fern-like red alga *C. serratus* (Figure 4.1) was collected from ‘Eua, Tonga, and examined in this study. The alga was identified by Dr Joe Zuccarello in the School of Biological Sciences. In a similar approach to that used in the isolation of the polyhalogenated indoles (Chapter 3), purification was tracked using HRESIMS in addition to ¹H NMR spectroscopy—fractions with data that displayed notable differences, when compared against the literature, were selected for further investigation. Using this strategy, a total of eight meroditerpenoids were purified—the two known macrolides, bromophycolides A (**181**) and T (**202**), and six new compounds, callophycol C (**227**), iodocallophycols E (**228**) and F (**229**),

iodocallophycoic acid B (**230**), deiiodocallophycoic acid B (**231**) and callophycoic acid I (**232**). Iodocallophycols E (**228**) and F (**229**) and iodocallophycoic acid B (**230**) incorporate iodine within their structure bringing the total number of isolated iodinated *Callophycus* metabolites to eight.

Salient features in the 1D and 2D NMR spectra of the 75% Me₂CO/H₂O (Figures 4.2 and 4.3) and 100% Me₂CO fractions (Figures 4.4 and 4.5) included several aromatic resonances and a deshielded *gem*-dimethyl at δ_{H} 1.81 and 1.79. The aromatic resonances of the major components were attributed to the 1,3,4-trisubstituted benzene ring present in the vast majority of *Callophycus* meroditerpenes, while the functionalised diterpene terminus in compounds such as bromophycolide A (**181**) accounted for the deshielded *gem*-dimethyl. At this stage, definitive structural progress of either moiety was impeded due to overlapping resonances of the many closely related compounds present within each sample, making identification of the major compound and detection of potentially new compounds difficult. Consequently, additional purification was required.

Figure 4.2. ¹H NMR spectrum of the 75% Me₂CO/H₂O screen fraction from *Callophycus serratus* (600 MHz, CD₃OD).

Figure 4.3. HMBC spectrum of the 75% Me₂CO/H₂O screen fraction from *Callophycus serratus* (600 MHz, CD₃OD).

Figure 4.4. ¹H NMR spectrum of the 100% Me₂CO screen fraction from *Callophycus serratus* (600 MHz, CDCl₃).

Figure 4.5. HMBC spectrum of the 100% Me₂CO screen fraction from *Callophycus serratus* (600 MHz, CDCl₃).

4.3.1 The Isolation of New Callophycoic Acids and Callophycols

Analysis of the NMR spectra of the 75% Me₂CO/H₂O and 100% Me₂CO fractions showed that the latter contained several more resonances (Figures 4.2–4.5) and for this reason, it was selected first for further examination. Following purification on silica gel, fractions were spotted onto a TLC plate, eluted with CH₂Cl₂, and visualised after dipping the plate into an H₂SO₄, then a vanillin solution, followed by heating. A series of early eluting fractions were visualised as a purple char, which turned to a vibrant blue upon cooling to room temperature (Figure 4.6). These fractions were combined to form three samples based on slight differences in R_f (retention factor) values. Analysis of the NMR spectra of these samples established the presence of a tetrasubstituted benzene ring, vastly reducing the number of potentially known *Callophycus* compounds to just seven. Fraction two appeared to contain a pure compound by ¹H NMR and HRESIMS but was later determined to be a mixture of callophycols A (**214**) and B (**215**) (2.4 mg), while fraction three was found to be a new chlorinated congener, callophycol C (**227**)

(5.2 mg). Fractions eluting from silica gel between 50% DCM/*n*-hexane and DCM showed aromatic resonances between δ_{H} 6.50–7.26 and were indicated by HRESIMS data to contain mixtures of halogenated C_{26} compounds. Subsequent purification using HILIC HPLC led to the isolation of iodocallophycols E (**228**) and F (**229**). Later eluting DCM fractions contained the major compounds of the original sample, which were tentatively identified as mixtures of bromophycolides on the basis of NMR and HRESIMS data.

Figure 4.6. TLC of silica fractions showing the colour transformation of the char from purple to blue. The blue char was divided into the three fractions circled in orange.

The isolation of **228** and **229** prompted the extraction of a second batch of the alga and a more cautious purification approach was taken by substituting silica with DIOL. Silica gel, possibly due to its acidic nature, has been reported to cause the decomposition of iodinated compounds.¹⁹ Purification of iodocallophycols E (**228**) and F (**229**) from the second extraction was achieved using C_{18} HPLC, which also resulted in pure bromophycolide T (**202**) (0.10 mg). Samples containing iodocallophycol E (**228**) were pooled together (combined mass: 1.3 mg), as were samples of iodocallophycol F (**229**) (combined mass: 1.8 mg). Iodocallophycic acid B (**230**) was purified from the relevant DIOL fraction on C_{18} HPLC (Scheme 4.1).

The 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ PSDVB fractions obtained from the first and second batch extractions were partitioned separately on DIOL. Selected fractions that eluted between 70% DCM/*n*-hexane and DCM were subjected to repeated chromatography on C_{18} HPLC, affording bromophycolide A (**181**) (1.2 mg) as the major compound.

The 20% EtOAc/DCM DIOL fraction (from first extraction) was further purified using C₁₈ HPLC to yield deiodocallophycoic acid B (**231**). The 10% EtOAc/DCM DIOL fraction of the first extract, was combined with fractions eluting between 10–20% EtOAc/DCM of the second extract and the resulting sample was finally purified using C₁₈ HPLC to afford iodo- and deiodocallophycoic acid B (**230** and **231**, respectively) and callophycoic acid I (**232**) (0.60 mg) (Scheme 4.1). The duplicate samples of iodocallophycoic acid B (**230**) were combined (combined mass: 0.80 mg), as were those of deiodocallophycoic acid B (**231**) (combined mass: 1.0 mg).

Scheme 4.1. Isolation scheme of compounds isolated from *Callophycus serratus*.

4.4 Callophycol C

Callophycol C (**227**) was isolated as a white amorphous solid. The most distinguishable feature observed in the ^1H NMR spectrum was a pair of aromatic proton *meta*-coupled doublet resonances that pointed to the presence of a 1,2,4,6-tetrasubstituted aromatic ring. Notable differences in the region δ_{H} 3.80–4.40 were observed in the ^1H NMR spectra of callophycols A–C (**214**, **215** and **227**, respectively), suggesting that **227** was a potentially new analogue.

Negative-ion mode HRESIMS data provided a deprotonated molecule at m/z 632.9787, consistent with the parent molecular formula $\text{C}_{26}\text{H}_{34}\text{Br}_3\text{ClO}$, which required eight double bond equivalents. The isotopic distribution pattern confirmed the presence of bromine and chlorine (Figure 4.7). All 26 carbons were observed in the ^{13}C NMR spectrum and a multiplicity-edited HSQC experiment accounted for 33 protons connected to 17 carbons. The remaining nine non-protonated carbon centres were comprised of seven olefinic/aromatic carbons (δ_{C} 149.5; 146.6; 131.5; 127.6; 127.4; 112.3; 110.9) and two quaternary carbons (δ_{C} 42.0; 40.0). The absence of one proton resonance in the HSQC experiment indicated this to be exchangeable.

Figure 4.7. Experimental and calculated mass spectra for callophycol C (**227**).

Structure elucidation of callophycol C (**227**) was executed in a successive manner beginning with the aromatic proton resonances. Two aromatic proton doublets, CH-3 (δ_{H} 7.39; δ_{C} 131.2) and CH-5 (δ_{H} 7.12; δ_{C} 132.2) exhibited small ^1H – ^1H scalar

coupling constants (2.3 Hz) suggesting a 1,2,4,6-tetrasubstituted benzene moiety with a *meta*-relationship between the two protons. Reciprocal COSY correlations were observed between each proton and additional correlations were present between H-5 and H₂-7 (δ_{H} 2.75) (long-range), and H₂-7 and H-8 (δ_{H} 2.16). The non-protonated carbon C-1 (δ_{C} 149.5) was designated to the *meta*-position relative to each aromatic proton on the basis of strong three-bond HMBC correlations from both H-3 and H-5 to C-1 and to each other's carbon. The deshielded chemical shift of C-1, combined with HMBC correlations from the exchangeable proton 1-OH (δ_{H} 5.75) to C-1, C-2 (δ_{C} 110.9) and C-6 (δ_{C} 131.5), indicated substitution of this carbon by a hydroxyl group. The placement of C-2 and C-6 to the appropriate *ortho*-positions relative to C-1 was established by the observation of a weak two-bond HMBC correlation from H-3 to C-2 and also HMBC correlations from H₂-7 to C-1, C-5 and C-6. The latter set of HMBC correlations placed the attachment point of CH₂-7 at C-6, establishing an aromatic ring. Additionally, an HMBC correlation was displayed from H-8 to C-6 (Figure 4.8).

Figure 4.8. Key COSY and HMBC correlations establishing the tetrasubstituted aromatic ring substructure of callophycol C (**227**).

Beginning from the highly deshielded proton resonances, CH₂-20 (δ_{H} 4.81, 4.61; δ_{C} 108.8), the partial structure outlined in Figure 4.8 was extended. From each proton resonance, HMBC correlations were observed to C-8 (δ_{C} 55.7), C-19

(δ_{C} 146.6) and C-21 (δ_{C} 37.9). The proton and carbon chemical shifts of CH₂-20 indicated an exomethylene (methylidene) motif that was confirmed by the HMBC correlation to the non-protonated C-19 carbon, establishing the internal segment of the olefinic bond. COSY correlations were observed between H₂-20 and the resonances of both H-8 and H₂-21 (δ_{H} 2.39, 2.00) and could thus be identified as long-range allylic coupling. Additional COSY correlations between H₂-21 and H₂-22 (δ_{H} 1.81, 1.48), and H₂-22 and H-23 (δ_{H} 1.45) led to further extensions of this segment, supported by HMBC correlations from H₂-21 to C-8, C-20, and C-23 (δ_{C} 50.2), H₂-22 to C-19 and C-23 and H-23 to C-21 and C-22 (Figure 4.9).

Figure 4.9. Key COSY and HMBC correlations establishing the extended substructure of callophycol C (**227**).

The substructure in Figure 4.9 was further extended to establish a cyclohexane moiety. Four HMBC correlations were observed from the proton resonance of the quaternary methyl singlet H₃-24 (δ_{H} 0.87) to C-8, C-9 (δ_{C} 40.0), C-10 (δ_{C} 40.3) and C-23 with the non-protonated carbon C-9 being its attachment point. These observations established the link between C-8 and C-23 through C-9, giving rise to a cyclohexane ring. HMBC correlations observed from H-8, H₂-10 (δ_{H} 1.88, 1.36) and H-23 to C-24 (δ_{C} 15.3) confirmed this connectivity (Figure 4.10).

Figure 4.10. Key COSY and HMBC correlations establishing a cyclohexane moiety in callophycol C (**227**).

Methylene H₂-10 was further connected to a methylene and a methine through observed COSY correlations between H₂-10 and H₂-11 (δ_{H} 2.26, 2.19) and H₂-11 and H-12 (δ_{H} 4.24). HMBC correlations observed from H₂-10 to C-11 (δ_{C} 31.3) and C-12 (δ_{C} 63.8) and from H-12 to C-10 and C-11 validated this connectivity (Figure 4.11).

Figure 4.11. Key COSY and HMBC correlations extending the cyclohexane substructure of callophycol C (**227**).

With six double bond equivalents established, two more were left to be determined as required by the molecular formula. Of those yet to be assigned, two olefinic carbons could account for only one of the two remaining double bond equivalents, a consequence of which implied the presence of a third ring within the structure. Evidence to support this was presented by HMBC correlations observed from H-23 to C-12 and C-13 (δ_{C} 42.0) giving rise to a decalin ring system. The methyl singlet H₃-25 (δ_{H} 0.97) displayed HMBC correlations to C-12, C-13, C-14 (δ_{C} 37.8) and

C-23, indicating its attachment to C-13 and supporting the elucidated decalin ring system (Figure 4.12).

Figure 4.12. Key HMBC correlations establishing the decalin ring system in callophycol C (**227**).

With the majority of carbons required by the molecular formula having been assigned within the structure, the assignment of two olefinic methyl singlets, two olefinic carbons and one methylene remained yet to be determined. A *gem*-dimethyl moiety was established based on the HMBC correlations from H₃-18 (δ_{H} 1.81) and H₃-26 (δ_{H} 1.78) to each other's carbon and the shared correlations to C-16 (δ_{C} 127.6) and C-17 (δ_{C} 127.4) (Figure 4.13). The near identical chemical shifts of the olefinic carbons caused the appearance of a single correlation in the HMBC experiment. Visualisation of this region at higher resolution generated by a band-selective HMBC acquired over the bandwidth δ_{C} 105–155 confirmed that the methyls were indeed correlating to both olefinic carbons (Figure 4.14). COSY correlations observed between H₂-14 (δ_{H} 1.69) and H₂-15 (δ_{H} 2.45, 2.12), in addition to HMBC correlations from H₂-15 to C-16 and C-17, provided the connection between the *gem*-dimethyl and decalin ring motifs. Finally, the halogens were left to be assigned: the bromines were assigned to C-2, C-4, and C-12 on the basis of almost identical carbon chemical shifts with **214** and **215**,¹⁸¹ which subsequently left chlorine to be positioned on the non-protonated olefinic C-16 carbon. The non-protonated chlorinated carbon chemical shift was in agreement with literature examples.¹⁸⁷ Thus, these assignments gave rise to the planar structure of **227** (Figure 4.13).

Figure 4.13. Key COSY and HMBC correlations establishing the planar structure of callophycol C (**227**).

Figure 4.14. (a) HMBC and (b) band-selective HMBC correlations from H₃-18 and H₃-26 to C-16 and C-17.

4.4.1 Relative Configuration

The congruent proton and carbon chemical shifts of the decalin ring systems in callophycols A–C (**214**, **215** and **227**, respectively, see Table 4.1) suggest that **227** has the same relative configuration. NOE correlations in a 1D NOE experiment were observed from H₃-24 to H₂-7, H₂-10a, H₂-11a, H₂-22b and H₃-25 and from H₃-25 to H₂-11a, H₂-22b and H₃-24 implying these to be on the same face of the bicyclic ring system. These assignments were indirectly supported through correlations observed on the opposite face, from H-12 to H₂-10b, H₂-11b, H₂-15a, and H-23. These observations implied a *trans*-fused decalin system (Figure 4.15)

consistent with those found in callophycols A (**214**) and B (**215**), whereby CH₃-24 and CH₃-25 assume axial configurations and the bulky bromine substituent an equatorial configuration—the proton multiplicity (doublet of doublets) and $^3J_{\text{HH}}$ coupling constants of H-12 (12.5, 4.2 Hz), which are indicative of an axial–axial and an axial–equatorial coupling,¹⁴⁰ support this assignment. Consequently, CH₂-7 and CH₂-14 were assigned to equatorial positions and the relative configuration of **227** is therefore provided as 8*R**,9*S**,12*R**,13*R**,23*S**. Full NMR data for callophycol C (**227**) is presented in Table 4.2.

Figure 4.15. 3D representation of callophycol C (**227**) with COSY correlations displaying W-coupling and selected NOE correlations. Double-headed arrows indicate reciprocal NOE correlations.

4.4.2 Tandem Mass Spectrometry

Fragmentation of **227** using tandem mass spectrometry (MSMS) afforded two major and one weak fragment from the parent ion. One of the major fragments was

observed at m/z 553.0520 and was attributed to an $[M - H - HBr]^-$ ion. The second major fragment ion observed was at m/z 78.9193 and was attributed to a bromide ion. The weakest fragment was observed at m/z 261.8617 and was attributed to the dibrominated phenol through an $[M - H - C_{19}H_{29}BrCl]^-$ ion. Although the MSMS fragments were not greatly informative towards verifying the proposed structure of **227**, the observation of fragments due to the loss of H-X is not unusual due to the weaker C-X bond relative to the C-C bond.

Table 4.1. Chemical Shift Differences Between the Decalin Ring Systems of Callophycols A (**214**),¹⁸¹ B (**215**)¹⁸¹ and C (**227**) ($CDCl_3$).

Pos.	214		215		227		Difference			
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	$\Delta\delta$ 214-227		$\Delta\delta$ 215-227	
	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	δ_C	δ_H	δ_C	δ_H
8	55.4	2.14	55.4	2.14	55.7	2.16	-0.3	-0.02	-0.3	-0.02
9	39.9		39.9		40.0		-0.1		-0.1	
10a	40.1	1.87	40.1	1.87	40.3	1.88	0.2	0.01	-0.2	-0.01
10b		1.35		1.37		1.36		-0.01		-0.01
11a		2.24		2.25		2.26		-0.02		-0.01
11b	31.2	2.18	31.1	2.19	31.3	2.19	0.1	-0.01	-0.2	0
12	63.2	4.15	63.8	4.21	63.8	4.24	-0.6	-0.09	0	-0.03
13	42.0		42.1		42.0		0		0.1	
19	146.3		146.4		146.6		-0.3		-0.2	
21a		2.35		2.35		2.39		-0.04		-0.04
21b	37.7	1.95	37.8	1.96	37.9	2.00	-0.2	-0.05	-0.1	-0.04
22a		1.67		1.75		1.81		-0.14		-0.06
22b	25.2	1.48	25.0	1.47	25.2	1.48	0	0.00	-0.2	-0.01
23	50.7	1.43	50.1	1.39	50.2	1.45	0.5	-0.02	-0.1	-0.06
24	15.1	0.86	15.2	0.86	15.3	0.87	-0.2	-0.01	-0.1	-0.01
25	19.8	0.99	19.7	0.99	19.6	0.97	0.2	0.02	0.1	0.02

Table 4.2. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Callophycol C (**227**) (CDCl_3).

Pos.	^{13}C		^1H		J (Hz)	COSY	HMBC (^1H to ^{13}C)	NOE [†]
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)				
1	149.5	C						
2	110.9	C						
3	131.2	CH	172	7.39	2.3	5	1,2,4,5	
4	112.3	C						
5	132.2	CH	165	7.12	2.3	3,7	1,2(w),3,4,7	
6	131.5	C						
7	24.6	CH ₂	128	2.75	6.8	5,8	1,2(w),5,6,8,9,19	5,8,10a,10b(w),20b,24
8	55.7	CH	126	2.16		7,20a,20b	6,7,9,13(w),19,20,21,23,24	
9	40.0	C						
10a	40.3	CH ₂	129	1.88	13.4,3.5	10b,11a,11b	9,11(w),12,23,24(w)	
10b			126	1.36	13.3,3.8	10a,11a,11b,24(w)	8(w),9,11,12(w),22(w),23,24	
11a	31.3	CH ₂	129	2.26	13.1,3.4	10a,10b,11b,12	9,12,13(w)	
11b			130	2.19	3.7	10a,10b,11a,12	9,12	
12	63.8	CH	148	4.24	12.5,4.2	11a,11b	10(w),11,13,14,25	10b,11b,15a,23
13	42.0	C						
14	37.8 [†]	CH ₂	128	1.69	9.3,7.8	15a,15b	12,13,15,16,23,25	
15a	29.3	CH ₂	128	2.45	13.4,8.6	14,15b	14,16	
15b			126	2.12	13.5,8.1	14,15a	16,17	
16	127.6 [†]	C						
17	127.4 [†]	C						
18	22.0	CH ₃	127	1.81 [†]			16,17,26	
19	146.6	C						
20a	108.8	CH ₂	157	4.81		8,20b,21a(w),21b	8,19(w),21	20b,21a
20b			155	4.61		8,20a,21b	8,19(w),21	5,7,20a,24(w)
21a			129	2.39	13.2,4.8,3.3	20a(w),21b,22a,22b	8,19,20,22(w),23	
21b	37.9 [†]	CH ₂	126	2.00	*	20a,20b,21a,22a,22b	19,20,22,23(w)	
22a	25.2	CH ₂	128	1.81 [†]	*	21a,21b,22b,23	19,21,23(w)	
22b			127	1.48	12.3,4.2	21a,21b,22b,23	23	
23	50.2	CH	121	1.45	11.2	22a,22b	8(w),9,12(w),13,21,22,24,25	
24	15.3	CH ₃	126	0.87		10b(w)	8,9,10,23	7,10a,11a,20b(w),22b,25
25	19.6	CH ₃	128	0.97			12,13,14,23	11a,14,22b,24
26	20.3	CH ₃	127	1.78			16,17,18	15a,15b(w),23
1-OH				5.75		br s	1,2,6	

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[†]Assignments interchangeable
[‡]Selected resonances
^{*}Undetermined
(w)Weak

4.5 Iodocallophycol E

Iodocallophycol E (**228**) was isolated as a white amorphous solid. A prominent feature of the ^1H NMR spectrum was the chemical shifts of the aromatic resonances that were all upfield of the residual CHCl_3 peak—all but three of the known *Callophycus* meroditerpenes possess an aromatic ring with proton chemical shifts spanning above and below δ_{H} 7.26. Most unusual however, was the presence of what initially appeared to be a phenolic exchangeable proton resonance; a broad singlet at δ_{H} 5.75. When the HSQC data was examined, this proton was surprisingly discovered to be connected to a carbon at δ_{C} 75.7 with a $^1J_{\text{CH}}$ of 192 Hz, suggesting this centre to be sp^2 -hybridised; together, these data were very odd and enticing. HRESIMS data alluded to the most likely cause of the peculiar NMR data; iodine.

A molecular formula of $\text{C}_{26}\text{H}_{34}\text{Br}_3\text{IO}$ was indicated by the observation of a deprotonated molecule at m/z 724.9131 in negative-ion mode HRESIMS, which required eight double bond equivalents. A characteristic ‘quartet’ composed of an approximate 1:3:3:1 ratio of the $[\text{M} - \text{H}]^-$ to $[\text{M} - \text{H} + 6]^-$ ions supported the presence of three bromine atoms (Figure 4.16). Due to its monoisotopic nature, iodine, unlike bromine and chlorine, lacks an isotopic distribution pattern in a mass spectrum, making its detection less obvious. However, the increased mass of the parent ion and although extremely weak, the observation of an in-source fragment ion at m/z 126.9052 both signified iodine, providing strong support for its presence (Figure 4.16). All 26 carbons were observed in the ^{13}C NMR spectrum, 19 of which were protonated by a total of 33 out of the 34 protons. The seven non-protonated carbons were attributed to five aromatic/olefinic centres (δ_{C} 152.3, 146.7, 145.3, 129.5 and 113.1) and two quaternary carbons (δ_{C} 44.1 and 41.9). The sole exchangeable proton was observed as a broad singlet at δ_{H} 4.87 in the ^1H NMR spectrum.

Beginning with the aromatic proton resonances, CH-2' (δ_{H} 7.10; δ_{C} 132.8), CH-5' (δ_{H} 6.59; δ_{C} 117.0) and CH-6' (δ_{H} 7.14; δ_{C} 130.0), a trisubstituted benzene ring

Figure 4.16. Experimental and calculated mass spectra for iodocallophycol E (**228**). The iodide ion is observed in the experimental spectrum.

was elucidated. The multiplicities and magnitude of the $^nJ_{\text{HH}}$ coupling constants established an *ortho*- and *meta*-relationship between H-5'/H-6' and H-2'/H-6', respectively, supported by COSY correlations between H-6' and both H-2' and H-5'. Additional COSY correlations were displayed between H-2' and H₂-1 (δ_{H} 3.03, 2.75) (long-range) and H₂-1 and H-2 (δ_{H} 2.64). Strong three-bond HMBC correlations were observed from both H-2' and H-6' to each other's carbon and to C-4' (δ_{C} 152.3), and from H-5' to the non-protonated carbons C-1' (δ_{C} 113.1) and C-3' (δ_{C} 129.5). The comparable carbon chemical shift of C-4' to the corresponding carbon in **227** and observed HMBC correlations from the exchangeable proton 4'-OH (δ_{H} 4.87) to C-3', C-4' and C-5' indicated the phenolic functionality was present in **228** with the hydroxyl positioned at this carbon. HMBC correlations from H₂-1 to C-2', C-3' and C-4' established its attachment point at C-3', which was confirmed by the HMBC correlation from H-2 to C-3'. Thus, C-1' was placed at the *ortho*-position relative to both H-2' and H-6' (Figure 4.17).

Elucidation of the second fragment was initiated from the methylenide, CH₂-18 (δ_{H} 4.94, 4.78; δ_{C} 109.8). Each proton resonance displayed HMBC correlations to the sp³-hybridised carbons C-10 (δ_{C} 53.6) and C-12 (δ_{C} 37.0) and to the non-protonated olefinic carbon C-11 (δ_{C} 145.3) establishing a double bond between C-11–C-18 and indicating the connectivity of C-11 to C-10 and C-12. This was supported by COSY correlations (allylic coupling) between H₂-18 and H-10 (δ_{H} 1.64) and H₂-12 (δ_{H} 2.34, 2.02). Additional COSY correlations between H-10 and H₂-9

Figure 4.17. Key COSY and HMBC correlations establishing the aromatic substructure of iodocallophycol E (**228**).

(δ_{H} 1.61, 1.56), H₂-9 and H₂-8 (δ_{H} 1.83, 1.20), H₂-12 and H₂-13 (δ_{H} 2.24, 2.02) and between H₂-13 and H-14 (δ_{H} 4.09) extended the substructure. This connectivity was supported by HMBC correlations observed from H₂-8 to C-10, H₂-9 to C-10 and C-11, H₂-12 to C-10, C-13 (δ_{C} 36.0) and C-14 (δ_{C} 67.0), H₂-13 to C-11, C-12 and C-14 and H-14 to C-12 and C-13 (Figure 4.18).

Figure 4.18. Key COSY and HMBC correlations establishing substructure two of iodocallophycol E (**228**).

The substructure outlined in Figure 4.18 was further extended through HMBC correlations from the methyl singlets, CH₃-19 (δ_{H} 1.13; δ_{C} 28.7) and CH₃-20 (δ_{H} 0.89; δ_{C} 17.5). Together the methyls formed a *gem*-dimethyl as evidenced by

HMBC correlations from the proton resonances of each methyl to the other's carbon, C-10, C-14 and C-15 (δ_C 41.9), establishing a link between C-10 and C-14 through C-15, to give rise to a cyclohexane moiety (Figure 4.19).

Figure 4.19. Key COSY and HMBC correlations establishing the cyclohexane substructure of iodocallophycol E (**228**).

Evidence connecting the aromatic ring (Figure 4.17) and cyclohexane (Figure 4.19) substructures was provided by the methyl singlet CH₃-17 (δ_H 1.06; δ_C 19.7). Four HMBC correlations were observed from H₃-17 to C-2, C-6 (δ_C 61.6), C-7 (δ_C 44.3), C-8, indicating its attachment to the non-protonated carbon C-7 and establishing the link between C-2 and C-8 through C-7 (Figure 4.20).

Figure 4.20. Key COSY and HMBC correlations establishing the link between the aromatic and cyclohexane substructures of iodocallophycol E (**228**).

Two double bond equivalents, four carbons, five protons, and the halogens were yet to be accounted for. The presence of the unassigned olefinic carbon C-3 (δ_C 146.7) accounted for one of the two double bond equivalents indicating the other had to be

satisfied by another ring. Sequential COSY correlations from H-6 (δ_{H} 4.41) through H₂-5 (δ_{H} 2.24, 2.12) to H₂-4 (δ_{H} 2.81, 2.18), and HMBC correlations from H-6 to C-4 (δ_{C} 35.0) and C-5 (δ_{C} 33.4) and H-5 to C-7, C-4 and C-6 established the connectivity between these centres. Three-bond HMBC correlations from H₂-1 and H₂-5 to C-3 placed its carbon between C-2 and C-4 forming a second cyclohexane motif. While the carbon chemical shift of C-3 was clearly indicative of a double bond, there was no obvious olefinic partner that remained (*i.e.* $> \delta_{\text{H}}$ 4.0 or $> \delta_{\text{C}}$ 100). Initially, the proton and carbon chemical shifts of the terminal end of the double bond were assumed to be identical to, and overlapping with, those of CH₂-18. However, this scenario would have presented too many carbons. The only candidate yet to be assigned was that of the unusual CH-16 (δ_{H} 5.75; δ_{C} 75.7) whose proton chemical shift was within the typical range for an alkenyl proton but the carbon chemical shift was not.¹⁴⁰ However, the magnitude of the $^1J_{\text{CH}}$ was measured at 192 Hz and was only consistent with an sp²-hybridised carbon,¹⁴⁰ giving rise to a terminal vinyl halide. Specifically, this combination of proton and carbon chemical shifts was consistent with those observed for the vinyl iodide methine in the structures of **213** and **216–219** only very recently reported.⁴⁴ Additional support towards the C-3–C-16 connectivity was obtained through HMBC correlations from H-16 to C-2, C-3 and C-4 and both H-2 and H₂-4 to C-16. Long-range allylic coupling between H-16 and H-2 was also observed. With all protons and carbons assigned within the structure, the placement of the halogens was determined based on chemical shift arguments. Iodine was placed at C-16 on the basis of similar proton and carbon chemical shifts to that of the iodinated *Callophycus* meroditerpenes.⁴⁴ C-1', CH-6 and CH-14 were substituted by bromine due to their comparable proton and carbon chemical shifts to **227**. With all the elements indicated by the molecular formula assigned, the planar structure of iodocallophycol E (**228**) is proposed (Figure 4.21).

Figure 4.21. Key COSY and HMBC correlations establishing the planar structure of iodocallophyscol E (**228**).

4.5.1 Relative Configuration within each Cyclohexane System

NOE data was obtained from a 1D NOE experiment and was used to establish the *E*-double bond geometry of $\Delta^{3,16}$ through an observed correlation between H-16 and H₂-1a. Reciprocal NOE correlations displayed between H-2 and H-6 in addition to NOE correlations from both of these protons to H₂-9 indicated these protons to be on the same face of the cyclohexane ring. H-5b and H₂-1 showed NOE correlations to H₃-17, suggesting these to be on the opposite face of the ring relative to H-2, H-6 and H₂-9. On the second cyclohexane, NOE correlations from H-14 to H-10, H-12b, H-13a and H₃-19, pointed towards these protons being on the same face, which was indirectly supported by an NOE correlation from H₃-20 to H-13b on the opposite face (Figure 4.22). These observations are consistent with those reported for **213** and **216–219**,⁴⁴ suggesting that the same relative configuration exists within each cyclohexane system of **228**, with the large bulky groups such as the aromatic ring, bromines and the ethylene bridge (containing the other cyclohexane ring) assuming equatorial positions (Figure 4.22). H-6 and H-14 both appear as a doublet of doublets with large (axial–axial) and small (axial–equatorial) coupling constants providing evidence of both bromines occupying equatorial positions.

Figure 4.22. 3D representation showing key NOE correlations establishing the relative configurations within each cyclohexane ring of iodocallophycol E (**228**). Double-headed arrows indicate a reciprocal NOE correlation.

4.5.2 Relative Configuration between the Cyclohexane Systems

With the relative configuration within each cyclohexane system established as identical to those reported in the literature for **213**, **216–219**,⁴⁴ it seemed likely that the relative configurations between the ring systems would also be the same. However, analysis of $^3J_{\text{HH}}$ coupling constants and NOE correlation data suggested that this was not the case. The multiplicities of H-8a (*td*, 12.9, 4.8 Hz), H-8b (*ddd*, 12.9, 12.1, 4.5 Hz) and H-10 (*d*, 10.7 Hz) suggested the molecule to be conformationally restricted and predominantly one conformation. The combination of large and small $^3J_{\text{HH}}$ coupling constants observed for each proton resonance of H₂-8 suggested an antiperiplanar conformation across C-8/C-9 with approximate dihedral angles of 180° and 60° between the *vicinally*-coupled protons. The apparent ‘doublet’ multiplicity of H-10 indicated 0 Hz (or close to) coupling to one proton of H₂-9 and a corresponding dihedral angle near 90°.† This further signified a dihedral angle close to 150° with the other proton of H₂-9, which was supported by the large coupling constant of 10.7 Hz. Visualised through C-10 across the C-9/C-10 bond, four conformations of the possible two configurations at C-10 could account for these observations (Figure 4.23). Further analysis of the NOE data was required to narrow

†Estimated using the vicinal Karplus correlation.¹⁴⁰

the possibilities to one.

Figure 4.23. Possible configurations and conformations (A, D, E, and H) deduced from the H-10 scalar coupling constant in iodocallophycol E (**228**). Options B, C, F and G could be discounted as possibilities based on the expected coupling constants.

NOE correlations were observed from H-2, H₃-19 and H₃-20 to H-9b and from H₃-19 to H-2' indicating that the *gem*-dimethyls were oriented towards the aromatic ring. An NOE correlation from H-8a to H-18b placed these two protons in close proximity to each other and indicated the methyldene moiety to be on the opposite side of the ethylene bridge, relative to the aromatic ring (and *gem*-dimethyls); an NOE correlation between H-1b and H-8b supported this assignment. These observations eliminated options 'A', 'D' and 'E' illustrated in Figure 4.23, indicating that option 'H' depicts the configuration at C-10 and preferred conformation across the C-9/C-10 bond. Additional evidence for this was provided by an NOE correlation observed from H-8b to H-10. These results suggest that the relative configurations between the two cyclohexane rings of **228** are different to those reported for **213** and **216–219** and therefore, the relative configuration of **228** is tentatively assigned as drawn in Figure 4.24. Key NOE correlations that established the relative configuration between the cyclohexane ring systems is also provided in Figure 4.24.

Finally, before a configurational assignment for **228** is proposed, International Union of Pure and Applied Chemistry (IUPAC) recommendations must be acknowledged. The IUPAC recommendation for relative configuration is that *R*^{*} is designated to

Table 4.3. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Iodocallophycol E (**228**) (CDCl_3).

Pos.	^{13}C		^1H			COSY	HMBC (^1H to ^{13}C)	NOE ‡
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1a	27.0	CH_2		3.03	dd	14.8, 11.8	2,3,7(w),2',3',4'	1b,16,17
1b				2.75	dd	14.8, 2.7	1a,2,2'	1a,8b,17,2'
2	51.8	CH		2.64	dd	11.8, 2.3	1a,1b,16	6,9b,16,2'
3	146.7	C						
4a	35.0	CH_2		2.81	ddd	12.6, 4.8, 3.5	4b,5a,5b	5a,5b
4b				2.18	dd	13.5, 4.1	4a,5a,5b	
5a	33.4	CH_2		2.24 †	m		4a,4b,5b,6	
5b				2.12	ddd	12.8, 8.6, 4.2	4a,4b,5a,6	17
6	61.6	CH	150	4.41	dd	12.8, 4.1	5a,5b	2,4b,5a,9a,9b
7	44.3	C						
8a	38.5	CH_2		1.83	td	12.9, 4.8	8b,9a,9b	8b,17,18b
8b				1.20	ddd	12.9, 12.1, 4.5	8a,9a,9b	1b,8a,10,17
9a	19.6 †	CH_2		1.61	*		8a,8b,9b	
9b				1.56	*		8a,8b,9a,10	
10	53.6	CH	129	1.64	d	10.7	8,9,11,14,15,18,19,20	
11	145.3	C						
12a	37.0	CH_2		2.34	dd	8.6, 3.5	12b,13a,13b	
12b				2.02 †	m		12a,13a,18a,18b	
13a	36.0	CH_2		2.24 †	m		12a,12b,13b,14	
13b				2.02 †	m		12a,13a,14	
14	67.0	CH	150	4.09	dd	10.4, 4.4	13a,13b	10,12b,13a,19
15	41.9	C						
16	75.7	CH	192	5.75	br s		2,3,4	1a,2
17	19.7 †	CH_3	126	1.06	s		2,6,7,8	
18a	109.8	CH_2	156	4.94	br s		10,11,12	
18b			156	4.78	br s		10,11,12	
19	28.7	CH_3	126	1.13	s		10,14,15,20	9b,10,14,20,2'
20	17.5	CH_3	126	0.89	s		10,14,15,19	9b,13b,19
1'	113.1	C						
2'	132.8	CH	165	7.10	d	2.5	1,1',4',6'	
3'	129.5	C						
4'	152.3	C						
4'-OH								
5'	117.0	CH	158	4.87	br s		3',4',5'	
6'	130.0	CH	167	6.59	d	8.5	1',3',4'	
				7.14	dd	8.5, 2.5	1',2',4'	

228

† Assignment interchangeable
 ‡ Selected resonances
 * Undetermined
(w)Weak

4.6 Iodocallophycol F

Iodocallophycol F (**229**) was isolated as a white amorphous solid. Negative-ion mode HRESIMS data provided a molecular formula of $C_{26}H_{33}Br_2IO$ (m/z 644.9880, $[M - H]^-$) indicating a difference of one additional double bond equivalent and the loss of one hydrogen and bromine from iodocallophycol E (**228**). A detailed analysis of the NMR data revealed close similarities to **228** and established that the *p*-bromophenol and bromo-(iodomethylene)cyclohexane moieties were conserved in **229**. Further examination of the NMR data signified that **229** contained a disubstituted olefin—from the *gem*-dimethyls H₃-19 (δ_H 0.96) and H₃-20 (δ_H 0.95), a reciprocal HMBC correlation was observed to each other’s carbon and additionally, each shared HMBC correlations to C-10 (δ_C 53.3), quaternary C-15 (δ_C 37.2) and the olefinic carbon C-14 (δ_C 136.5) establishing that C-15 served as the attachment point. Extensions to this segment were initiated through COSY correlations between H-14 (δ_H 5.34) and H-13 (δ_H 5.50), and H-13 and H₂-12 (δ_H 2.80, 2.56). HMBC correlations from the methylenic protons H₂-18 (δ_H 4.86, 4.69) to C-10, the non-protonated olefinic carbon C-11 (δ_C 145.6) and C-12 (δ_C 31.8) provided evidence for a link between C-10 and C-12 through C-11 establishing a methylene-cyclohexene motif. As with **228**, an ethylene bridge was found to connect the two 6-membered ring systems.

NOE data was comparable to that found in **228** leading to an identical *E*-geometry of the vinyl iodide and the tentative assignment of relative configuration of 2*S**,6*S**,7*S**,10*S** for **229**. Full NMR data is outlined in Table 4.4.

Table 4.4. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Iodocallophycol F (**229**) (CDCl_3).

Pos.	^{13}C		^1H			COSY	HMBC (^1H to ^{13}C)	NOE [†]
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult	J (Hz)		
1a								
1b	26.9	CH_2	124	3.00	dd	14.8, 11.2	2,3,2',3',4'	1b,16,17,2'
2	51.6	CH	124	2.74	dd	14.8, 3.2	2,3,2',3',4'	1a,8b,17,2'
3	147.1	C		2.66	dd	10.9, 2.7	1,3,4,6,7,16,3'	6,9a,2'
4a				2.80 [†]	m		4b,5a,5b,6(w)	4b,5a
4b	36.1	CH_2	*	2.11	m		4a,5a	
5a	33.6	CH_2	135	2.22	ddd	11.8, 8.4, 4.4	3,6,7	
5b			135	2.08	m		4a,5a,6	
6	62.0	CH	150	4.37	dd	9.5, 4.3	2(w),5,7(w),8,17	2,4b,5a,9a,9b
7	44.3	C					3,6	
8a				1.60	td	12.9, 4.1	2,6,7	
8b	37.5	CH_2	125	1.21	td	13.1, 3.7	2,6,7,9,17	
9a	19.4 [†]	CH_2	125	1.40	tt	13.1, 3.3	10,11,15	
9b			125	1.30	qd	12.2, 4.6	10,11,15	
10	53.3	CH	127	1.70	dd	11.2, 3.2	9,12,14,15,18,19	8b,18b,19
11	145.6	C						
12a				2.80 [†]	m		12b,13,14,18a,18b,	
12b	31.8	CH_2	124	2.56	d	19.7	12a,13,14,18a	12b,13
13	123.5	CH	165	5.50	dt	9.9, 3.4	12a,12b,14	
14	136.5	CH	161	5.34	d	9.9	10,12a,12b,13	
15	37.2	C						
16	75.4	CH	192	5.85	br s		2	1a,2
17	19.3 [†]	CH_3	126	1.00	s		2,6,7,8	1b,5b,8a
18a				4.86	br s		12a,12b,18b	12b,18b
18b	110.3	CH_2	156	4.69	br s		10,11,12	
19	30.8	CH_3	126	0.96 [†]	s		10,11(w),12	10,18a
20	25.9	CH_3	126	0.95 [†]	s		10,14,15,20	9a,10,14
1'	113.2	C					10,14,15,19	9a,14
2'	133.0	CH	162	7.12	d	2.3	1,1',4',6'	
3'	129.7	C						
4'	152.3	C						
5'	117.1	CH	158	6.60	d	8.4	1',3',4'	
6'	130.0	CH	167	7.14	dd	8.4, 2.5	1',2',4'	

229

[†]Assignment interchangeable
[‡]Selected resonances
 *Undetermined
 (w)Weak

4.7 Iodocallophycoic Acid B

Iodocallophycoic acid B (**230**), which was isolated as a white amorphous solid, dissolved readily in CDCl_3 , however, broad signals were observed in the NMR spectra. When the sample was dissolved in CD_3OD all the peaks resolved to provide a spectrum like that of previously isolated callophycols. A singlet peak at δ_{H} 6.00, δ_{C} 75.3 indicated another vinyl iodide-containing metabolite.

Negative-ion mode HRESIMS produced a deprotonated molecule at m/z 611.0668 consistent with the parent molecular formula $\text{C}_{27}\text{H}_{34}\text{BrIO}_3$ for which ten double bond equivalents were required—one more than **229** and with the addition of a carboxyl group. The multiplicity-edited HSQC established that 32 protons were connected to 19 carbons indicating the presence of two exchangeable protons. When acquired using default parameters, only 25 out of 27 carbon resonances were observed and some appeared extremely weak in the ^{13}C NMR spectrum. Also, the HMBC experiment did not provide the chemical shift of one out of the two missing carbons. To overcome this issue, the ^{13}C acquisition parameters were altered and this is discussed in more detail in Section 4.10.

An analysis of the NMR data of **230** established the presence of the methylene-cyclohexene moiety connected to the bromo-(iodomethylene)cyclohexane *via* the ethylene bridge, analogous to the structure of **229**. The structural change from **229** was found in the aromatic ring where a carboxylic acid replaced the bromine. COSY correlations were observed between H-2' (δ_{H} 7.76) and H-6' (δ_{H} 7.68) and H-5' (δ_{H} 6.78) and H-6'. The multiplicity and $^nJ_{\text{HH}}$ coupling constants indicated an *ortho*-relationship between H-5'/H-6' and a *meta*-relationship between H-2'/H-6. HMBC correlations from both H-2' and H-6' to C-4' (δ_{C} 160.1) established the *meta*-position of the hydroxyl group, consistent with the previously isolated compounds. From H-5' two HMBC correlations were observed, one to C-4' and the other, which was much stronger in magnitude, to C-3' (δ_{C} 127.8). Within the aromatic/olefinic region of the ^{13}C NMR spectrum, one carbon was clearly absent. The assignment

of the majority of aromatic/olefinic carbons within the structure left only that of C-1'. The apparent lack of an HMBC correlation to C-1' from H-5' led to the conclusion that the chemical shifts of C-1' and C-3' were overlapping making the correlation appear more intensely in the HMBC spectrum; no additional carbon resonance appeared in the specified spectral region when the ^{13}C NMR experimental parameters were altered to increase signal-to-noise (Section 4.10). As with the previous elucidations (*i.e.* **227–229**), HMBC correlations from the methylene H₂-1 (δ_{H} 3.02, 2.77) to C-2' (δ_{C} 132.9), C-3' and C-4' established the connection of the aromatic ring to the remainder of the structure. This left a carboxylic acid functional group (C-7': δ_{C} 171.8) to be assigned to the structure, which could only be placed on the non-protonated C-1' carbon.

NOE correlations were analogous to those observed for **228** and **229** leading to the assignment of an *E*-double bond geometry of the vinyl iodide and a 2*S**,6*S**,7*S**,10*S** relative configuration for iodocallophycoic acid B (**230**). NMR data is presented in Table 4.5.

Table 4.5. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Iodocallophycoic acid B (**230**) (CD_3OD).

Pos.	^{13}C		^1H			COSY	HMBC (^1H to ^{13}C)	NOE [†]
	δ (ppm)	mult	$^1J_{\text{CH}}$ (Hz)	δ (ppm)	mult			
1a	27.5	CH ₂		3.02 [†]	m	1b	2,3,7,2',3',4'	1b,16,17
1b				2.77	m	1a,2	2,3,7,2',3',4'	
2	51.1	CH		3.01 [†]	m	1b,16	1,3,4,6,7,8,16,17,3'	
3	148.8	C						
4a	38.4 [†]	CH ₂		2.80	dt	4b,5a,5b	2,3,5,6,16	
4b				2.08	td	4a,5a,5b,16	3,5,6,16	4a,6
5a	35.0	CH ₂		2.25	ddd	4a,4b,5b,6	3,6,7	4b,5b,6
5b				2.00	qd	4a,4b,5a,6	4,6	
6	63.4	CH		4.49	dd	5a,5b	2,4,5,7,8,17	2,5a,9b
7	45.5	C						
8a	38.4 [†]	CH ₂		1.67 [†]	td	8b,9a,9b	2,6,7,9	
8b				1.28	m	8a,9a,9b	6,7,9,10	
9a	20.3	CH ₂		1.56	tt	8a,8b,9b,10	8,10	2,9b,20
9b				1.35	qd	8a,8b,9a,10	8	
10	54.6	CH		1.68 [†]	d	9a,9b,14,18b	9,11,12,14,15,18	8b,18b,19,20
11	147.1	C						
12a	32.7	CH ₂		2.86	dquin	12b,13,14,18a,18b	11,13,14,18	12b
12b				2.54	ddd	12a,13,14,18a	10,11,13,14,18	12a,13,18a
13	124.3	CH		5.50	dt	12a,12b,14	11,12,15	
14	137.6	CH		5.34	d	10,12a,12b,13	10,12,13,15,16	
15	38.0	C						
16	75.3	CH	192 [§]	6.00	s	2,4b	2,3,4	1a,2
17	18.4	CH ₃		0.94	s		2,6,7,8	1a,1b,5b
18a	110.4	CH ₂		4.83	s	12a,12b,18b	10,11(w),12	12b,18b
18b				4.70	s	10,12a,18a	10,12	9b,10,18a
19	31.2	CH ₃		0.93	s		10,14,15,20	9a,10,14
20	26.3	CH ₃		0.95	s	10,14,15,19	9a,10,14	
1'	127.8 [†]	C						
2'	132.9	CH		7.76	s	6'	1,4',6'	
3'	127.8 [†]	C						
4'	160.1	C						
5'	115.5	CH		6.78	d	6'	1',3',4'	
6'	130.3	CH		7.68	d	2',5'	2',4'	
7'	171.8	C						

[†]Assignments interchangeable

[‡]Selected resonances

[§]Measured from the unsuppressed $^1J_{\text{CH}}$ in the HMBC experiment
(w)Weak

4.8 Deiodocallophycoic Acid B

Deiodocallophycoic acid B (**231**) was isolated as a white amorphous solid. Of interest in the ^1H NMR spectrum of **231** was two pairs of overlapping methylenic protons, deceptively masked as doublets. The occurrence of two methylenes on the same molecule is less common among closely related compounds reported in the literature,^{44,175–181} and further examination of the NMR data pointed towards that of a new meroditerpene. With the exception of the additional methylene, and the lack of a singlet at δ_{H} 5.75, the proton NMR spectrum resembled that of **230** (Figure 4.25).

Deiodocallophycoic acid B (**231**) formed a deprotonated molecule at m/z 485.1684 in negative-ion mode HRESIMS. A significant $[\text{M} - \text{H} + 2]^-$ indicated the presence of bromine, giving rise to the molecular formula $\text{C}_{27}\text{H}_{35}\text{BrO}_3$ for which ten double bond equivalents were required and differing from **230** by the replacement of iodine with an additional hydrogen. All COSY and HMBC correlations determined the carbon framework to be analogous to **230** and a complete methylene motif (CH_2 -16: δ_{H} 4.87, 4.73; δ_{C} 110.6) was found in **231** at the position where the vinyl iodide was observed in **230**.

The relative configuration of **231** is proposed to be $2R^*,6S^*,7S^*,10S^*$ on the basis of analogous NOE correlations to **228–230** and also the close agreement of proton and carbon chemical shifts in the cyclohexane and cyclohexene systems of **229** and **231** (Table 4.6). NMR data for **231** is presented in Table 4.7.

Figure 4.25. Comparison of the ^1H NMR spectra (600 MHz, CDCl_3) of iodocallophycoic acid B (**230**) (blue) and deiodocallophycoic acid B (**231**) (black).

Table 4.6. Chemical Shift Differences Between the Cyclohexane Ring Systems of Iodocallophycol F (**229**) and Deiodocallophycoic acid B (**231**) (CDCl_3).

Pos.	229		231		Difference $\Delta\delta$ 229 – 231	
	δ_{C} (ppm)	δ_{H} (ppm)	δ_{C} (ppm)	δ_{H} (ppm)	δ_{C} (ppm)	δ_{H} (ppm)
2	51.6	2.66	47.4	2.67	4.2	-0.01
6	62.0	4.37	63.0	4.34	-1.0	0.03
7	44.3		44.3		0	
8a	37.5	1.60	37.3	1.70	0.2	-0.10
8b		1.21		1.29		-0.08
9a	19.4	1.40	19.1	1.40	0.3	0
9b		1.30		1.30		0
10	53.3	1.70	53.3	1.67	0	0.03
15	37.2		37.1		0.1	
17	19.3	1.00	17.8	0.97	1.5	0.03
19	30.8	0.96	30.8	0.91	0	0.05
20	25.9	0.95	25.8	0.87	0.1	0.08

Table 4.7. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Deiodocallophycoic acid B (**231**) (CDCl_3).

Pos.	^{13}C		^1H		COSY	HMBC (^1H to ^{13}C)	NOE ‡
	δ (ppm)	mult	δ (ppm)	J (Hz)			
1a	26.5	CH_2	2.98	14.9, 10.2	1b, 2, 2'	3, 7, 2', 3', 4'	1b, 16b
1b			2.81	14.9, 2.1	1a, 2, 2'	3'	1a
2	47.4	CH	2.67	10.2	1a, 1b, 16a, 16b		
3	146.2	C					
4a	*	CH_2	2.35 §	7.8	4b, 5a, 5b, 6		
4b			2.07 § ‡		4a, 5a, 16a		
5a	35.4	CH_2	2.26		4a, 4b, 5b, 6		
5b			2.09 ‡		4a, 5a	3, 6	
6	63.0	CH	4.34	6.6, 4.4	4a, 5a, 5b		2, 4b, 5a, 9b
7	44.3	C					
8a	37.3	CH_2	1.70		8b, 9a, 9b	7	
8b			1.29 ‡		8a, 9a, 9b	6, 9	
9a	19.1	CH_2	1.40		8a, 8b, 9b, 10		
9b			1.30 ‡		8a, 8b, 9a, 10	10	
10	53.3	CH	1.67		9a, 9b, 14, 18b		8b, 18b, 19
11	145.9	C					
12a	31.8	CH_2	2.87	19.5	12b, 13, 14, 18a, 18b		
12b			2.57	19.5	12a, 13, 14, 18a, 18b	11, 13, 14	
13	123.6	CH	5.50	9.9, 3.4	12a, 12b, 14		
14	136.5	CH	5.32	9.9	10, 12a, 12b, 13	12	
15	37.1	C					
16a	110.6	CH_2	4.87		2, 4b, 16b	2	
16b			4.73		2, 16a		
17	17.8	CH_3	0.97			2, 6, 7, 8	
18a	110.1	CH_2	4.85		12a, 12b, 18b	10, 12	
18b			4.71		10, 12a, 12b, 18a	10, 12	
19	30.8	CH_3	0.91			10, 14, 15, 20	10, 14
20	25.8	CH_3	0.87			10, 14, 15, 19	9a, 10, 14
1'	121.8	C					
2'	132.7	CH	7.88		1a, 1b, 6'	1, 4', 6', 7'	
3'	128.0	C					
4'	158.4	C					
5'	115.4	CH	6.78	8.3	6'	1', 3'	
6'	130.0	CH	7.82	8.3	2', 5'	2', 4'	
7'	170.1	C					

‡ Assignments interchangeable
 ‡ Selected resonances
 § Tentatively assigned from the COSY experiment
 * Undetermined

4.9 Callophycoic Acid I

Callophycoic acid I (**232**) was isolated as a white amorphous solid. A prominent feature observed in the ^1H NMR spectrum of **232** was that of a highly deshielded pair of proton resonances at δ_{H} 6.08 and 5.83, neither of which matched with signals reported in any other *Callophycus* meroditerpene.^{44,175–181}

A deprotonated molecule at m/z 501.1650 was detected using HRESIMS which was suitable for the molecular formula $\text{C}_{27}\text{H}_{35}\text{BrO}_4$. An approximate 1:1 ratio of the $[\text{M} - \text{H}]^-$ and the $[\text{M} - \text{H} + 2]^-$ ions verified the presence of bromine. Ten double bond equivalents were required by the molecular formula.

Sequential COSY and HMBC correlations led to the elucidation of the trisubstituted *p*-hydroxybenzoic acid and a decalin ring system analogous to that in callophycol C (**227**). Compared with **227**, the isoprene head group presented a point of difference. In compound **232**, the deshielded methyl H_3 -16 (δ_{H} 1.88) protons correlated to the ketone carbonyl C-14 (δ_{C} 201.9), the non-protonated olefin C-15 (δ_{C} 144.2) and the methyldiene C-27 (δ_{C} 125.2). From H_2 -27 (δ_{H} 6.08, 5.83), HMBC correlations were observed to C-14, C-15 and C-16 (δ_{C} 17.7). These observations established the attachment of CH_3 -16 and CH_2 -27 to C-15 with the ketone carbonyl carbon adjacent to C-15 forming an α,β -unsaturated ketone.

Similarities in chemical shift data about the decalin ring systems of **211**, **212** and **232** (see Table 4.8) suggest that **232** contains an identical relative configuration to the former two compounds and this is supported by analogous NOE correlations. As such, the relative configuration of $6R^*,7S^*,10R^*,13R^*,23S^*$ is proposed. NMR data is provided in Table 4.9.

Table 4.8. Chemical Shift Difference Between the Decalin Ring Systems of Callophycoic acids G (**211**), ¹⁸¹H (**212**)¹⁸¹ and I (**232**) (CDCl₃).

Pos.	211		212		232		Difference			
							$\Delta\delta$ 211–232		$\Delta\delta$ 212–232	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
6	55.6	2.25	55.3	2.25	55.8	2.24	-0.2	0.01	-0.5	0.01
7	41.9		42.0		40.0		1.9		2.0	
8a		1.91		1.90		1.92		-0.01		-0.02
8b	40.2	1.38	40.1	1.40	40.1	1.39	0.1	-0.01	0	-0.01
9a		2.27		2.27		2.26		0.01		0.01
9b	31.2	2.19	31.2	2.19	31.4	2.20	-0.2	-0.01	-0.2	-0.01
10	64.0	4.32	64.0	4.33	63.5	4.20	0.5	0.12	0.5	0.13
11	41.5		41.9		41.8		0.3		0.1	
20	147.3		146.6		148.4		-1.1		-0.2	
22a		2.38		2.36		2.36		0.02		0
22b	37.7	2.03	37.7	1.99	37.7	1.98	0	0.05	0	0.01
23a		1.72		1.72		1.70		0.02		0.02
23b	25.1	1.48	25.1	1.48	25.0	1.46	0.1	0.02	0.1	0.02
24	49.8	1.54	49.9	1.54	50.2	1.44	-0.4	0.1	-0.3	0.1
25	15.0	0.87	15.0	0.87	15.2	0.90	-0.2	-0.03	-0.2	-0.03
26	19.6	0.94	19.6	0.94	19.6	1.00	0	-0.06	0	-0.06

Table 4.9. ^{13}C (150 MHz) and ^1H (600 MHz) NMR Data for Callophycoic acid I (**232**) (CDCl_3).

Pos.	$^{13}\text{C}^\dagger$		^1H		COSY	HMBC (^1H to ^{13}C)	NOE §
	δ (ppm)	mult	δ (ppm)	mult			
1	169.5	C					
2	121.4	C					
3	132.3	CH	7.83	d	5b,17	1,17,19	
4	128.1	C					
5a	23.4	CH_2	2.81 ‡	dd	6	4	
5b			2.71	dd	3,6	4,6	5a,8a,25
6	55.8	CH	2.24	m	5a,5b,21a,21b		3,24
7	40.0	C					
8a			1.92	dt	8b,9a,9b		
8b	40.1	CH_2	1.39	td	8a,9b		
9a			2.26	m	8a,8b,9b,10		
9b	31.4	CH_2	2.20	m	8a,8b,9a,10		
10	63.5	CH	4.20	dd	9a,9b		9b,13a,24
11	41.8	C					
12	34.6	CH_2	1.76	m	13a,13b		
13a			2.80 ‡	dd	12,13b		
13b	30.8	CH_2	2.37	dd	12,13a		
14	201.9	C					
15	144.2	C					
16	17.7	CH_3	1.88	s	27a,27b	14,15,27	
17	129.7	CH	7.80	dd	3,18		
18	115.1	CH	6.76	d	17	2,4	
19	158.3	C					
20	148.4 *	C					
21a	108.6	CH_2	4.83	s	6,21b,22b	6,22	
21b			4.71	s	6,21a	6,22	
22a			2.36	m	22b,23b		
22b	37.7	CH_2	1.98	m	21a,22a,23a		
23a	25.0	CH_2	1.70	m	22b,23b,24		
23b			1.46	m	23a		
24	50.2	CH	1.44	m	23a	6,10,13b	
25	15.2	CH_3	0.90	s		6,7,8,24	26
26	19.6	CH_3	1.00	s		10,11,12,24	25
27a			6.08	s	16,27b	14,16	
27b	125.2	CH_2	5.83	d	16,27a	14,16	
OH			5.45	br s			

† Assigned from the HSQC and HMBC experiments
 ‡ Assignments interchangeable
 * Tentatively assigned by comparison to the CD_3OD data
 § Determined from CD_3OD acquired data
(w)Weak

4.10 Weak resonances in the ^{13}C NMR Spectra of Callophycoic Acids

Some of the carbon signals in **230–232** were either extremely weak or absent entirely from the ^{13}C NMR spectrum (Figure 4.26). Variation in spin-lattice (longitudinal) relaxation (T_1) and enhanced signals of protonated carbons through NOE are two major factors that influence ^{13}C NMR peak intensities.¹⁴⁰ Spin-lattice relaxation occurs as the nuclei net magnetisation, following irradiation by a radio-frequency pulse to excite and flip the nuclei net magnetisation onto the xy -plane, relaxes back along the z -axis in alignment with the externally-applied magnetic field. While T_1 relaxation times are short for proton nuclei (on the order of seconds to fractions of a second), they are much longer and vary greatly for carbon nuclei, meaning that those with long T_1 times often result in only partial recovery of the signal.¹⁴⁰ As a consequence, the nuclei of non-protonated carbons, such as carbonyls, which generally have longer T_1 relaxation times and are not affected by NOE because of the lack of proton attachment, typically exhibit weaker signals in a ^{13}C NMR spectrum than those of protonated carbons.¹⁴⁰ This explanation, however, did not account for the very weak signals in the ^{13}C NMR spectrum of **230–232** that were attributed to methines, methylenes and even methyls (Figure 4.27). As may be expected, the carbonyl carbon resonances of the carboxylic acids were not observed. This was particularly problematic in the structure elucidation of iodocallophycoic acid B (**230**) because evidence of a carboxylic acid carbonyl was also lacking in the HMBC experiment. Although the functionality of a carboxylic acid was certain in **230**, as all atoms required by the molecular formula were established within the molecule except for ‘COOH’, a carbon signal needed to be observed in the ^{13}C spectrum to unequivocally prove its existence.

Signals in a ^{13}C spectrum resulting from nuclei with long T_1 times, can be amplified by increasing the relaxation delay in the pulse sequence. The longer delay allows more time for the nuclei net magnetisation to re-establish along the z -axis, which

Figure 4.26. Stacked ^{13}C NMR spectra of iodocallophycoic acid B (**230**) (blue, CD_3OD), deiodocallophycoic acid B (**231**) (black, CDCl_3) and callophycoic acid I (**232**) (red, CD_3OD) (150 MHz). Each should contain 27 resonances but many are either absent or extremely weak.

Figure 4.27. ^{13}C NMR spectrum of iodocallophycoic acid B (**230**) (CDCl_3) magnified to show the weak resonances (150 MHz).

consequently leads to more coherent magnetisation being flipped onto the xy -plane when the next 90° pulse-width is applied. As a result, when compared to a ^{13}C NMR spectrum acquired from default parameters, the same overall experimental time (at the expense of the number of iterative scans) leads to signal amplification of nuclei with long T_1 times. In an attempt to increase the weakened signals in **230** and also to observe the carbonyl carbon of the carboxylic acid, the relaxation delay was increased to 2.00 s (default: 0.01 s) and the experiment was acquired

over the same period of time as the default ^{13}C NMR experiment. Unexpectedly, instead of increasing the signals this adjustment proved to be detrimental with some of the weaker signals worsening (relative to other carbons) and thus indicated that the problem was not related to long T_1 relaxation times. This result suggested that the T_1 relaxation was occurring at a faster rate than the average carbon. To test this theory, the acquisition time was decreased from 1.60 s to 0.20 s and the overall experimental time kept the same. The shorter acquisition time meant that more scans could be acquired in the same period of time. The resulting ^{13}C NMR spectrum showed that the weaker resonances were stronger (relative to the other carbon resonances) and although very weak and broad, evidence of the carbonyl carbon appeared at δ_{C} 171.6 (Figure 4.28). To validate the carbonyl carbon as the carboxylic acid, both sets of ^{13}C NMR spectra (one with a long relaxation delay and the other with a decreased acquisition time) were acquired for deiodocallophycoic acid B (**231**). The ^{13}C NMR spectrum acquired with a shorter acquisition time provided the resonance corresponding to the carbonyl carbon (Figure 4.29) and was consistent with the carbon chemical shift (pertaining to a carboxylic acid carbonyl) observed in the HMBC experiment. Similarities in carbon chemical shifts and the weak broad signal that arose in the spectrum for **230** and **231**, supported that the carbon observed at δ_{C} 171.6 in the ^{13}C NMR spectrum of **230** indeed belonged to the carboxylic acid.

The short T_1 relaxation times can be explained by the tumbling rate of the molecule. Hydrogen bonding leads to the dimerisation of carboxylic acids in solution, thus increasing the molecule's effective size. Larger species resulting from this intermolecular association substantially decreases the tumbling rate in solution and enables more efficient T_1 relaxation, thus shortening the T_1 time of the nuclei, particularly that of the carbonyl carbon.¹⁸⁹ Reducing the acquisition time in the pulse sequence enabled more scans to be sampled in the same overall experimental time, which led to stronger ^{13}C signals of those with short T_1 times. It should be noted that for **230–232**, a combination of the short T_1 relaxation (possibly caused by dimerisation) and paucity of material were likely contributing factors to

the weaker signals observed in the ^{13}C NMR spectrum for each compound.

Figure 4.28. ^{13}C NMR spectra of iodocallophycoic acid B (**230**) acquired from default parameters (black), increased relaxation delay (2.0 s; blue) and decreased acquisition time (0.2 s; red) (150 MHz).

Figure 4.29. ^{13}C NMR spectra of deiodocallophycoic acid B (**231**) acquired from default parameters (black), increased relaxation delay (2.0 s; blue) and decreased acquisition time (0.2 s; red) (150 MHz).

4.11 Biological Activity

The samples of bromophycolide A (**181**) and T (**202**) isolated during this study, in addition to iodocallophycols E (**228**) and F (**229**) were submitted to the School of

Biological Sciences to test for cytotoxicity against the HL-60 cell line. The results showed moderate cytotoxicity for (**181**), (**202**) and (**228**) with IC₅₀ values of 5.1, 6.2 and 6.0 μ M ($n=3$ experimental repeats), respectively. At this stage, the bioassay result for **229** is inconclusive as it showed a similar level of activity to **228** in the first assay, but lacked activity in the last two assays.

4.12 Proposed Biogenesis

There are two pathways by which a meroterpene can be biosynthesised. One involves a combination of polyketide and terpene biosynthesis and the other is non-polyketide (Shikimate) combined with terpene biosynthesis. The meroditerpenes of *Callophycus* fall into the latter category.

A putative biogenesis has been proposed by Kubanek and co-workers for **181**–**226**.^{44,175–181} Coupling between geranylgeranyl pyrophosphate (GGPP) and an aromatic moiety *via* electrophilic aromatic substitution generates the meroditerpene carbon backbone present in the *Callophycus* meroditerpenes. Biogenesis of the aromatic moiety is suggested to arise through the Shikimate pathway.^{175,181} The proposed biogenesis depicts terpene cyclisation after the C–C coupling reaction with their recent isolation of 3-geranylgeranyl-4-hydroxybenzoic acid (**191**)⁴⁴ offering support towards this notion. The large structural diversity exhibited by compounds **181**–**226** indicates the presence of promiscuous terpenoid cyclisation processes and as is typical in the cyclisation of brominated terpenes,^{80,81} those containing bromine in their structure are thought to follow a bromonium ion induced terpene cyclisation.^{175,177–181} The site of bromination is consistent with sites of unsaturation in the acyclic diterpene precursor.¹⁸⁰

A common structural framework is observed for the known compounds callophycols A (**214**), B (**215**), callophycoic acids G (**211**) H (**212**) and the new compounds, callophycol C (**227**) and callophycoic acid I (**232**), suggesting a mutual precursor exists between them. The 2,4-dibromophenol functionality of **214**, **215** and **227**

is thought to arise from a decarboxylation of the benzoic acid functionality, such as that observed in **232**, during the electrophilic aromatic substitution step in the biogenesis.^{44,181} Terpene cyclisation is proposed¹⁸¹ to be initiated by the selective addition of an electrophilic bromonium ion across the $\Delta^{10,11}$ double bond[†] similarly to that previously reported in the literature by Butler and co-workers.^{80,81,83} This addition induces a cascade cyclisation of the terpene to form the brominated decalin ring system observed in **211**, **212**, **214**, **215**,¹⁸¹ **227** and **232**. Callophycol B (**215**) could be the precursor to callophycol C (**227**) through a dehydrobromination (Scheme 4.2). Callophycoic acid I (**232**) is possibly derived from callophycoic acid G (**211**) through a C-14/C-15 epoxide, followed by β -elimination of a methyl proton, then oxidation of the allylic alcohol to form the ketone (Scheme 4.2). Epoxidation is envisioned to happen either via a bromohydrin intermediate formed by nucleophilic attack of a hydroxide on a bromonium ion in a similar fashion to that proposed for the epoxide-containing bromophycolides such as **183**,¹⁸⁰ or through a biologically equivalent Prilezhaev-type (also referred to as Prileschajew) reaction[‡] with epoxidation occurring directly from the $\Delta^{14,15}$ olefin. In this proposed biogenesis, β -elimination of a methylene CH₂-13 proton is conceivable, which would generate a tertiary allylic alcohol. Such a structural motif is exhibited in the acyclic terpene portion of bromophycoic acid B (**221**), while bromophycoic acids C (**222**) and D (**223**) contain a hydroperoxide and α,β -unsaturated ketone,¹⁷⁵ respectively, lending support to the proposed biogenesis.

The newly found iodocallophycols E (**228**) and F (**229**) and iodo- and deiiodocallophycoic acid B (**230**) and (**231**, respectively) isolated in this study closely resemble callophycoic acids C–E (**207–209**, respectively), albeit without the ether linkage to the aromatic ring. The proposed biogenesis of **207–209** provides a convincing possible route to their formation,¹⁸¹ although it is tempting to suggest the cyclic ether derives from a *p*-hydroxybenzoic acid-diterpene equivalent that contains a vinyl iodide motif such as that found in **230** (Scheme 4.3).

[†]The numbering system here is for the acyclic geranylgeranyl pyrophosphate and should not be confused with that used for compounds **227–232**.

[‡]The Prilezhaev reaction involves olefin oxidation *via* reaction with a peroxy acid, typically *m*-chloroperoxybenzoic acid, to form an epoxide.¹⁹⁰

Scheme 4.2. Possible biogenesis of callophycol C (**227**) and callophycoic acid I (**232**).

Scheme 4.3. Hypothetical biogenesis of the formation of the cyclic ether functionality in callophycoic acids C–E (**207–209**, respectively) from a vinyl iodide precursor.

A putative biogenesis can be readily deduced for deiodocallophycoic acid B (**231**) *via* typical bromonium induced cyclisation (two such events would be required to form the separate cyclohexane motifs) and then dehydrobromination. From a biosynthetic perspective, the question thus appears to be, how is the iodine in **228**, **229** and **230** incorporated? Vanadium haloperoxidase (V-HPOs) enzymes are believed to be responsible for biohalogenation in marine algae. These enzymes oxidise halides in the presence of H_2O_2 , rendering a source of an electrophilic halonium ion that proceeds to react with either an organic substrate or a second equivalent of H_2O_2 (see Scheme 1.1).^{80,82} As was mentioned in Section 1.2.4, three such V-HPOs exist (*i.e.* V-ClPO, V-BrPO and V-IPO) and are named on the basis of the enzymes ability to oxidise the most electronegative halide.^{80,82}

Unlike V-BrPOs, research carried out on V-IPOs is scarce and little is known about iodination of natural products.^{85,87,191} Two pathways leading to the vinyl iodide formation in **228**, **229** and **230** can be envisioned, one incorporating iodine through an electrophilic iodonium ion (Scheme 4.4) and the other through a nucleophilic iodide (Scheme 4.5). Compound **230** could be derived from **231** by the electrophilic addition of an iodonium ion across the $\Delta^{3,16}$ olefin in a similar fashion to that observed for bromonium ion formation.^{80,81,83} This could be ring-opened to form either a halohydrin or dihalide, which would then proceed through a dehydration

or dehydrohalogenation reaction, respectively, to generate the vinyl iodide (Scheme 4.4). A hypothetical precursor containing the cyclised terpene of **231** but with a *p*-bromophenol would lead to **228** and **229**. This pathway is in agreement with that proposed in the literature.⁴⁴ The alternative pathway could lead to the iodinated compounds from a cyclised precursor, such as callophycoic acid E (**209**), through a selective anti-Markovnikov hydroiodination across the enol ether, then subsequent proton abstraction and re-establishment of the phenol moiety to form the vinyl iodide (Scheme 4.5). The implication of nucleophilic iodide in this scheme is somewhat questionable because biosynthetically, halogenation is generally believed to occur *via* an electrophilic source.^{80,82} However, this potential pathway should not be excluded as it indicates that little knowledge exists on the iodination of organic substrates in Nature.

Interestingly, this study resulted in the isolation of both macrolide and non-macrolide meroditerpenes from a single specimen of *Callophycus serratus*. The presence of both types of meroditerpenes from a single organism rules out two out of three suggestions made in the literature¹⁸¹ to explain experimental findings of only one type of compound, pointing towards environmental differences between their collections as the probable underlying cause.¹⁸¹

4.13 Concluding Remarks

The investigation of a Tongan specimen of *Callophycus serratus* has yielded six new meroditerpenes, callophycol C (**227**), iodocallophycols E (**228**) and F (**229**), iodocallophycoic acid B (**230**), deiiodocallophycoic acid B (**231**) and callophycoic acid I (**232**), bringing the total number of *Callophycus* meroditerpene isolates to 52. The isolation was guided using a combination of MS and NMR spectroscopy and was exceptionally successful from a dereplication perspective when considering the large number of metabolites reported from *C. serratus*. Taking into consideration the putative biogenesis proposed by Kubanek and co-workers, an alternative pathway is

Scheme 4.4. Possible biogenesis of iodocallophyscols E (**228**) and F (**229**) and iodo- and deiodocallophyscoic acids B (**230** and **231**, respectively).

Scheme 4.5. Alternative possible biogenesis of iodocallophycols E (**228**) and F (**229**) and iodocallophycoic acids B (**230**).

proposed that leads to the cyclisation of callophycoic acids C–E through a vinylic iodide motif precursor like that observed in **228**, **229** and **230**. Interestingly, this study is the first to result in the isolation of macrolide meroditerpenes alongside those of non-macrolide meroditerpenes suggesting that in previous literature reports, environmental, rather than genotypical differences were the probable reason behind their observations of finding one type of compound exclusive of the other. To the best of the author's knowledge, the organism under study was that of a single species identified as *Callophycus serratus*.

Chapter 5

Concluding Remarks

This study involved an extensive investigation into the halogenated secondary metabolites of the New Zealand red alga *Rhodophyllis membranacea*. From this organism, a total of 21 compounds were isolated, of which 16 were found to be new. These consisted of 11 tetra-, four penta- and one hexahalogenated indole. The most exceptional compounds isolated from this alga, were four tetrahalogenated indoles substituted with bromine, chlorine and iodine—a rare feature observed among halogenated natural products. The biohalogenation processes leading to the production of polyhalogenated indoles in this alga are assumed to be catalysed by a vanadium-dependent haloperoxidase. It is particularly intriguing that the alga would produce so many congeners, including regioisomers and hexahalogenated indoles. This could be of interest to researchers working in the field of biohalogenation.

While an extensive investigation was carried out on the polyhalogenated indoles of *R. membranacea* further work is envisioned. Although the experimental data supports the proposed structures of the tetra- and pentahalogenated indoles isolated in this study, X-ray crystallographic data would solidify the arguments put forward and should be considered in future work. At this stage, the hexahalogenated indole is simply identified as a tribromo-trichloroindole and the constitution remains unsolved. Future directions for the elucidation of this compound would primarily involve isolating more mass to crystallise the compound so that an X-ray crystallographic analysis can be carried out. More mass would also mean that ^{13}C , ^{15}N and HMBC (acquired in DMSO- d_6 to potentially observe correlations from the NH proton) NMR data could be obtained. Also, the antifungal activity exhibited by the polyhalogenated indoles deserves further consideration. Those that were isolated as pure compounds, but not included in antifungal testing, should be submitted for biological testing. This would give insight into the SAR, if any exists. Furthermore, if these tests were to be performed, it would be interesting to compare the activity of

the pure compounds with that of the mixtures of tri-, penta- and hexahalogenated indoles.

An investigation of *Callophycus serratus* resulted in the isolation of six new halogenated meroditerpenes, of which three were found to be iodinated. NMR spectroscopic data suggests that the relative configuration of four of the new compounds (**228–231**), differs from that of literature reports of closely related compounds isolated from *Callophycus*. As a result, the relative configuration of compounds **228–231** is tentatively assigned based on ^1H — ^1H scalar couplings and NOE spectroscopic data. Additional work to investigate this further could involve molecular modelling so that comparisons can be made with experimental findings. Also, any future work should attempt to determine the absolute configurations.

Overall, this work resulted in nine new iodine-containing secondary metabolites from two different species of algae. At first, this seemed to be an extraordinary result because of the ostensible rarity of iodinated marine natural products reported in the literature. However, this is not unusual at all. Considering the relative halide concentrations in seawater,³⁷ the proportion of iodinated to brominated and chlorinated metabolites, should be significantly lower than that which is actually present in the literature. Biohalogenation processes that incorporate halides into organic substrates and the higher reactivity of the iodide ion due to its enhanced oxidation potential are responsible for the greater proportion of iodinated marine natural products.⁴⁴

The success of this research is accredited to the use of both NMR and MS as complementary tools during the isolation process. Specifically, the deceptively simple NMR spectra of the polyhalogenated indoles would have undoubtedly led to the algae being discounted from this study if an exclusively NMR-guided isolation strategy was employed. It is often challenging for a natural product chemist to find new secondary metabolites from an organism that has well established chemistry, as was the case with *Callophycus*. With over forty meroditerpenes sourced from this algae and reported in the literature, the challenge of dereplication was achieved

through the use of both NMR and HRESIMS data and by careful examination and comparison of these data to those in the literature.

Chapter 6

Experimental

6.1 General Experimental Procedures

Optical rotations were recorded on a Rudolph Research analytical Autopol IV polarimeter. UV spectra were obtained on an Agilent 8453 UV-visible spectrophotometer. IR spectra were recorded on a Bruker Alpha Platinum ATR FT-IR spectrometer. A 600 MHz Varian Direct Drive spectrometer equipped with a triple resonance HCN cryogenic probe operating at 25 K was used to record all NMR spectra (600 MHz for ^1H nuclei, 150 MHz for ^{13}C nuclei, and 60 MHz for ^{15}N nuclei). The residual solvent peak was used as an internal reference for ^1H (δ_{H} 7.26, CDCl_3 ; 3.31, CD_3OD ; 2.50, $\text{DMSO}-d_6$) and ^{13}C (δ_{C} 77.16, CDCl_3 ; 49.00, CD_3OD ; 39.52, $\text{DMSO}-d_6$) chemical shifts. ^{15}N chemical shifts were internally calibrated by the spectrometer to CH_3NO_2 (δ scale, $^{15}\text{NO}_2$ δ_{N} 0). Samples were quantified by ^1H NMR spectroscopy using an internal CH_3NO_2 standard and the acquisition parameters described by West.⁹⁰ ^1H , ^{13}C , 1D NOE, TOCSY, COSY, HSQC, HMBC, band-selective HMBC and CIGAR NMR spectroscopic experiments were used throughout this research. High-resolution (ESI) mass spectrometric data were obtained with an Agilent 6530 Accurate-Mass Q-TOF LC-MS equipped with a 1260 Infinity binary pump. Low-resolution mass spectrometric data were acquired from a Shimadzu GC-2010 operating with a GCMS-QP2010 MS detector. HPLC purifications were carried out using a Rainin Dynamax SD-200 solvent delivery system with 25 mL pump heads (analytical and semi-preparative chromatography) and a Varian Prostar 335 diode array detector. Samples were submitted to Associate Professor Martyn Coles for analysis by single-crystal X-ray crystallography and the data obtained on an Agilent SuperNova diffractometer.

TLC analyses were carried out using Machery-Nagel Polygram Sil G/UV₂₅₄ plates.

Developed plates were visualised under UV light ($\lambda = 254$ nm) before analysing by dipping in 5% $\text{H}_2\text{SO}_4/\text{MeOH}$ followed by 0.1% vanillin/EtOH and heating. The mobile phase used for TLC was 5% MeOH/DCM unless otherwise stated. Normal-phase bench-top column chromatography was performed using Pure Science silica gel 60, 40-63 μm or YMC DIOL (2,3-dihydroxypropoxy-propyl-derivatised silica) 12 nm, S-50 μm . Reversed-phase bench-top column chromatography was carried out using Mitsubishi Dianon HP20 or Supelco Dianon HP20SS poly(styrene-divinylbenzene) (PSDVB). Size-exclusion column chromatography was performed using Sigma Sephadex LH20. Normal-phase HPLC was performed using Phenomenex Lichrospher DIOL (analytical: 4.0×250 mm, 5 μm ; semi-preparative: 10×250 mm, 5 μm) or Phenomenex Luna hydrophilic interaction liquid chromatography (HILIC) (analytical: 4.6×250 mm, 5 μm). Reversed-phase HPLC was carried out with Phenomenex Luna octyl-derivatised silica gel (C_8) (analytical: 4.6×250 mm, 5 μm) or Phenomenex Prodigy octadecyl-derivatised silica gel (C_{18}) (analytical: 4.6×250 mm, 5 μm ; semi-preparative: 10×250 mm, 5 μm). Analytical HPLC was performed at a flowrate of 1 mL/min and semi-preparative HPLC at 5 mL/min. All solvents used for column chromatography were of HPLC grade, and H_2O was glass distilled. Solvent mixtures are reported as % v/v unless otherwise stated.

Bioassays: Samples were submitted to the School of Biological Sciences, where they were tested for biological activity. A standard 48 h MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) cell proliferation assay was used to evaluate cytotoxic activity against the HL-60 promyelocytic leukaemia cell line ($n=1-3$ independent experiments with duplicate wells per experiment). Cells were treated with compound at various concentrations, and a dose-response generated relative to a control of untreated HL-60 cells. A bakers yeast (*Saccharomyces cerevisiae*) wild-type strain BY4741 was used to assess antifungal activity. Concentrations that inhibited growth of wild-type yeast by 50% relative to untreated controls, were determined in liquid synthetic complete media *via* measurements of cell density at A_{600} . Positive controls were peloruside A (HL-60: IC_{50} 10 ± 4 nM)

and cycloheximide (*S. cerevisiae*: IC₅₀ 40 nM).

6.2 Isolation of Compounds from PTN4_08B

PTN4_08B was collected from Vava'u, Tonga in 2009. The sample was stored at $-20\text{ }^{\circ}\text{C}$ until extraction. A voucher specimen is held at the School of Chemical and Physical Sciences.

A section (19 g, wet weight) of PTN4_08B was extracted overnight in MeOH (100 mL). The extract was filtered and set aside and the sponge re-extracted in MeOH (100 mL) overnight. The second extract was passed through PSDVB (80 mL) and then the first extract was passed through the same column. The eluents were combined, diluted with 200 mL H₂O (100% v/v) and re-cycled through the PSDVB column. The eluent was collected and diluted with 400 mL H₂O (100% v/v) and re-cycled through the column. The column was washed with H₂O (240 mL) and then eluted with 1) 30% Me₂CO/H₂O, 2) 75% Me₂CO/H₂O and 3) Me₂CO (**A1–A3**). Fraction **A2** was cyclic loaded onto HP20ss (7 mL) until a 4-fold dilution was achieved and then the column was washed with NaCl solution (150 mL, 2 M), then H₂O (600 mL) before eluting with MeOH (100 mL). Once dry, the sample seemed to flake and was consequently cyclic loaded back onto HP20ss (7 mL) and washed with more H₂O (900 mL). The column was eluted with MeOH (200 mL), which dried to form a glass-like film (**B1**). Fraction **B1** was loaded onto DIOL (10 mL) that was pre-equilibrated in DCM, and eluted with 30 mL portions of DCM, 50% EtOAc/DCM, EtOAc, 5%, 10%, 20%, 50% MeOH/EtOAc and 20% MeOH/DCM. This provided 48 fractions that were combined based on TLC to give five fractions (**C1–C5**). Further purification was carried out on fraction **C4** using C₁₈ HPLC (50% MeCN/0.1 M NH₄COOH), which yielded five fractions (**D1–D5**). Fraction **D4** (5.3 mg) was found to contain a 1:1 mixture of the sulfated steroids halistanol sulfate (**65**) and topsentinol K trisulfate (**66**).

6.3 Isolation of Compounds from PTN3_19C

PTN3_19C was collected by hand from ‘Eua, Tonga in 2009. The sample was stored at $-20\text{ }^{\circ}\text{C}$ until extraction. A voucher specimen is held at the School of Chemical and Physical Sciences.

A sample of the sponge PTN3_19C (68 g, wet weight) was sectioned and extracted twice in MeOH (300 mL) overnight. The second, followed by the first extracts were passed through PSDVB (100 mL) and the eluents were combined, then diluted with H_2O (100% v/v) and re-cycled through the column. The eluent was collected, diluted with H_2O and passed through the column again. The column was washed with H_2O and eluted with 1) 30% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$, 2) 75% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ and 3) Me_2CO (**A1–A3**). **A3** was fractionated by flash column chromatography on silica gel (10 mL) eluting with *n*-hexane, 50% DCM/*n*-hexane, DCM, 1%, 2%, 5%, 10%, and 20% MeOH/DCM, which yielded the α -exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**) (2.7 mg) and α -exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**) (13.4 mg) compounds from 50% DCM/*n*-hexane and 1% MeOH/DCM, respectively.

α -Exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (69): White amorphous solid; ^1H and ^{13}C NMR data see Table 2.1; HRESIMS (15 V) m/z (% relative intensity) 309.2793 $[\text{M} + \text{H}]^+$ (20.6), 291.2681 (23.1), 273.2569 (16.6), 263.2732 (57.0), 137.1315 (20.1), 123.1164 (40.9), 109.1012 (72.3), 97.1014 (33.0), 95.0858 (100), 83.0853 (27.3), 81.0703 (68.2), 57.0703 (28.5); HRESIMS m/z 309.2788 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{20}\text{H}_{36}\text{O}_2$, 309.2788).

α -Exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (70): White amorphous solid; ^1H and ^{13}C NMR data see Table 2.2; HRESIMS (15 V) m/z (% relative intensity) 307.2636 (100), 289.2529 (33.3), 261.2570 (67.4), 149.1321 (32.7), 135.1164 (46.7), 125.0604 (32.5), 121.1014 (29.5), 109.1007 (35.3), 107.0848 (24.8), 97.1008 (28.4), 95.0861 (54.9), 89.0594 (28.6), 85.1015 (27.0), 83.0859 (29.8), 81.0703 (50.7), 57.0703 (36.3); HRESIMS m/z 325.2729 $[\text{M} + \text{H}]^+$ (calcd

for C₂₀H₃₆O₃, 325.2737).

6.4 Isolation of Indoles from *Rhodophyllis membranacea*

Specimens of *Rhodophyllis membranacea* were collected by hand using SCUBA at a depth of 3–10 m from Moa Point, Wellington, New Zealand in January 2012 and in 2014 and stored at –20 °C until extraction. The alga was identified by Dr Joe Zuccarello at the School of Biological Sciences, Victoria University of Wellington, Wellington, where a voucher specimen (PTN4.20A) is also stored.

The alga (230 g wet weight) was extracted with MeOH (2 × 800 mL) twice overnight. The second extract, followed by the first was passed through a PSDVB column (200 mL) pre-equilibrated in MeOH. The combined eluents were loaded back onto the PSDVB column by successively diluting with H₂O until a two-fold dilution was achieved after which point the column was washed with H₂O. Elution of the column was performed with 1) 30% Me₂CO/H₂O, 2) 75% Me₂CO/H₂O and 3) Me₂CO (fractions **A1–A3**, respectively). Fraction **A1** was subjected to flash column chromatography on Diol (10 mL) eluting with DCM, 50% DCM/EtOAc, EtOAc, 50% DCM/MeOH then MeOH which yielded 4-chloro-3-hydroxyl-3-(2-oxopropyl)-2-oxindole (**139**) (0.70 mg) in the 50% DCM/EtOAc fractions. Fraction **A3** was loaded onto silica (20 mL) and batch eluted with DCM (60 mL), then 20% MeOH/DCM (60 mL). The DCM fraction was then partitioned on Sephadex LH20 with 50% MeOH/DCM, and the resulting fractions were combined on the basis of TLC (fractions **B1–B17**). Fraction **B9** was further purified on a semi-preparative C₁₈ HPLC column (55% MeCN/H₂O) yielding the major compound 4,7-dibromo-2,3-dichloroindole (**119**) (1.3 mg, *t*_R = 42.3 min) and 7-bromo-2,3-dichloro-6-iodoindole (**129**) (0.70 mg, *t*_R = 59.5 min).

Second, third and fourth extractions of the alga (154, 781 and 951 g, respectively)

were treated in a similar fashion. Fractions eluting from the Sephadex LH20 column that contained the compounds of interest (determined using LC-MS) were dissolved in 75% MeOH/MeCN, centrifuged, and the supernatant was separated and combined as one sample (fraction **C1**). A portion (280 mg) was partitioned on C₁₈ HPLC (85% MeOH/H₂O) providing six fractions (**D1–D6**).

6.4.1 Fractionation of **D3**—Tetrahalogenated Indoles

Further semi-preparative HPLC (C₁₈, 55% MeCN/H₂O) on fraction **D3** provided 17 fractions (**E1–E17**), including more of 4,7-dibromo-2,3-dichloroindole (**119**) (11.7 mg) and 7-bromo-2,3-dichloro-6-iodoindole (**129**) (5.6 mg) in addition to 5-bromo-2,3,4-trichloroindole (**133**) (1.3 mg, $t_R = 36.2$ min). Fraction **E4**, was purified on C₈ HPLC using 80% MeCN/H₂O to give 7-bromo-2,3,4-trichloroindole (**121**) as the major compound (1.5 mg, $t_R = 6.8$ min). The following fractions were subjected to further purification on Diol HPLC (1% IPA/*n*-hexane): Fraction **E8**, provided compounds 2,3,4-trichloro-7-iodoindole (**123**) (1.3 mg, $t_R = 20.1$ min) and 2,3,4,7-tetrabromoindole (**116**) (1.0 mg, $t_R = 23.1$ min); Fraction **E9** afforded 6,7-dibromo-2,3-dichloroindole (**128**) (7.6 mg, $t_R = 19.0$ min) and 2-bromo-3,4-dichloro-7-iodoindole (**124**) (0.13 mg, $t_R = 24.0$ min); Fraction **E11** gave rise to 2,3,6,7-tetrabromoindole (**125**) (1.2 mg, $t_R = 24.4$ min). Fraction **E10**, provided 2,3,7-trichloro-6-iodoindole (**132**) (0.06 mg, $t_R = 24.8$ min) in addition to two earlier eluting fractions, one of which was purified twice using C₁₈ HPLC (70% MeOH/H₂O) to afford compounds 3,6,7-tribromo-2-chloroindole (**126**) (1.3 mg, $t_R = 53.2$ min) and 2,6,7-tribromo-3-chloroindole (**127**) (3.7 mg, $t_R = 67.5$ min). Fractions **E13–E15** were fractionated on Diol HPLC (1% IPA/*n*-hexane) to afford four fractions each (**F1–F12**). Based on the ¹H NMR spectrum and mass spectrometric analysis, fractions **F3**, **F4**, **F8**, and **F12** were combined and subjected to further chromatography on C₁₈ HPLC (70% MeOH/H₂O) affording fractions **G1–G5**. Fractions **G1** and **G2** were purified further using C₁₈ HPLC and eluting from 60% MeCN/H₂O to provide compounds 2,7-dibromo-3-chloro-6-iodoindole (**130**)

(0.70 mg, $t_R = 58.4$ min) and 3,7-dibromo-2-chloro-6-iodoindole (**131**) (0.08 mg, $t_R = 62.8$ min) as the major compounds.

6.4.2 Fractionation of D4—Pentahalogenated Indoles

Semi-preparative HPLC of fraction **D4** using C_{18} eluting with MeOH/H₂O (85% MeOH/H₂O 0–31 min, ramped to 100% from 31–31.5 min and eluted at 100% from 31.5–40 min) generated two fractions (**H1** and **H2**). Fraction **H1** was purified further using semi-preparative C_{18} HPLC (55% MeCN/H₂O) as a single continuous run and sample was injected at 45 min intervals, which provided 14 fractions (**I1–I14**). Using the same column and conditions, fraction **I6** was purified to provide 5,7-dibromo-2,3,4-trichloroindole (**137**) (1.2 mg, $t_R = 81.8$ min). Further HPLC purification of fraction **I7** was performed on DIOL with 1% IPA/*n*-hexane to yield fractions **J1–J3**. Fractionation of **J2** on C_{18} HPLC (semi-preparative; 80% MeOH/H₂O) provided pure 4,5,7-tribromo-2,3-dichloroindole (**136**) (1.7 mg, $t_R = 32.0$ min). Fractions **I9** and **I12** were purified separately from 80% MeOH/H₂O using C_{18} HPLC to provide 2,3,4,5,7-pentabromoindole (**134**) (1.6 mg, $t_R = 53.9$ min) and 2,3,4,6,7-pentabromoindole (**135**) (0.30 mg, $t_R = 56.7$ min), respectively.

6.4.3 Fractionation of D5—Hexahalogenated Indoles

Fraction **D5** was subjected to chromatography on C_{18} HPLC (semi-preparative) eluting with MeCN/H₂O (80% MeCN/H₂O 0–26 min, ramped to 100% between 26–27 min and eluted at 100% from 27–41 min) to yield two fractions (**K1** and **K2**). Fraction **K1** was further purified using C_{18} HPLC (55% MeCN/H₂O) in a continuous chromatographic run with sample being injected at *ca.* 35 min intervals. This provided five fractions (**L1–L5**) each containing a mixture of hexahalogenated indoles—based on the ¹H NMR spectrum and the HRESIMS data, fraction **L3** appeared to consist of mainly one hexahalogenated indole with the major compound

corresponding to a tribromo-trichloroindole.

6.4.4 Isolation of 4-Chloroisatin

A separate sample of the alga (96 g) was extracted in MeOH (2×400 mL) and loaded onto PSDVB (80 mL) using the same methodology described above. After washing with H₂O, the column was eluted with: 1) 20% MeOH/H₂O, 2) 40% MeOH/H₂O, 3) 60% MeOH/H₂O, 4) 80% MeOH/H₂O, 5) MeOH and 6) 20% MeOH/DCM (**M1–M6**). Further fractionation of **M3** was performed on Diol (10 mL) eluting with DCM, 50% EtOAc/DCM, EtOAc, then MeOH. The DCM fraction was purified on C₁₈ HPLC using a gradient from acidified H₂O to 55% acidified MeOH/H₂O (each with 0.2% HCOOH) over 10 min, after which point the column was run isocratically at 55% acidified MeOH/H₂O for a further 10 min. This provided 4-chloroisatin (**138**) as the major compound (1.3 mg, $t_R = 15.0$ min; tautomer ratio: 1:2.5, CD₃OD).

2,3,4,7-Tetrabromoindole (116): White film; UV (MeOH): $\lambda_{\max}$ (log ϵ) 290 (3.60), 237 (4.53); ¹H and ¹³C NMR data see Tables 3.13–3.16; GCMS m/z (% relative intensity) 436 (14.5), 434 (47.9), 432 (71.5), 430 (56.6), 428 [M – 1]⁺ (18.7), 350 (16.3), 271 (23.8), 192 (29.9), 165 (19.3), 113 (42.3), 112 (46.5), 86 (100), 85 (61.4); HRESIMS (30 V) m/z (% relative intensity) 427.6867 [M – H][–] (6.4), 347.7635 (9.9), 78.9187 (100.0); HRESIMS m/z 427.6920 [M – H][–] (calcd for C₈H₂Br₄N, 427.6926).

4,7-Dibromo-2,3-dichloroindole (119): White crystals; UV (MeOH): $\lambda_{\max}$ (log ϵ) 290 (4.23), 229 (4.77); IR $\nu_{\max}$ 3378 cm^{–1}; ¹H and ¹³C NMR data see Tables 3.13–3.16; GCMS m/z (% relative intensity) 349 (8.2), 347 (26.1), 345 (81.0), 343 (100.0), 341 [M]⁺ (30.6), 262 (32.6), 183 (49.0), 182 (26.6), 148 (53.0), 121 (34.3), 112 (25.8), 86 (44.9), 85 (38.4); HRESIMS (30 V) m/z (% relative intensity) 339.7857 [M – H][–] (3.7), 259.8681 (17.4), 78.9188 (91.9); HRESIMS m/z 339.7938 [M – H][–] (calcd for C₈H₂Br₂Cl₂N, 339.7937).

7-Bromo-2,3,4-trichloroindole (121): White film; UV (MeOH): $\lambda_{\max}$ ($\log \varepsilon$) 288 (4.07), 229 (4.59); ^1H and ^{13}C NMR data see Tables 3.13–3.16; GCMS m/z (% relative intensity) 303 (15.4), 301 (55.3), 299 (100.0), 297 $[\text{M}]^+$ (48.4), 262 (12.5), 218 (45.9), 191 (10.2), 183 (24.5), 182 (35.5), 148 (42.9), 121 (38.5), 112 (20.8), 109 (20.2), 91 (29.0), 86 (47.7) 85 (38.6); HRESIMS (30 V) m/z (% relative intensity) 295.8403 $[\text{M} - \text{H}]^-$ (3.7), 215.9186 (11.9), 78.9190 (98.5); HRESIMS m/z 295.8444 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{BrCl}_3\text{N}$, 295.8442).

2,3,4-Trichloro-7-iodoindole (123): White film; UV (MeOH): $\lambda_{\max}$ ($\log \varepsilon$) 290 (3.98), 235 (4.24) 226 (4.43); ^1H and ^{13}C NMR data see Tables 3.13–3.16; GCMS m/z (% relative intensity) 351 (0.3), 349 (31.4), 347 (100.0), 345 $[\text{M}]^+$ (99.5), 218 (92.3), 191 (33.2), 183 (43.8), 148 (67.9), 121 (42.0), 113 (5.8), 112 (22.6), 86 (43.8) 85 (34.5); HRESIMS (30 V) m/z (% relative intensity) 343.8230 $[\text{M} - \text{H}]^-$ (5.3), 216.9244 (13.9), 126.9043 (100), 34.9672 (9.2); HRESIMS m/z 343.8313 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{Cl}_3\text{IN}$, 343.8303).

2-Bromo-3,4-dichloro-7-iodoindole (124): White film; UV (MeOH): $\lambda_{\max}$ ($\log \varepsilon$) 290 (4.35), 234 (4.81) 227 (4.83); ^1H and ^{13}C NMR data see Tables 3.13–3.16; GCMS m/z (% relative intensity) 394 (5.1), 392 (40.4), 390 (100.0), 388 $[\text{M} - 1]^+$ (53.4), 262 (29.6), 183 (37.1), 182 (13.2), 148 (53.1), 121 (29.6), 113 (6.8), 112 (19.1), 86 (34.4) 85 (24.4); HRESIMS (30 V) m/z (% relative intensity) 387.7816 $[\text{M} - \text{H}]^-$ (8.0), 260.8752 (22.3), 181.9576 (14.4), 126.9049 (100), 78.9194 (30.6), 34.9698 (2.4); HRESIMS m/z 387.7804 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{BrCl}_2\text{IN}$, 387.7798).

2,3,6,7-Tetrabromoindole (125): White film; UV (MeOH): $\lambda_{\max}$ ($\log \varepsilon$) 289 (4.06), 231 (4.66); IR $\nu_{\max}$ 3400 cm^{-1} ; ^1H and ^{13}C NMR data see Tables 3.3 and 3.4; GCMS m/z (% relative intensity) 436 (16.7), 434 (66.4), 432 (100.0), 430 (71.0), 428 $[\text{M} - 1]^+$ (18.0), 349 (11.5), 271 (18.0), 192 (23.7), 165 (16.5), 113 (25.7), 112 (39.1), 86 (54.0), 85 (35.0); HRESIMS (30 V) m/z (% relative intensity) 427.6919 $[\text{M} - \text{H}]^-$ (17.6), 348.7729 (1.9), 347.7639 (4.1), 78.9190 (74.33); HRESIMS m/z 427.6928 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{Br}_4\text{N}$, 427.6926).

3,6,7-Tribromo-2-chloroindole (126): White film; UV (MeOH): $\lambda_{\max}$ ($\log \epsilon$) 288 (3.93), 230 (4.57); ^1H and ^{13}C NMR data see Tables 3.5 and 3.6; GCMS m/z (% relative intensity) 392 (8.5), 390 (49.3), 388 (100.0), 386 (86.7), 384 $[\text{M} - 1]^+$ (26.7), 305 (14.7), 227 (30.1), 226 (14.7), 148 (30.9), 121 (18.6), 113 (19.5), 112 (28.6), 86 (36.1), 85 (24.9); HRESIMS (30 V) m/z (% relative intensity) 383.7421 $[\text{M} - \text{H}]^-$ (12.7), 303.8141 (5.5), 225.9014 (1.6), 78.9188 (89.3); HRESIMS m/z 383.7438 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{Br}_3\text{ClN}$, 383.7431).

2,6,7-Tribromo-3-chloroindole (127): White film; UV (MeOH): $\lambda_{\max}$ ($\log \epsilon$) 289 (4.01), 231 (4.61); ^1H and ^{13}C NMR data see Tables 3.7 and 3.8; GCMS m/z (% relative intensity) 392 (8.3), 390 (47.4), 388 (100.0), 386 (88.1), 384 $[\text{M} - 1]^+$ (26.1), 305 (21.0), 227 (28.3), 226 (15.1), 148 (23.5), 121 (18.6), 113 (17.9), 112 (22.1), 86 (26.2), 85 (17.5); HRESIMS (30 V) m/z (% relative intensity) 383.7391 $[\text{M} - \text{H}]^-$ (13.9), 303.8133 (6.7), 225.8992, (2.4), 78.9185 (100); HRESIMS m/z 383.7435 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{Br}_3\text{ClN}$, 383.7431).

6,7-Dibromo-2,3-dichloroindole (128): White film; UV (MeOH): $\lambda_{\max}$ ($\log \epsilon$) 288 (4.00), 229 (4.63); IR $\nu_{\max}$ 3415 cm^{-1} ; ^1H and ^{13}C NMR data see Tables 3.9 and 3.10; GCMS m/z (% relative intensity) 348 (4.6), 346 (24.4), 344 (100.0), 342 (75.4), 340 $[\text{M} - 1]^+$ (26.9), 262 (21.7), 183 (54.4), 182 (20.2), 148 (39.8), 121 (38.6), 113 (12.8), 112 (33.3), 86 (49.1), 85 (36.4); HRESIMS (30 V) m/z (% relative intensity) 339.7938 $[\text{M} - \text{H}]^-$ (9.9), 259.8639 (6.7), 78.9188 (98.1); HRESIMS m/z 339.7936 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{Br}_2\text{Cl}_2\text{N}$, 339.7937).

7-Bromo-2,3-dichloro-6-iodoindole (129): White crystals; UV (MeOH): $\lambda_{\max}$ ($\log \epsilon$) 289 (4.42), 235 (4.98); $\nu_{\max}$ 3397 cm^{-1} ; ^1H and ^{13}C NMR data see Tables 3.11 and 3.12; GCMS m/z (% relative intensity) 394 (6.2), 392 (41.2), 390 (89.2), 388 $[\text{M} - 1]^+$ (58.6), 262 (35.6), 226 (14.7), 183 (100.0), 148 (55.4), 121 (45.4), 113 (12.0), 112 (43.9), 86 (59.2), 85 (41.0); HRESIMS (30 V) m/z (% relative intensity) 387.7782 $[\text{M} - \text{H}]^-$ (17.6), 260.8744 (33.2), 181.9546 (9.1), 78.9191 (97.9); HRESIMS m/z 387.7799 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{BrCl}_2\text{IN}$, 387.7798).

2,7-Dibromo-3-chloro-6-iodoindole (130): White film; UV (MeOH): $\lambda_{\max}$ (log ϵ) 291 (4.13), 237 (4.71); IR $\nu_{\max}$ 3403 cm^{-1} ; ^1H and ^{13}C NMR data see Tables 3.13–3.16; GCMS m/z (% relative intensity) 438 (14.1), 436 (72.1), 434 (100.0), 432 $[\text{M} - 1]^+$ (45.4), 306 (14.06), 227 (38.5), 148 (25.4), 121 (20.1), 113 (11.0), 112 (25.8), 86 (28.7) 85 (18.4); HRESIMS (30 V) m/z (% relative intensity) 431.7319 $[\text{M} - \text{H}]^-$ (27.0), 304.8246 (26.8), 126.9045 (77.8), 78.9191 (98.2); HRESIMS m/z 431.7283 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{Br}_2\text{ClIN}$, 431.7293).

3,7-Dibromo-2-chloro-6-iodoindole (131): White film; UV (MeOH): $\lambda_{\max}$ (log ϵ) 290 (4.23), 237 (4.84); ^1H and ^{13}C NMR data see Tables 3.13–3.16; GCMS m/z (% relative intensity) 439 (13.4), 437 (58.8), 435 (100.0), 433 $[\text{M}]^+$ (46.6), 306 (14.06), 227 (38.5), 148 (25.4), 121 (20.1), 113 (11.0), 112 (25.8), 86 (28.7) 85 (18.4); HRESIMS (30 V) m/z (% relative intensity) 431.7220 $[\text{M} - \text{H}]^-$ (29.0), 304.8211 (24.3), 126.9034 (72.4), 78.9192 (89.1); HRESIMS m/z 431.7280 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{Br}_2\text{ClIN}$, 431.7293).

2,3,7-Trichloro-6-iodoindole (132): White film; UV (MeOH): $\lambda_{\max}$ (log ϵ) 288 (4.47), 234 (5.05); ^1H and ^{13}C NMR data see Tables 3.13–3.16; GCMS m/z (% relative intensity) 351 (0.3), 349 (32.1), 347 (97.4), 345 $[\text{M}]^+$ (100.0), 218 (73.2), 191 (15.6), 183 (24.2), 148 (31.0), 121 (29.3), 112 (18.3), 87 (42.1) 86 (25.0) 74 (65.2); HRESIMS (30 V) m/z (% relative intensity) 343.8292 $[\text{M} - \text{H}]^-$ (6.6), 216.9238 (20.3), 126.9042 (100.0), 34.9696 (6.6); HRESIMS m/z 343.8302 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{Cl}_3\text{IN}$, 343.8303).

5-Bromo-2,3,4-trichloroindole (133): White film; UV (MeOH): $\lambda_{\max}$ (log ϵ) 280 (4.59), 227 (5.17); ^1H and ^{13}C NMR data see Table 3.13 and 3.14; GCMS m/z (% relative intensity) 305 (2.2), 303 (17.8), 301 (60.7), 299 (100), 297 $[\text{M}]^+$ (54.1), 262 (15.2), 218 (56.8), 191 (11.4), 183 (25.2), 182 (23.5), 148 (42.6), 121 (39.3), 113 (5.1), 112 (15.3), 91 (39.9), 86 (32.1) 85 (26.4); HRESIMS (30 V) m/z (% relative intensity) 295.8431 $[\text{M} - \text{H}]^-$ (9.0), 215.9174 (18.9), 78.9193 (98.3); HRESIMS m/z 295.8444 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{H}_2\text{BrCl}_3\text{N}$, 295.8442).

2,3,4,5,7-Pentabromoindole (134): White needles; UV (MeOH): $\lambda_{\max}$ (log ε) 294 (3.78), 236 (4.39); IR $\nu_{\max}$ 3407 cm^{-1} ; ^1H and ^{13}C NMR data see Tables 3.17 and 3.18; HRESIMS (40 V) m/z (% relative intensity) 513.5984 (31.9), 511.6010 (98.2), 509.6028 (100), 507.6041 (46.7), 505.6083 $[\text{M} - \text{H}]^-$ (6.8), 432.6807 (44.7), 430.6836 (77.9), 429.6771 (42.8), 428.6853 (58.5), 427.6774 (28.6), 351.7643 (47.5), 349.7676 (59.2), 347.7684 (20.5), 80.9172 (59.4), 78.9190 (72.6); HRESIMS m/z 505.6029 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{HBr}_5\text{N}$, 505.6031).

2,3,4,6,7-Pentabromoindole (135): White needles; UV (MeOH): $\lambda_{\max}$ (log ε) 297 (4.96), 235 (5.54); IR $\nu_{\max}$ 3394 cm^{-1} ; ^1H and ^{13}C NMR data see Tables 3.17 and 3.18; HRESIMS (40 V) m/z (% relative intensity) 513.5970 (30.1), 511.6016 (94.9), 509.6012 (100), 507.6024 (46.4), 505.5997 $[\text{M} - \text{H}]^-$ (7.2), 432.6818 (39.8), 430.6830 (67.5), 429.6750 (54.9), 428.6843 (50.9), 427.6799 (41.1), 351.7658 (30.1), 349.7640 (22.0), 80.9177 (70.1), 78.9191 (74.1); HRESIMS m/z 505.6032 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{HBr}_5\text{N}$, 505.6031).

4,5,7-Tribromo-2,3-dichloroindole (136): White needles; UV (MeOH): $\lambda_{\max}$ (log ε) 296 (4.34), 234 (4.92); IR $\nu_{\max}$ 3423 cm^{-1} ; ^1H and ^{13}C NMR data see Tables 3.17 and 3.18; HRESIMS (40 V) m/z (% relative intensity) 423.7008 (23.9), 421.7035 (34.2), 419.7049 (20.8), 417.7089 $[\text{M} - \text{H}]^-$ (4.4), 343.7774 (12.7), 342.7843 (39.8), 341.7776 (38.8), 340.7858 (39.6), 339.7790 (38.5), 338.7881 (15.1), 337.7804 (12.3), 263.8644 (22.5), 261.8672 (58.0), 259.8699 (34.7), 80.9172 (97.2), 78.9193 (100); HRESIMS m/z 417.7031 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{HBr}_3\text{Cl}_2\text{N}$, 417.7042).

5,7-Dibromo-2,3,4-trichloroindole (137): White needles; UV (MeOH): $\lambda_{\max}$ (log ε) 295 (4.45), 234 (5.02); IR $\nu_{\max}$ 3423 cm^{-1} ; ^1H and ^{13}C NMR data see Tables 3.17 and 3.18; HRESIMS (40 V) m/z (% relative intensity) 377.7509 (21.2), 375.7539 (18.0), 373.7564 $[\text{M} - \text{H}]^-$ (4.2), 298.9318 (25.9), 297.8259 (41.7), 296.8350 (40.1), 295.8281 (61.7), 294.8367 (18.0), 293.8301 (25.7), 80.9172 (100), 78.9192 (98.5); HRESIMS m/z 373.7557 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_8\text{HBr}_2\text{Cl}_3\text{N}$, 373.7547).

6.5 Isolation of Compounds from *Callophycus serratus*

A specimen of *Callophycus serratus* was collected by hand from ‘Eua, Tonga in June 2016 and stored at $-20\text{ }^{\circ}\text{C}$ until extraction. The alga was identified by Dr Joe Zuccarello at the School of Biological Sciences, Victoria University of Wellington, where a voucher specimen (PTN4_34F) is also stored.

The alga (102.5 g wet weight) was extracted in MeOH (400 mL) twice overnight. The second, then first extracts were cyclic loaded onto PSDVB (80 mL) and eluted with 1) 30% Me₂CO/H₂O 2) 75% Me₂CO/H₂O 3) 100% Me₂CO (**A1–A3**). Fraction **A2** was loaded onto DIOL (15 mL) that was pre-equilibrated in *n*-hexane overnight, and batch-eluted with 45 mL portions of DCM, 10%, 20%, 50% EtOAc/DCM, EtOAc and MeOH (**B1–B6**). **B3** was further purified using C₁₈ HPLC, 85% MeOH/H₂O isocratic, to afford deiiodocallophycoic acid B (**231**) ($t_{\text{R}} = 12.2\text{ min}$). Fraction **A3** was dry-loaded onto silica gel (20 mL) and flash column chromatography was performed eluting with 60 mL portions of *n*-hexane, 5%, 10%, 20%, 50% DCM/*n*-hexane, DCM, 25%, 50% EtOAc/DCM and 20% MeOH/EtOAc, which yielded 84 fractions that were combined on the basis of TLC to provide 22 fractions (**C1–C22**). Both **C12** and **C13** were separately subjected to further purification on a HILIC HPLC column, eluting with 10% EtOAc/*n*-hexane. This resulted in the purification of iodocallophycol F (**229**) from fraction **C12** ($t_{\text{R}} = 24.4\text{ min}$) and both iodocallophycols E (**228**) and F (**229**) ($t_{\text{R}} = 16.6\text{ min}$) from **C13**.

A second batch of the alga (79 g) was extracted in MeOH (400 mL), cyclic loaded onto PSDVB and eluted with 1) 30% Me₂CO/H₂O 2) 75% Me₂CO/H₂O 3) 100% Me₂CO (**D1–D3**) in a similar fashion to that described above. The 100% Me₂CO fraction (**D3**) was further purified on a DIOL (15 mL) column from 45 mL volumes of *n*-hexane, 5%, 10%, 20%, 50% DCM/*n*-hexane, DCM, EtOAc, MeOH to generate 47 fractions that were combined, following a TLC analysis, into 14 samples (**E1–E14**). Samples **E5–E7** were recombined due to similarities observed in their ¹H

NMR spectra and the resulting sample was purified by HPLC (C_{18}) using 80% MeCN/ H_2O to afford iodocallophycoic acid B (**230**) ($t_R = 16.6$ min). **E9** was fractionated using C_{18} HPLC, eluting isocratically with 90% MeCN/ H_2O , which afforded pure bromophycolide T (**202**) (0.10 mg, $t_R = 4.8$ min) and additional mass of **228** ($t_R = 22.5$ min) and **229** ($t_R = 21.6$ min). All samples identified as **228** were combined (1.3 mg), as were those of **229** (1.8 mg). The 75% Me₂CO/ H_2O fraction (**D2**) was further fractionated by flash column chromatography on DIOL, eluting with *n*-hexane, 30%, 50%, 70% DCM/*n*-hexane, DCM, 10%, 20%, 50% EtOAc/DCM, EtOAc and MeOH, which resulted in 50 fractions that were combined to form 11 samples (**F1–F11**). Additional purification of **F5** was performed on C_{18} HPLC, eluting at 80% MeCN/ H_2O isocratically for 12.5 min, then ramping to 100% MeCN between 12.5–13.0 min and eluting for 12 min, providing fractions **G1–G3**. Bromophycolide A (**181**) was finally purified from **G1** using C_{18} HPLC, 75% MeCN/ H_2O (1.2 mg, $t_R = 14.5$ min). Fractions **B2** (first batch extraction) and **G7** were subjected to HPLC C_{18} chromatography, eluting with 80% MeCN/ H_2O to afford iodocallophycoic acid B (**230**) ($t_R = 16.6$ min), deiiodocallophycoic B (**231**) ($t_R = 11.6$ min) and callophycoic acid I (0.60 mg) (**232**) ($t_R = 7.0$ min). Fractions that contained **230** were combined (0.80 mg) as were those that contained **231** (1.0 mg).

Bromophycolide A (181): White amorphous solid; All NMR data as previously described.¹⁸⁰

Bromophycolide T (202): White amorphous solid; All NMR data as previously described.¹⁷⁷

Callophycol C (227): White amorphous solid; ¹H and ¹³C NMR data see Table 4.2; HRESIMS (40 V) *m/z* (% relative intensity) 638.9696 (0.4), 636.9715 (0.8), 634.9743 (0.7), 632.9812 [M – H][–] (0.2), 559.0454 (9.5), 557.0483 (50.8), 555.0503 (76.2), 553.0520 (29.0), 265.8586 (4.6), 263.8604 (10.6), 261.8617 (5.7), 80.9171 (96.3), 78.9193 (100); HRESIMS *m/z* 632.9787 [M – H][–] (calcd for C₂₆H₃₃Br₃ClO, 632.9776).

Iodocallophycol E (228): White amorphous solid; $[\alpha]_{\text{D}}^{20} -27.1$ (c 0.17, CHCl_3); IR ν_{max} 3378, 2953, 1267 cm^{-1} ; ^1H and ^{13}C NMR data see Table 4.3; HRESIMS (5 V) m/z (% relative intensity) 730.9107 (30.8), 728.9128 (100), 726.9143 (93.2), 724.9153 $[\text{M} - \text{H}]^-$ (20.4), 126.9054 (93.6), 80.91711 (7.3), 78.9192 (7.9); HRESIMS m/z 724.9131 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{26}\text{H}_{33}\text{Br}_3\text{IO}$, 724.9132).

Iodocallophycol F (229): White amorphous solid; $[\alpha]_{\text{D}}^{20} -49.2$ (c 0.12, CHCl_3); IR ν_{max} 3378, 2956, 1265 cm^{-1} ; ^1H and ^{13}C NMR data see Table 4.4; HRESIMS (5 V) m/z (% relative intensity) 648.9877 (28.4), 646.9896 (53.3), 644.9900 $[\text{M} - \text{H}]^-$ (17.5), 126.9058 (100), 80.9173 (3.6), 78.9195 (3.4); HRESIMS m/z 644.9880 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{26}\text{H}_{32}\text{Br}_2\text{IO}$, 644.9870).

Iodocallophycoic Acid B (230): White amorphous solid; $[\alpha]_{\text{D}}^{20} -27.5$ (c 0.08, CHCl_3); IR ν_{max} 3320, 3068, 2925, 1683, 1273 cm^{-1} ; ^1H and ^{13}C NMR data see Table 4.5; HRESIMS (5 V) m/z (% relative intensity) 615.0654 (2.0), 613.0625 (30.3), 611.0630 $[\text{M} - \text{H}]^-$ (17.7), 126.9053 (100), 80.9173 (13.2), 78.9172 (14.3); HRESIMS m/z 611.0668 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{27}\text{H}_{33}\text{BrIO}_3$, 611.0663).

Deiodocallophycoic Acid B (231): White amorphous solid; $[\alpha]_{\text{D}}^{20} +46.0$ (c 0.1, CHCl_3); IR ν_{max} 3291, 3068, 2952, 1683, 1274 cm^{-1} ; ^1H and ^{13}C NMR data see Table 4.7; HRESIMS (30 V) m/z (% relative intensity) 487.1723 (1.4), 485.1722 $[\text{M} - \text{H}]^-$ (1.0), 405.2445 (49.9), 150.0356 (24.8), 80.9171 (100), 78.9190 (78.9); HRESIMS m/z 485.1684 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{27}\text{H}_{34}\text{BrO}_3$, 485.1697).

Callophycoic Acid I (232): White amorphous solid; $[\alpha]_{\text{D}}^{20} +19.4$ (c 0.06, MeOH); IR ν_{max} 3330, 3068, 2925, 1679, 1275 cm^{-1} ; ^1H and ^{13}C NMR data see Table 4.9; HRESIMS (30 V) m/z (% relative intensity) 505.1576 (0.8), 503.1650 (3.5), 501.1658 $[\text{M} - \text{H}]^-$ (2.2), 423,2413 (9.7), 422.2405 (37.0), 421.2378 (87.1), 377.2477 (8.4), 80.9173 (100), 78.9194 (85.9); HRESIMS m/z 501.1650 $[\text{M} - \text{H}]^-$ (calcd for $\text{C}_{27}\text{H}_{34}\text{BrO}_4$, 501.1646).

Appendix A

Screening and NMR Protocol

The screening protocol utilised in this research was initially developed by West⁹⁰ for New Zealand sponges.[†] Where appropriate, masses and volumes were scaled accordingly.

Equipment

- 1 × screen column loaded with 80 mL of HP20 equilibrated in MeOH.
- 1 × backloading column loaded with 40 mL of HP20 equilibrated in MeOH.

Extraction

- Extract *ca.* 100 g of sponge material in 400 mL MeOH overnight.
- Filter the first extract and set aside. Re-extract the sponge material (and filter paper/celite as necessary) in 400 mL of MeOH overnight.
- Filter the second extract.
- Keep all sponge material (and filter paper/celite as necessary) until the screen is complete at which time it may be discarded.

Cyclic Loading

- Pass the second extract through the screen column with a flow rate of *ca.* 10 mL/min.
- Pass the first extract through the screen column with a flow rate of *ca.* 10 mL/min. Combine the eluent with that of the second extract.

[†]Last updated 13/11/2006

- Dilute the combined eluents with 800 mL of distilled H₂O. Pass the diluted eluents back through the screen column as a flow rate of *ca.* 10 mL/min.
- Dilute the eluent with 1.6 L of distilled H₂O. Pass the diluted eluent back through the screen column at a flow rate of *ca.* 10 mL/min.
- The eluent should be kept until the screen is complete at which time it may be discarded.

Elution

- Elute the screen column with 250 mL of distilled H₂O at a flow rate of *ca.* 10 mL/min. The H₂O eluent can be discarded immediately.
- Elute the screen column with 250 mL of 30% Me₂CO/H₂O (75 mL Me₂CO to 175 mL H₂O) at a flow rate of *ca.* 10 mL/min.
- Elute the screen column with 250 mL of 75% Me₂CO/H₂O (187.5 mL Me₂CO to 62.5 mL H₂O) at a flow rate of *ca.* 10 mL/min.
- Elute the screen column with 250 mL of Me₂CO at a flow rate of *ca.* 10 mL/min.
- The eluent should be kept until the screen is complete at which time it may be discarded.

Backloading the 75% Me₂CO/H₂O Fraction

- Dilute the 75% Me₂CO/H₂O fraction with 250 mL of distilled H₂O. Pass the diluted eluent through the backloading column at a flow rate of *ca.* 8 mL/min.
- Dilute the eluent with 500 mL of distilled H₂O. Pass the diluted eluent back through the backloading column at a flow rate of *ca.* 8 mL/min.
- The eluent should be kept aside until the screen is complete at which time it may be discarded.
- Elute the backloading column with 150 mL of Me₂CO.

Processing the 75% Me₂CO/H₂O Fraction

- Concentrate the Me₂CO eluent of the backloading column to dryness and transfer to a pre-weighed sample vial. Concentrate to dryness and record the mass.
- Subsample *ca.* 30 mg of material for NMR analysis if necessary.
- Prepare an NMR sample in *ca.* 700 μ L of CD₃OD in a 5 mm NMR tube.

NMR Analysis of the 75% Me₂CO/H₂O Fraction

- Run a ¹H NMR spectrum of the sample on the 600 MHz instrument using the standard Screen1H parameter set (experiment time: *ca.* 4 min).
- NMR parameters for ¹H NMR screen
  - increments per scan (ni) = 64.
  - acquisition time (at) = 3.407 s.
  - relaxation delay (d1) = 0.01 s.
  - ¹H spectral width: -1.0–9.0 ppm.
  - total experimental time: 3 min, 39 s.
- Run a COSY spectrum of the sample using the standard ScreenCOSY parameter set (experiment time: *ca.* 20 min).
- NMR parameters for COSY screen
  - increments per scan (ni) = 1.
  - number of scans (nt) = 512.
  - acquisition time (at) = 0.150 s.
  - relaxation delay (d1) = 2.0 s.
  - ¹H spectral width: -1.0–9.0 ppm.
  - window functions: sinebell.

- total experimental time: 19 min, 56 s.
- Run an HSQC spectrum of the sample using the standard ScreenHSQC parameter set (experiment time: *ca.* 4 h).
- NMR parameters for HSQC screen
  - increments per scan (ni) = 8.
  - number of scans (nt) = 512.
  - acquisition time (at) = 0.150 s.
  - One-Bond coupling constant = 146 Hz.
  - relaxation delay (d1) = 1.5 s.
  - ^1H spectral width: -1.0 – 9.0 ppm.
  - ^{13}C spectral width: 0 – 160 ppm
  - ^1H – ^{13}C multiplicity editing: yes
  - window functions: cosine.
  - total experimental time: 3 h, 51 min.
- Run an HMBC spectrum of the sample using the default gHMBC parameter set (experiment time: *ca.* 8 h)
- NMR parameters for HMBC screen
  - increments per scan (ni) = 16.
  - number of scans (nt) = 512.
  - acquisition time (at) = 0.213 s.
  - coupling constant = 8 Hz.
  - relaxation delay (d1) = 1.5 s.
  - ^1H spectral width: -1.0 – 9.0 ppm.
  - ^{13}C spectral width: 0 – 220 ppm
  - window functions: sinebell.

- total experimental time: 8 h, 12 min.

Backloading the 30% Me₂CO/H₂O Fraction

- Dilute the 30% Me₂CO/H₂O fraction with 250 mL of distilled H₂O. Pass the diluted eluent through the backloading column at a flow rate of *ca.* 8 mL/min.
- Dilute the eluent with 500 mL of distilled H₂O. Pass the diluted eluent back through the backloading column at a flow rate of *ca.* 8 mL/min.
- The eluent should be kept aside until the screen is complete at which time it may be discarded.
- Elute the backloading column with 150 mL of Me₂CO.

Processing the 30% Me₂CO/H₂O Fraction

- Concentrate the Me₂CO eluent of the backloading column to dryness and transfer to a pre-weighed sample vial. Concentrate to dryness and record the mass.
- Subsample *ca.* 30 mg of material for NMR analysis if necessary.
- Prepare an NMR sample in *ca.* 700 μ L of CD₃OD in a 5 mm NMR tube.

NMR Analysis of the 30% Me₂CO/H₂O Fraction

- Run a ¹H NMR spectrum of the sample on the 600 MHz instrument using the standard Screen1H parameter set (experiment time: *ca.* 4 min).

Processing the 100% Me₂CO/H₂O Fraction

- Concentrate the Me₂CO eluent of the backloading column to dryness and transfer to a pre-weighed sample vial. Concentrate to dryness and record the mass.
- Subsample *ca.* 30 mg of material for NMR analysis if necessary.

- Prepare an NMR sample in *ca.* 700 μL of CD_3OD in a 5 mm NMR tube.

NMR Analysis of the 100% $\text{Me}_2\text{CO}/\text{H}_2\text{O}$ Fraction

- Run a ^1H NMR spectrum of the sample on the 600 MHz instrument using the standard Screen1H parameter set (experiment time: *ca.* 4 min).

Appendix B

NMR Spectra of Known Compounds

2,3,4,7-Tetrabromoindole

116

^1H NMR spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 2,3,4,7-tetrabromoindole (**116**) (150 MHz, CDCl_3)

COSY spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, CDCl_3)

HMBC spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, CDCl₃)

¹⁵N CIGARAD spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, CDCl₃)

^1H NMR spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 2,3,4,7-tetrabromoindole (**116**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, DMSO- d_6)

HMBC spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, DMSO-d₆)

¹⁵N CIGARAD spectrum of 2,3,4,7-tetrabromoindole (**116**) (600 MHz, DMSO-d₆)

4,7-Dibromo-2,3-dichloroindole

119

^1H NMR spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (150 MHz, CDCl_3)

COSY spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, CDCl_3)

HMBC spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, CDCl_3)

^1H NMR spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, DMSO- d_6)

HMBC spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, DMSO- d_6)

^{15}N CIGARAD spectrum of 4,7-dibromo-2,3-dichloroindole (**119**) (600 MHz, DMSO- d_6)

7-Bromo-2,3,4-trichloroindole

121

¹H NMR spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, CDCl₃)

¹³C NMR spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (150 MHz, CDCl₃)

COSY spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, CDCl_3)

HMBC spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, CDCl_3)

^1H NMR spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, DMSO- d_6)

HMBC spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, DMSO- d_6)

^{15}N CIGARAD spectrum of 7-bromo-2,3,4-trichloroindole (**121**) (600 MHz, DMSO- d_6)

Indole

140

^1H NMR spectrum of indole (**140**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of indole (**140**) (150 MHz, CDCl_3)

COSY spectrum of indole (**140**) (600 MHz, CDCl₃)

Coupled HSQC spectrum of indole (**140**) (600 MHz, CDCl₃)

HMBC spectrum of indole (**140**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of indole (**140**) (600 MHz, CDCl_3)

^1H NMR spectrum of indole (**140**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of indole (**140**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of indole (**140**) (600 MHz, DMSO-d₆)

Coupled HSQC spectrum of indole (**140**) (600 MHz, DMSO-d₆)

HMBC spectrum of indole (**140**) (600 MHz, $\text{DMSO}-d_6$)

^{15}N CIGARAD spectrum of indole (**140**) (600 MHz, $\text{DMSO}-d_6$)

Bromophycolide A

181

^1H NMR spectrum of bromophycolide A (**181**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of bromophycolide A (**181**) (150 MHz, CDCl_3)

Bromophycolide T

202

^1H NMR spectrum of bromophycolide T (**202**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of bromophycolide T (**202**) (150 MHz, CDCl_3)

Appendix C

NMR Spectra of α -Exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone

^1H NMR spectrum of α -exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of α -exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**) (150 MHz, CDCl_3)

COSY spectrum of α -exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**) (600 MHz, CDCl_3)

HSQC spectrum of α -exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**) (600 MHz, CDCl_3)

HMBC spectrum of α -exomethylene- γ -methyl- γ -tetradecyl- γ -butyrolactone (**69**) (600 MHz, CDCl_3)

Appendix D

NMR Spectra of α -Exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone

^1H NMR spectrum of α -exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of α -exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**) (150 MHz, CDCl_3)

COSY spectrum of α -exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**) (600 MHz, CDCl_3)

HSQC spectrum of α -exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**) (600 MHz, CDCl_3)

HMBC spectrum of α -exomethylene- β -hydroxyl- γ -methyl- γ -tetradecyl- γ -butyrolactone (**70**) (600 MHz, CDCl_3)

Appendix E

NMR Spectra of 2,3,4-Trichloro-7-iodoindole

123

¹H NMR spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, CDCl₃)

¹³C NMR spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (150 MHz, CDCl₃)

COSY spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, CDCl_3)

HMBC spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, CDCl_3)

^1H NMR spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, DMSO- d_6)

HMBC spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, DMSO-d₆)

¹⁵N CIGARAD spectrum of 2,3,4-trichloro-7-iodoindole (**123**) (600 MHz, DMSO-d₆)

Appendix F

NMR Spectra of 2-Bromo-3,4-dichloro-7-iodoindole

124

^1H NMR spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (150 MHz, CDCl_3)

COSY spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, CDCl_3)

HMBC spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, CDCl₃)

¹⁵N CIGARAD spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, CDCl₃)

^1H NMR spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, DMSO- d_6)

HMBC spectrum of 2-bromo-3,4-dichloro-7-iodoindole (**124**) (600 MHz, $\text{DMSO}-d_6$)

Appendix G

NMR Spectra of 2,3,6,7-Tetrabromoindole

125

^1H NMR spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 2,3,6,7-tetrabromoindole (**125**) (150 MHz, CDCl_3)

COSY spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, CDCl_3)

HMBC spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, CDCl_3)

^1H NMR spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 2,3,6,7-tetrabromoindole (**125**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, DMSO- d_6)

HMBC spectrum of 2,3,6,7-tetrabromoindole (**125**) (600 MHz, (DMSO- d_6))

Appendix H

NMR Spectra of 3,6,7-Tribromo-2-chloroindole

126

^1H NMR spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (150 MHz, CDCl_3)

COSY spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, CDCl_3)

HMBC spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, CDCl_3)

^1H NMR spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, DMSO- d_6)

HMBC spectrum of 3,6,7-tribromo-2-chloroindole (**126**) (600 MHz, ($\text{DMSO}-d_6$))

Appendix I

NMR Spectra of 2,6,7-Tribromo-3-chloroindole

127

^1H NMR spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (150 MHz, CDCl_3)

COSY spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, CDCl_3)

HMBC spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, CDCl_3)

^1H NMR spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, DMSO- d_6)

HMBC spectrum of 2,6,7-tribromo-3-chloroindole (**127**) (600 MHz, DMSO- d_6)

Appendix J

NMR Spectra of 6,7-Dibromo-2,3-dichloroindole

128

^1H NMR spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 6,7-Dibromo-2,3-dichloroindole (**128**) (150 MHz, CDCl_3)

COSY spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, CDCl_3)

HMBC spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, CDCl_3)

^1H NMR spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, DMSO- d_6)

HMBC spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, DMSO- d_6)

^{15}N CIGARAD spectrum of 6,7-dibromo-2,3-dichloroindole (**128**) (600 MHz, DMSO- d_6)

Appendix K

NMR Spectra of 7-Bromo-2,3-dichloro-6-iodoindole

129

^1H NMR spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (150 MHz, CDCl_3)

COSY spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, CDCl_3)

HMBC spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, CDCl_3)

^1H NMR spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, DMSO- d_6)

HMBC spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, DMSO-d₆)

¹⁵N CIGARAD spectrum of 7-bromo-2,3-dichloro-6-iodoindole (**129**) (600 MHz, DMSO-d₆)

Appendix L

NMR Spectra of 2,7-Dibromo-3-chloro-6-iodoindole

130

^1H NMR spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (150 MHz, CDCl_3)

COSY spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, CDCl_3)

HMBC spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, CDCl_3)

^1H NMR spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, DMSO- d_6)

Coupled HSQC spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, DMSO- d_6)

HMBC spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, DMSO-d₆)

¹⁵N CIGARAD spectrum of 2,7-dibromo-3-chloro-6-iodoindole (**130**) (600 MHz, DMSO-d₆)

Appendix M

NMR Spectra of 3,7-Dibromo-2-chloro-6-iodoindole

131

^1H NMR spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (150 MHz, CDCl_3)

COSY spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (600 MHz, CDCl_3)

HSQC spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (600 MHz, CDCl_3)

HMBC spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (600 MHz, CDCl_3)

¹H NMR spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (150 MHz, $\text{DMSO}-\text{d}_6$)

COSY spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (600 MHz, $\text{DMSO}-\text{d}_6$)

Coupled HSQC spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (600 MHz, DMSO- d_6)

HMBC spectrum of 3,7-dibromo-2-chloro-6-iodoindole (**131**) (600 MHz, DMSO- d_6)

Appendix N

NMR Spectra of 2,3,7-Trichloro-6-iodoindole

132

^1H NMR spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (150 MHz, CDCl_3)

COSY spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (600 MHz, CDCl_3)

HMBC spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (600 MHz, CDCl_3)

^1H NMR spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (150 MHz, $\text{DMSO}-d_6$)

COSY spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (600 MHz, $\text{DMSO}-d_6$)

Coupled HSQC spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (600 MHz, DMSO- d_6)

HMBC spectrum of 2,3,7-trichloro-6-iodoindole (**132**) (600 MHz, DMSO- d_6)

Appendix O

NMR Spectra of 5-Bromo-2,3,4-trichloroindole

133

¹H NMR spectrum of 5-bromo-2,3,4-trichloroindole (**133**) (600 MHz, CDCl₃)

¹³C NMR spectrum of 5-bromo-2,3,4-trichloroindole (**133**) (150 MHz, CDCl₃)

COSY spectrum of 5-bromo-2,3,4-trichloroindole (**133**) (600 MHz, CDCl_3)

Coupled HSQC spectrum of 5-bromo-2,3,4-trichloroindole (**133**) (600 MHz, CDCl_3)

HMBC spectrum of 5-bromo-2,3,4-trichloroindole (**133**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 5-bromo-2,3,4-trichloroindole (**133**) (600 MHz, CDCl_3)

Appendix P

NMR Spectra of 2,3,4,5,7-Pentabromoindole

134

^1H NMR spectrum of 2,3,4,5,7-pentabromoindole (**134**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 2,3,4,5,7-pentabromoindole (**134**) (150 MHz, CDCl_3)

Coupled HSQC spectrum of 2,3,4,5,7-pentabromoindole (**134**) (600 MHz, $CDCl_3$)

HMBC spectrum of 2,3,4,5,7-pentabromoindole (**134**) (600 MHz, $CDCl_3$)

^{15}N CIGARAD spectrum of 2,3,4,5,7-pentabromoindole (**134**) (600 MHz, CDCl_3)

^1H NMR spectrum of 2,3,4,5,7-pentabromoindole (**134**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 2,3,4,5,7-pentabromoindole (**134**) (150 MHz, DMSO- d_6)

Coupled HSQC spectrum of 2,3,4,5,7-pentabromoindole (**134**) (600 MHz, DMSO- d_6)

HMBC spectrum of 2,3,4,5,7-pentabromoindole (**134**) (600 MHz, DMSO- d_6)

^{15}N CIGARAD spectrum of 2,3,4,5,7-pentabromoindole (**134**) (600 MHz, DMSO- d_6)

Appendix Q

X-ray Diffraction Data for 2,3,4,5,7-Pentabromoindole

The following data was collected and solved by Associate Professor Martyn Coles

Data collection SuperNova, Dual, Cu at zero, Atlas , Program package WinGX, Abs. correction 'MULTISCAN' Refinement using SHELXL-97 , Drawing using ORTEP-3 for Windows

Empirical formula	$\text{C}_8\text{H}_2\text{Br}_5\text{N}$
Formula weight	511.66 g/mol
Temperature/K	120.01(10)
Crystal system	monoclinic
Space group	$P2_1/c$ (No.14)
a/Å	16.1303(8)
b/Å	3.9804(2)
c/Å	17.4061(9)
$\alpha/^\circ$	90
$\beta/^\circ$	105.163(5)
$\gamma/^\circ$	90
Volume/Å ³	1078.66(10)
Z	4
$\rho_{\text{calc}}/\text{cm}^3$	3.151
μ/mm^{-1}	22.239

F(000)	928.0
Crystal size/mm ³	0.11 × 0.03 × 0.03
Radiation	Cu K α (γ = 1.54184)
2 Θ range for data collection/°	10.5316 to 143.421
Index ranges	$-16 \leq h \leq 19$, $-4 \leq k \leq 4$, $-20 \leq l \leq 20$
Reflections collected	4636
Independent reflections	2079 ($R_{\text{int}} = 0.03$, $R_{\text{sigma}} = 0.048$)
Data/restraints/parameters	2079/0/127
Goodness-of-fit on F^2	1.097
Final R indexes [$I \geq 2\sigma(I)$]	$R_1 = 0.036$, $wR_2 = 0.088$
Final R indexes [all data]	$R_1 = 0.040$, $wR_2 = 0.091$
Largest diff. peak/hole / e Å ⁻³	0.98/−0.74

Fractional Atomic Coordinates ($\times 10^4$) and Equivalent Isotropic Displacement Parameters ($\text{\AA}^2 \times 10^3$). U_{eq} is defined as 1/3 of the trace of the orthogonalised U_{IJ} tensor.

Atom	x	y	z	$U(\text{eq})$
Br1	9054.0(4)	7059.2(17)	1894.0(3)	25.76(16)
Br2	9417.9(4)	6808.9(16)	3980.1(3)	26.47(16)
Br3	8082.0(4)	3828.3(17)	5103.4(3)	27.28(17)
Br4	6176.8(4)	−52.4(17)	4822.9(3)	27.51(17)
Br5	5769.8(4)	−275.2(15)	1502.9(3)	23.01(16)
N1	7583(3)	3725(13)	2060(3)	23.4(10)
C1	8330(4)	5224(15)	2448(3)	21(1)
C2	8446(4)	5130(15)	3251(3)	22.7(11)
C3	7711(4)	3446(14)	3403(3)	20.8(11)
C4	7425(4)	2650(15)	4075(3)	23.2(11)
C5	6644(4)	1056(15)	3962(3)	23.3(11)
C6	6146(4)	162(15)	3196(4)	25.5(12)
C7	6422(4)	927(16)	2542(3)	24.8(11)

Bond Lengths

Atom	Atom	Length/Å
Br1	C1	1.848(5)
Br2	C2	1.863(6)
Br3	C4	1.885(5)
Br4	C5	1.898(5)
Br5	C7	1.900(6)
N1	C1	1.356(8)
N1	C8	1.371(7)
C1	C2	1.362(8)
C2	C3	1.446(8)
C3	C4	1.402(8)
C3	C8	1.428(8)
C4	C5	1.377(8)
C5	C6	1.411(8)
C6	C7	1.361(8)
C7	C8	1.390(9)

Bond Angles.

Atom	Atom	Atom	Length/°
C1	N1	C8	107.3(5)
N1	C1	C2	111.4(5)
N1	C1	Br1	121.0(4)
C2	C1	Br1	127.6(5)
C1	C2	C3	107.5(5)
C1	C2	Br2	123.7(4)
C3	C2	Br2	128.8(4)
C4	C3	C8	120.0(5)
C4	C3	C2	136.2(5)
C8	C3	C2	103.8(5)
C5	C4	C3	118.2(5)
C5	C4	Br3	121.1(4)
C3	C4	Br3	120.7(4)
C4	C5	C6	121.7(5)
C4	C5	Br4	122.1(4)
C6	C5	Br4	116.2(4)
C7	C6	C5	120.2(6)
C6	C7	C8	120.0(5)
C6	C7	Br5	121.3(5)
C8	C7	Br5	118.7(4)
N1	C8	C7	130.2(5)
N1	C8	C3	110.1(5)
C7	C8	C3	119.8(5)

Appendix R

NMR Spectra of 2,3,4,6,7-Pentabromoindole

135

^1H NMR spectrum of 2,3,4,6,7-pentabromoindole (**135**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 2,3,4,6,7-pentabromoindole (**135**) (150 MHz, CDCl_3)

Coupled HSQC spectrum of 2,3,4,6,7-pentabromoindole (**135**) (600 MHz, $CDCl_3$)

HMBC spectrum of 2,3,4,6,7-pentabromoindole (**135**) (600 MHz, $CDCl_3$)

^{15}N CIGARAD spectrum of 2,3,4,6,7-pentabromoindole (**135**) (600 MHz, CDCl_3)

Appendix S

NMR Spectra of 4,5,7-Tribromo-2,3-dichloroindole

136

^1H NMR spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (150 MHz, CDCl_3)

Coupled HSQC spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (600 MHz, CDCl_3)

HMBC spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (600 MHz, CDCl_3)

^{15}N CIGARAD spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (600 MHz, CDCl_3)

^1H NMR spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (600 MHz, $\text{DMSO}-d_6$)

^{13}C NMR spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (150 MHz, $\text{DMSO}-d_6$)

HSQC spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (600 MHz, $\text{DMSO}-d_6$)

HMBC spectrum of 4,5,7-tribromo-2,3-dichloroindole (**136**) (600 MHz, DMSO- d_6)

Appendix T

NMR Spectra of 5,7-Dibromo-2,3,4-trichloroindole

137

^1H NMR spectrum of 5,7-dibromo-2,3,4-trichloroindole (**137**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of 5,7-dibromo-2,3,4-trichloroindole (**137**) (150 MHz, CDCl_3)

HSQC spectrum of 5,7-dibromo-2,3,4-trichloroindole (**137**) (600 MHz, CDCl₃)

HMBC spectrum of 5,7-dibromo-2,3,4-trichloroindole (**137**) (600 MHz, CDCl₃)

^{13}C NMR spectrum of 5,7-dibromo-2,3,4-trichloroindole (**137**) (150 MHz, $\text{DMSO}-d_6$)

HSQC spectrum of 5,7-dibromo-2,3,4-trichloroindole (**137**) (600 MHz, $\text{DMSO}-d_6$)

HMBC spectrum of 5,7-dibromo-2,3,4-trichloroindole (**137**) (600 MHz, DMSO- d_6)

Appendix U

NMR Spectra of Callophycol C

^1H NMR spectrum of callophycol C (**227**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of callophycol C (**227**) (150 MHz, CDCl_3)

COSY spectrum of callophycol C (**227**) (600 MHz, CDCl₃)

HSQC spectrum of callophycol C (**227**) (600 MHz, CDCl_3).

HMBC spectrum of callophycol C (**227**) (600 MHz, CDCl_3)

Appendix V

NMR Spectra of Iodocallophycol E

¹H NMR spectrum of iodocallophycol E (**228**) (600 MHz, CDCl₃)

^{13}C NMR spectrum of iodocallophycol E (**228**) (150 MHz, CDCl_3)

COSY spectrum of iodocallophycol E (**228**) (600 MHz, CDCl₃)

HSQC spectrum of iodocallophycol E (**228**) (600 MHz, CDCl_3).

HMBC spectrum of iodocallophycol E (**228**) (600 MHz, CDCl_3)

Appendix W

NMR Spectra of Iodocallophycol F

^{13}C NMR spectrum of iodocallophycol F (**229**) (150 MHz, CDCl_3)

COSY spectrum of iodocallophycol F (**229**) (600 MHz, CDCl₃)

HSQC spectrum of iodocallophycol F (**229**) (600 MHz, CDCl_3).

HMBC spectrum of iodocallophycol F (**229**) (600 MHz, CDCl_3)

Appendix X

NMR Spectra of Iodocallophycoic acid B

^1H NMR spectrum of iodocallophycoic acid B (**230**) (600 MHz, CD_3OD)

^{13}C NMR spectrum of iodocallophycoic acid B (**230**) (150 MHz, CD_3OD)

COSY spectrum of iodocallophycoic acid B (**230**) (600 MHz, CD₃OD)

HSQC spectrum of iodocallophycoic acid B (**230**) (600 MHz, CD_3OD).

HMBC spectrum of iodocallophycoic acid B (**230**) (600 MHz, CD_3OD)

Appendix Y

NMR Spectra of Deiodocallophycoic acid B

^1H NMR spectrum of deiodocallophycoic acid B (**231**) (600 MHz, CDCl_3)

^{13}C NMR spectrum of deiodocallophycoic acid B (**231**) (150 MHz, CDCl_3)

COSY spectrum of deiodocallophycoic acid B (**231**) (600 MHz, CDCl_3)

HSQC spectrum of deiodocallophycoic acid B (**231**) (600 MHz, CDCl_3).

HMBC spectrum of deiodocallophycoic acid B (**231**) (600 MHz, CDCl_3)

Appendix Z

NMR Spectra of Callophycoic acid I

¹H NMR spectrum of callophycoic acid I (**232**) (600 MHz, CDCl₃)

^{13}C NMR spectrum of callophycoic acid I (**232**) (150 MHz, CD_3OD)

COSY spectrum of callophycoic acid I (**232**) (600 MHz, CDCl₃)

HSQC spectrum of callophycoic acid I (**232**) (600 MHz, CDCl_3).

HMBC spectrum of callophycoic acid I (**232**) (600 MHz, CDCl_3)

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