

**ISOLATION, STRUCTURAL CHARACTERISATION AND  
EVALUATION OF CYTOTOXIC ACTIVITY OF NATURAL  
PRODUCTS FROM SELECTED SOUTH AFRICAN MARINE  
RED ALGAE**

A thesis submitted in fulfilment of the  
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## Abstract

The medicinal chemistry of selected marine algae indigenous to South Africa was investigated. Following the isolation and characterisation of a number of new and known compounds, the associated *in vitro* cytotoxic profiles of these new compounds was investigated.

*Plocamium maxillosum* yielded two new cyclic polyhalogenated monoterpenes which were characterised as 2*E*-chloromethine-4*E*-chlorovinyl-4-methyl-5-cyclohexen-1-one (**2.1**) and 2*Z*-chloromethine-4*E*-chlorovinyl-4-methyl-5-cyclohexen-1-one (**2.2**) on the basis of one and two dimensional NMR spectroscopic data and MS analysis. These compounds were also found to have good cytotoxic activity against breast cancer cell lines. Although these compounds are based on a regular monoterpene skeleton, they represent an uncommon feature not often seen in cyclic halogenated monoterpenes from marine algae.

*Plocamium robertiae* yielded one new cyclic polyhalogenated monoterpene identified as 4,5-dibromo-5-chloromethyl-1-chlorovinyl-2-chloro-methylcyclohexane (**2.6**) and one known compound called 2,4-dichloro-1-chlorovinyl-1-methylcyclohexane-5-ene or Plocamene D (**2.9**).

*Portieria hornemannii* was collected from Port Edward in Natal and yielded three new compounds, namely; 3*Z*-1,6-dibromo-3-(bromomethylidene)-2,7-dichloro-7-methyloctane (**3.1**), 1*E*,3*Z*-1,6-dibromo-3-(bromomethylidene)-7-chloro-7-methyloct-1-ene (**3.2**), 1*Z*,3*Z*-1,6-dibromo-3-(bromomethylidene)-7-chloro-7-methyloct-1-ene (**3.3**), and one known compound, namely; 3*S*,6*R*-6-bromo-3-(bromomethyl)-3,7-dichloro-7-methyloct-1-ene (**3.4**). Compounds **3.1** and **3.2** showed no cytotoxic activity against breast cancer cells.

Another *Portieria hornemannii* sample was collected from Noordhoek in the Eastern Cape, it yielded one known compound referred to as 3Z-6-bromo-3-(bromomethylidene)-2,7-dichloro-7-methyloct-1-ene (**3.5**), as well as one new compound called portieric acid A (**3.6**) or 5-bromo-2-(bromomethylidene)-6-chloro-6-methylheptanoic acid. Portieric acid A showed slight cytotoxic activity and also represents a new class of compound within the genus *Portieria*.

The isolation of secondary metabolites from the South African red alga, *Laurencia glomerata*, yielded two known compounds; 7-hydroxylaurene (**4.9**) and *cis*-neolaurencenyne (**4.12**), as well as one chamigrane related compound (**4.11**). *Laurencia flexuosa* yielded one known compound called 3Z-bromofucin (**4.13**). Using <sup>1</sup>H NMR, GC and molecular systematics, a novel method for identifying different species of *Laurencia* was also investigated.

To my wife, Harriet Maria Knott, here is to making dreams a reality...



## ***Searching Seaweed***

*by Michael Knott*

*Oh the mystery of your leaves, will they ever cure nature's disease?*

*Your unknown chemistry is magic, but the hours in the lab can be tragic.*

*Pure compounds still unwritten and new, will you hold the key to Man's pale hue?*

*Novel, new, naughty and nice, will my proton look clean at a price?*

*Terpenes dance together on a crude stage, but a silica column makes single file come of age.*

*Hunting the unseen impossible dream, make order from chaos on a merry stream.*

*Explain the invisible on a TLC plate, only then will you understand your very own fate.*

*Search the carbons, search your soul, you'll find weird things in this black hole.*

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# Table of Contents

|                                   |              |
|-----------------------------------|--------------|
| <b>Abstract.....</b>              | <b>ii</b>    |
| <b>Acknowledgments.....</b>       | <b>vi</b>    |
| <b>Table of Contents.....</b>     | <b>vii</b>   |
| <b>List of Figures.....</b>       | <b>xii</b>   |
| <b>List of Schemes.....</b>       | <b>xvii</b>  |
| <b>List of Tables.....</b>        | <b>xviii</b> |
| <b>List of Abbreviations.....</b> | <b>xxii</b>  |

|                                                                                                                       |          |
|-----------------------------------------------------------------------------------------------------------------------|----------|
| <b>Chapter one: Introduction</b>                                                                                      | <b>1</b> |
| 1.1 Introduction to marine algae.....                                                                                 | 1        |
| 1.2 The natural product chemistry of South African marine algae.....                                                  | 5        |
| 1.3 Rationale behind the study and project objectives.....                                                            | 8        |
| 1.4 Review of cytotoxic compounds isolated from <i>Plocamium</i> , <i>Portieria</i> and <i>Laurencia</i> species..... | 12       |
| 1.5 References.....                                                                                                   | 58       |

|                                                                                                                                      |           |
|--------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>Chapter two: Polyhalogenated cyclic monoterpenes from the red alga <i>Plocamium maxillosum</i> and <i>Plocamium robertiae</i></b> | <b>65</b> |
| 2.1 Abstract.....                                                                                                                    | 65        |
| 2.2 Introduction.....                                                                                                                | 65        |
| 2.3 Chapter aims.....                                                                                                                | 66        |
| 2.4 Results and discussion.....                                                                                                      | 66        |
| 2.4.1 Collection, extraction and isolation.....                                                                                      | 67        |
| 2.4.2 Characterisation of compounds from <i>Plocamium maxillosum</i> and <i>Plocamium robertiae</i> .....                            | 69        |
| 2.5 Experimental section.....                                                                                                        | 86        |
| 2.5.1 General experimental procedures.....                                                                                           | 86        |
| 2.5.2 Plant material.....                                                                                                            | 87        |
| 2.5.3 Extraction and isolation.....                                                                                                  | 87        |
| 2.5.4 Compounds.....                                                                                                                 | 88        |
| 2.5.5 Cytotoxicity assays.....                                                                                                       | 90        |
| 2.6 References.....                                                                                                                  | 91        |
| 2.7 Supplementary data.....                                                                                                          | 93        |
| 2.8 Appendix 1.....                                                                                                                  | 97        |

**Chapter three: The isolation of halomon related secondary metabolites from the red alga *Portieria hornemannii*** **116**

|       |                                                                                      |     |
|-------|--------------------------------------------------------------------------------------|-----|
| 3.1   | Abstract.....                                                                        | 116 |
| 3.2   | Introduction.....                                                                    | 117 |
| 3.3   | Chapter aims.....                                                                    | 118 |
| 3.4   | Results and discussion.....                                                          | 118 |
| 3.4.1 | Collection, extraction and isolation.....                                            | 118 |
| 3.4.2 | Characterisation of halogenated monoterpenes from <i>Portieria hornemannii</i> ..... | 123 |
| 3.5   | Experimental section.....                                                            | 142 |
| 3.5.1 | General experimental procedures.....                                                 | 142 |
| 3.5.2 | Plant material.....                                                                  | 143 |
| 3.5.3 | Extraction and isolation.....                                                        | 143 |
| 3.5.4 | Compounds.....                                                                       | 145 |
| 3.5.5 | Cytotoxicity assays.....                                                             | 145 |
| 3.6   | References.....                                                                      | 147 |
| 3.7   | Supplementary data.....                                                              | 149 |
| 3.8   | Appendix 1.....                                                                      | 149 |

|                                                                                                       |            |
|-------------------------------------------------------------------------------------------------------|------------|
| <b>Chapter four: The natural product chemistry of selected South African <i>Laurencia</i> species</b> | <b>164</b> |
| 4.1 Abstract.....                                                                                     | 164        |
| 4.2 Introduction.....                                                                                 | 164        |
| 4.3 Chapter aims.....                                                                                 | 167        |
| 4.4 Results and discussion.....                                                                       | 167        |
| 4.4.1 Collection, extraction and isolation.....                                                       | 167        |
| 4.4.2 Characterisation of metabolites from selected <i>Laurencia</i> species.....                     | 170        |
| 4.5 Experimental section.....                                                                         | 183        |
| 4.5.1 General experimental procedures.....                                                            | 183        |
| 4.5.2 Plant material.....                                                                             | 183        |
| 4.5.3 Extraction and isolation.....                                                                   | 183        |
| 4.5.4 Compounds.....                                                                                  | 185        |
| 4.6 References.....                                                                                   | 186        |

|                                                                                                                     |                |
|---------------------------------------------------------------------------------------------------------------------|----------------|
| <b>Chapter five: Molecular systematics and chemical taxonomy of selected South African <i>Laurencia</i> species</b> | <b>188</b>     |
| 5.1 Abstract.....                                                                                                   | 188            |
| 5.2 Introduction.....                                                                                               | 188            |
| 5.3 Chapter aims.....                                                                                               | 193            |
| 5.4 Results and discussion.....                                                                                     | 193            |
| 5.4.1 Collection and extraction.....                                                                                | 193            |
| 5.4.2 NMR and GC crude extracts from selected <i>Laurencia</i> species.....                                         | 194            |
| 5.5 Experimental section.....                                                                                       | 210            |
| 5.5.1 General experimental procedures.....                                                                          | 210            |
| 5.5.2 Plant material.....                                                                                           | 210            |
| 5.5.3 Extraction and isolation.....                                                                                 | 210            |
| 5.6 References.....                                                                                                 | 212            |
| 5.7 Appendix 1.....                                                                                                 | 213            |
| <br><b>Chapter six: Conclusion</b>                                                                                  | <br><b>227</b> |
| 6.1 Conclusive remarks.....                                                                                         | 227            |
| 6.2 References.....                                                                                                 | 231            |

## List of Figures

|                    |                                                                                                                                                                       |    |
|--------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Figure 2.1</b>  | $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>2.1</b> .....                                                                                   | 69 |
| <b>Figure 2.2</b>  | $^{13}\text{C}$ ( $\text{CDCl}_3$ , 100 MHz) NMR spectra of compound <b>2.1</b> .....                                                                                 | 70 |
| <b>Figure 2.3</b>  | Selected COSY, HSQC and HMBC correlations (a); HMBC correlations (b).....                                                                                             | 70 |
| <b>Figure 2.4</b>  | HMBC correlations from the diastereotopic methylene signals at $\delta$ 2.96 and 2.66 to the olefinic carbon at $\delta$ 134.0 (amongst others).....                  | 71 |
| <b>Figure 2.5</b>  | HMBC correlations from the olefinic methine at $\delta$ 7.26 to carbon signals at $\delta$ 134.0 and 184.9.....                                                       | 72 |
| <b>Figure 2.6</b>  | Selected NOESY correlations and proposed numbering system for compound <b>2.1</b> .....                                                                               | 72 |
| <b>Figure 2.7</b>  | The anisotropic effect of the carbonyl deshields <i>trans</i> $\beta$ -chloroenones, while upfield shift is observed in known <i>cis</i> - $\beta$ -chloroenones..... | 73 |
| <b>Figure 2.8</b>  | $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>2.2</b> .....                                                                                   | 74 |
| <b>Figure 2.9</b>  | $^{13}\text{C}$ ( $\text{CDCl}_3$ , 100 MHz) NMR spectra of compound <b>2.2</b> .....                                                                                 | 74 |
| <b>Figure 2.10</b> | Different cyclic monoterpene skeletons (A-C) present in marine algae.....                                                                                             | 76 |
| <b>Figure 2.11</b> | Dose-dependent inhibition of breast cancer cell viability by compounds <b>2.1</b> and <b>2.2</b> .....                                                                | 78 |
| <b>Figure 2.12</b> | $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>2.6</b> .....                                                                                   | 79 |
| <b>Figure 2.13</b> | $^{13}\text{C}$ ( $\text{CDCl}_3$ , 100 MHz) NMR spectra of compound <b>2.6</b> .....                                                                                 | 79 |
| <b>Figure 2.14</b> | Proposed numbering system for compound <b>2.6</b> .....                                                                                                               | 80 |
| <b>Figure 2.15</b> | $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>2.9</b> .....                                                                                   | 84 |
| <b>Figure 2.16</b> | $^{13}\text{C}$ ( $\text{CDCl}_3$ , 100 MHz) NMR spectra of compound <b>2.9</b> .....                                                                                 | 84 |
| <b>Figure 2.17</b> | HR-GCTOFMS data for compound <b>2.1</b> .....                                                                                                                         | 93 |
| <b>Figure 2.18</b> | HR-GCTOFMS data for compound <b>2.2</b> .....                                                                                                                         | 94 |



|                                                                                                                                                              |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Figure 2.19</b> HR-GCTOFMS data for compound <b>2.6</b> .....                                                                                             | 95  |
| <b>Figure 2.20</b> A previous study on <i>P. maxillosum</i> using gas chromatography-mass spectrometry (GC-MS).....                                          | 95  |
| <b>Figure 2.21</b> $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of the crude $\text{CH}_2\text{Cl}_2$ extract of <i>Plocamium maxillosum</i> ..... | 96  |
| <b>Figure 2.22</b> $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of the crude hexane extract of <i>Plocamium robertiae</i> .....                    | 96  |
| <b>Figure 3.1</b> Fraction 1 - Hexane extract of PE07-4A.....                                                                                                | 120 |
| <b>Figure 3.2</b> Fraction 2 - $\text{CH}_2\text{Cl}_2$ extract of PE07-4B.....                                                                              | 120 |
| <b>Figure 3.3</b> Fraction 1 - Hexane extract of NDK081212.....                                                                                              | 122 |
| <b>Figure 3.4</b> Fraction 2 - $\text{CH}_2\text{Cl}_2$ extract of NDK081212.....                                                                            | 122 |
| <b>Figure 3.5</b> $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>3.1</b> .....                                                        | 123 |
| <b>Figure 3.6</b> HSQC correlations of compound <b>3.1</b> .....                                                                                             | 124 |
| <b>Figure 3.7</b> HMBC correlations for compound <b>3.1</b> .....                                                                                            | 125 |
| <b>Figure 3.8</b> Selected HMBC correlations for compound <b>3.1</b> .....                                                                                   | 125 |
| <b>Figure 3.9</b> $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>3.2</b> .....                                                        | 128 |
| <b>Figure 3.10</b> $^{13}\text{C}$ NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of compound <b>3.2</b> .....                                                    | 128 |
| <b>Figure 3.11</b> $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>3.3</b> .....                                                       | 131 |
| <b>Figure 3.12</b> $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>3.4</b> .....                                                       | 133 |
| <b>Figure 3.13</b> $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>3.5</b> .....                                                       | 135 |
| <b>Figure 3.14</b> $^{13}\text{C}$ NMR ( $\text{CDCl}_3$ , 100 MHz) spectra of compound <b>3.5</b> .....                                                     | 135 |
| <b>Figure 3.15</b> $^1\text{H}$ NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound <b>3.6</b> .....                                                       | 137 |
| <b>Figure 3.16</b> Common fragment found in halomon related compounds.....                                                                                   | 138 |
| <b>Figure 3.17</b> Selected HMBC correlations data for compound <b>3.6</b> .....                                                                             | 139 |

|                                                                                                                                       |     |
|---------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Figure 3.18</b> Possible mechanism of synthesis of portieric acid A.....                                                           | 139 |
| <b>Figure 3.19</b> PE07-4A1 (Fraction 3 - 100% hexane fraction obtained from the silica column).....                                  | 149 |
| <b>Figure 3.20</b> PE07-4A2 (Fraction 4 - 90% hexane fraction obtained from the silica column).....                                   | 149 |
| <b>Figure 3.21</b> IR spectra for compound <b>3.6</b> .....                                                                           | 150 |
| <b>Figure 3.22</b> LREIMS data for compound <b>3.1</b> .....                                                                          | 150 |
| <b>Figure 3.23</b> HR-GCTOFMS data for compound <b>3.1</b> .....                                                                      | 151 |
| <b>Figure 3.24</b> LREIMS and HREIMS data for compound <b>3.2</b> .....                                                               | 152 |
| <b>Figure 3.25</b> HR-GCTOFMS data for compound <b>3.2</b> .....                                                                      | 153 |
| <b>Figure 3.26</b> LR-TOFMS data of portieric acid A ( <b>3.6</b> ).....                                                              | 154 |
| <b>Figure 3.27</b> Worldwide distribution of the number of different kinds of <i>Portieria hornemannii</i> found in each country..... | 155 |
| <b>Figure 4.1</b> <sup>1</sup> H NMR spectrum (CDCl <sub>3</sub> , 400 MHz) of compound <b>4.9</b> .....                              | 170 |
| <b>Figure 4.2</b> <sup>13</sup> C (CDCl <sub>3</sub> , 100 MHz) NMR spectra of compound <b>4.9</b> .....                              | 171 |
| <b>Figure 4.3</b> <sup>1</sup> H NMR spectrum (CDCl <sub>3</sub> , 400 MHz) of compound <b>4.11</b> .....                             | 173 |
| <b>Figure 4.4</b> Numbering and selected HMBC for compound <b>4.11</b> .....                                                          | 174 |
| <b>Figure 4.5</b> <sup>1</sup> H NMR spectrum (CDCl <sub>3</sub> , 400 MHz) of compound <b>4.12</b> .....                             | 177 |
| <b>Figure 4.6</b> <sup>13</sup> C (CDCl <sub>3</sub> , 100 MHz) NMR spectra of compound <b>4.12</b> .....                             | 177 |
| <b>Figure 4.7</b> <sup>1</sup> H NMR spectrum (CDCl <sub>3</sub> , 400 MHz) of compound <b>4.13</b> .....                             | 180 |
| <b>Figure 4.8</b> <sup>1</sup> H NMR spectrum (CDCl <sub>3</sub> , 400 MHz) of bromofucin.....                                        | 180 |
| <b>Figure 5.1</b> <sup>1</sup> H NMR spectra of the D928 ( <i>L. flexuosa</i> ) crude extract.....                                    | 195 |
| <b>Figure 5.2</b> <sup>1</sup> H NMR spectra of the D930 ( <i>L. natalensis</i> ) crude extract.....                                  | 195 |
| <b>Figure 5.3</b> <sup>1</sup> H NMR spectra of the D943 ( <i>L. glomerata</i> ) crude extract.....                                   | 196 |

|                                                                                                                                                       |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Figure 5.4</b> $^1\text{H}$ NMR spectra of the D947 ( <i>Laurencia</i> sp. (growing as a clump) – perhaps <i>L. glomerata</i> ) crude extract..... | 197 |
| <b>Figure 5.5</b> $^1\text{H}$ NMR spectra of the D903 ( <i>Laurencia</i> sp. (attenuated)) crude extract.....                                        | 198 |
| <b>Figure 5.6</b> $^1\text{H}$ NMR spectra of the D922 ( <i>Laurencia</i> sp.) crude extract.....                                                     | 198 |
| <b>Figure 5.7</b> $^1\text{H}$ NMR spectra of the D926 ( <i>L. glomerata</i> ) crude extract.....                                                     | 199 |
| <b>Figure 5.8</b> $^1\text{H}$ NMR spectra of the D927 crude extract.....                                                                             | 199 |
| <b>Figure 5.9</b> GC trace of D928 ( <i>L. flexuosa</i> ) crude extract.....                                                                          | 201 |
| <b>Figure 5.10</b> GC trace of D930 ( <i>L. natalensis</i> ) crude extract.....                                                                       | 201 |
| <b>Figure 5.11</b> GC trace of D943 ( <i>L. glomerata</i> ) crude extract.....                                                                        | 202 |
| <b>Figure 5.12</b> GC trace of D947 ( <i>Laurencia</i> sp. growing as a clump – perhaps <i>L. glomerata</i> ) crude extract.....                      | 202 |
| <b>Figure 5.13</b> GC trace of D903 ( <i>Laurencia</i> sp. (attenuated)) crude extract.....                                                           | 203 |
| <b>Figure 5.14</b> GC trace of D922 ( <i>Laurencia</i> sp.) crude extract.....                                                                        | 203 |
| <b>Figure 5.15</b> GC trace of D926 ( <i>L. glomerata</i> ) crude extract.....                                                                        | 204 |
| <b>Figure 5.16</b> GC trace of D927 crude extract.....                                                                                                | 204 |
| <b>Figure 5.17</b> Crude extract of an unknown species of <i>Laurencia</i> (KOS 110121).....                                                          | 207 |
| <b>Figure 5.18</b> Crude extract of an unknown species of <i>Laurencia</i> (D1024).....                                                               | 208 |
| <b>Figure 5.19</b> Crude extract of <i>L. complanata</i> (D1053).....                                                                                 | 208 |
| <b>Figure 5.20</b> $^1\text{H}$ NMR data of the D930 ( <i>L. natalensis</i> ) crude extract.....                                                      | 214 |
| <b>Figure 5.21</b> $^1\text{H}$ NMR data of the D943 ( <i>L. glomerata</i> ) crude extract.....                                                       | 215 |
| <b>Figure 5.22</b> $^1\text{H}$ NMR data of the D947 ( <i>Laurencia</i> sp. (growing as a clump) – perhaps <i>L. glomerata</i> ) crude extract.....   | 216 |
| <b>Figure 5.23</b> $^1\text{H}$ NMR data of the D903 ( <i>Laurencia</i> sp. (attenuated)) crude extract.....                                          | 217 |
| <b>Figure 5.24</b> $^1\text{H}$ NMR data of the D922 ( <i>Laurencia</i> sp.) crude extract.....                                                       | 218 |

|                    |                                                                                              |     |
|--------------------|----------------------------------------------------------------------------------------------|-----|
| <b>Figure 5.25</b> | $^1\text{H}$ NMR data of the D926 ( <i>L. glomerata</i> ) crude extract.....                 | 219 |
| <b>Figure 5.26</b> | $^1\text{H}$ NMR data of the D927 crude extract.....                                         | 220 |
| <b>Figure 5.27</b> | $^1\text{H}$ NMR spectra of the D928 ( <i>L. flexuosa</i> ) crude extract.....               | 221 |
| <b>Figure 5.28</b> | $^1\text{H}$ NMR spectra of the D930 ( <i>L. natalensis</i> ) crude extract.....             | 222 |
| <b>Figure 5.29</b> | $^1\text{H}$ NMR spectra of the D903 ( <i>Laurencia</i> sp. (attenuated)) crude extract..... | 223 |
| <b>Figure 5.30</b> | $^1\text{H}$ NMR spectra of the D922 ( <i>Laurencia</i> sp.) crude extract.....              | 224 |
| <b>Figure 5.31</b> | $^1\text{H}$ NMR spectra of the D926 ( <i>L. glomerata</i> ) crude extract.....              | 225 |
| <b>Figure 5.32</b> | $^1\text{H}$ NMR spectra of the D927 crude extract.....                                      | 226 |

## List of Schemes

|                                                                                                                          |     |
|--------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Scheme 2.1</b> Extraction and isolation of metabolites from <i>Plocamium maxillosum</i> .....                         | 67  |
| <b>Scheme 2.2</b> Extraction and isolation of metabolites from <i>Plocamium robertiae</i> .....                          | 68  |
| <b>Scheme 2.3</b> Proposed biosynthesis of <b>2.1</b> and <b>2.2</b> from putative precursor <b>2.4</b> .....            | 77  |
| <b>Scheme 3.1</b> Extraction and isolation of metabolites from <i>P. hornemannii</i><br>(Port Edward, January 2007)..... | 119 |
| <b>Scheme 3.2</b> Extraction and isolation of metabolites from <i>P. hornemannii</i><br>(Noordhoek, December 2008).....  | 121 |
| <b>Scheme 4.1</b> Extraction and isolation of metabolites from <i>L. glomerata</i> (Keurbooms,<br>September 2010).....   | 168 |
| <b>Scheme 4.2</b> Extraction and isolation of metabolites from <i>L. flexuosa</i> (Keurbooms,<br>September 2010).....    | 169 |
| <b>Scheme 4.3</b> Proposed mechanism for the synthesis of 3Z-bromofucin from<br>laurencenyne.....                        | 179 |

## List of Tables

|                                                                                                                                                                              |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Table 1.1</b> Marine derived experimental anti-cancer agents.....                                                                                                         | 9  |
| <b>Table 1.2</b> Minimal inhibitory concentration (MIC) [ $\mu\text{M}$ ] of test compounds <b>1.17-1.25</b><br>on several mammalian cell lines.....                         | 13 |
| <b>Table 1.3</b> Selected structural features and cytotoxic effects of compounds <b>1.34-1.40</b><br>on oesophageal cancer cells.....                                        | 17 |
| <b>Table 1.4</b> Results of comparative testing of compounds <b>1.8, 1.42-1.50</b> in<br>the NCI <i>in vitro</i> primary anti-tumour screen.....                             | 20 |
| <b>Table 1.5</b> Cytotoxic activity results ( $\text{IC}_{50}$ reported in $\mu\text{g/ml}$ ).....                                                                           | 24 |
| <b>Table 1.6</b> <i>In vitro</i> cytotoxicity of compounds <b>1.64-1.68</b> are expressed as $\text{IC}_{50}$ in $\mu\text{M}$ .....                                         | 25 |
| <b>Table 1.7</b> Anti-tumour activity ( $\text{IC}_{50}$ in $\mu\text{M}$ ) of selected cuparene sesquiterpenes isolated<br>from <i>Laurencia</i> species.....               | 29 |
| <b>Table 1.8</b> <i>In vitro</i> cytotoxic activities of two metabolites ( $\text{IC}_{50}$ $\mu\text{M}$ ).....                                                             | 30 |
| <b>Table 1.9</b> <i>In vitro</i> anti-tumour activities of compounds <b>1.91-1.93</b> against various cell lines<br>( $\text{IC}_{50}$ in $\mu\text{M}$ ).....               | 32 |
| <b>Table 1.10</b> <i>In vitro</i> anti-tumour activities of thyrсенol A-B and dehydrothyrсенiferol against<br>various cell lines ( $\text{IC}_{50}$ in $\mu\text{M}$ ).....  | 33 |
| <b>Table 1.11</b> $\text{IC}_{50}$ values of biological assays of pure polyether squalene derivatives from<br><i>Laurencia viridis</i> .....                                 | 36 |
| <b>Table 1.12</b> Selective cytotoxic activity of Laurefurenynes C and F.....                                                                                                | 37 |
| <b>Table 1.13</b> $\text{IC}_{50}$ [ $\mu\text{M}$ ] data for cytotoxic compounds isolated from <i>Laurencia</i> species<br>using A-431 cell lines.....                      | 40 |
| <b>Table 1.14</b> $\text{IC}_{50}$ [ $\mu\text{M}$ ] data for cytotoxic compounds isolated from <i>Portieria</i> and<br><i>Laurencia</i> species using A-549 cell lines..... | 43 |

|                                                                                                                                                                                |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Table 1.15</b> IC <sub>50</sub> [μM] data for cytotoxic compounds isolated from <i>Plocamium</i> and <i>Laurencia</i> species using CHO cell lines.....                     | 45  |
| <b>Table 1.16</b> IC <sub>50</sub> [μM] data for cytotoxic compounds isolated from <i>Plocamium</i> and <i>Laurencia</i> species using HeLa cell lines.....                    | 46  |
| <b>Table 1.17</b> IC <sub>50</sub> [μM] data for cytotoxic compounds isolated from <i>Laurencia</i> species using HT-29 cell lines.....                                        | 48  |
| <b>Table 1.18</b> IC <sub>50</sub> [μM] data for cytotoxic compounds isolated from <i>Laurencia</i> species using MCF-7 cell lines.....                                        | 51  |
| <b>Table 1.19</b> IC <sub>50</sub> [μM] data for cytotoxic compounds isolated from <i>Portieria</i> and <i>Laurencia</i> species using P-388 cell lines.....                   | 53  |
| <b>Table 1.20</b> IC <sub>50</sub> [μM] data for cytotoxic compounds isolated from <i>Plocamium</i> species using WHCO1 cell lines.....                                        | 55  |
| <b>Table 2.1</b> <sup>1</sup> H NMR (400 MHz), <sup>13</sup> C NMR (100 MHz), and HMBC data for compound <b>2.1</b> and <b>2.2</b> in CDCl <sub>3</sub> .....                  | 76  |
| <b>Table 2.2</b> <sup>1</sup> H NMR (400 MHz), <sup>13</sup> C NMR (100 MHz), COSY and HMBC data for compound <b>2.6</b> in CDCl <sub>3</sub> .....                            | 82  |
| <b>Table 2.3</b> <sup>13</sup> C (100MHz, CDCl <sub>3</sub> ) NMR data for compound <b>2.6</b> , as well as the literature values for compound <b>2.7</b> and <b>2.8</b> ..... | 83  |
| <b>Table 2.4</b> <sup>13</sup> C NMR data and <sup>1</sup> H NMR data for Plocamene D.....                                                                                     | 85  |
| <b>Table 2.5</b> Metabolites isolated from <i>Plocamium angustum</i> .....                                                                                                     | 97  |
| <b>Table 2.6</b> Metabolites isolated from <i>Plocamium brasiliense</i> .....                                                                                                  | 97  |
| <b>Table 2.7</b> Metabolites isolated from <i>Plocamium cartilagineum</i> .....                                                                                                | 97  |
| <b>Table 2.8</b> Metabolites isolated from <i>Plocamium coccineum</i> .....                                                                                                    | 99  |
| <b>Table 2.9</b> Metabolites isolated from <i>Plocamium corallorhiza</i> .....                                                                                                 | 99  |
| <b>Table 2.10</b> Metabolites isolated from <i>Plocamium cornutum</i> .....                                                                                                    | 100 |
| <b>Table 2.11</b> Metabolites isolated from <i>Plocamium costatum</i> .....                                                                                                    | 100 |

|                   |                                                                                                                                           |     |
|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Table 2.12</b> | Metabolites isolated from <i>Plocamium cruciferum</i> .....                                                                               | 100 |
| <b>Table 2.13</b> | Metabolites isolated from <i>Plocamium hamatum</i> .....                                                                                  | 101 |
| <b>Table 2.14</b> | Metabolites isolated from <i>Plocamium mertinsii</i> .....                                                                                | 101 |
| <b>Table 2.15</b> | Metabolites isolated from <i>Plocamium oregonum</i> .....                                                                                 | 101 |
| <b>Table 2.16</b> | Metabolites isolated from <i>Plocamium suhrii</i> .....                                                                                   | 102 |
| <b>Table 2.17</b> | Metabolites isolated from <i>Plocamium telfairae</i> .....                                                                                | 102 |
| <b>Table 2.18</b> | Metabolites isolated from <i>Plocamium violaceum</i> .....                                                                                | 102 |
| <b>Table 2.19</b> | Metabolites isolated from <i>Plocamium</i> species.....                                                                                   | 103 |
| <b>Table 3.1</b>  | <sup>1</sup> H NMR (400 MHz), <sup>13</sup> C NMR (100 MHz), COSY, HMBC and NOESY data for compound <b>3.1</b> in CDCl <sub>3</sub> ..... | 127 |
| <b>Table 3.2</b>  | <sup>1</sup> H NMR (400 MHz) and <sup>13</sup> C NMR (100 MHz) data for compound <b>3.1</b> and <b>3.2</b> in CDCl <sub>3</sub> .....     | 130 |
| <b>Table 3.3</b>  | <sup>1</sup> H NMR (400 MHz) and <sup>13</sup> C NMR (100 MHz) data for compound <b>3.2</b> and <b>3.3</b> in CDCl <sub>3</sub> .....     | 132 |
| <b>Table 3.4</b>  | <sup>1</sup> H NMR (CDCl <sub>3</sub> , 400 MHz) data for compound <b>3.4</b> and 2-dechlorohalomon....                                   | 134 |
| <b>Table 3.5</b>  | <sup>1</sup> H NMR (400 MHz) and <sup>13</sup> C NMR (100 MHz) data for compound <b>3.5</b> , compared with known literature values.....  | 136 |
| <b>Table 3.6</b>  | <sup>1</sup> H NMR (400 MHz), <sup>13</sup> C NMR (100 MHz), COSY and HMBC data for compound <b>3.6</b> in CDCl <sub>3</sub> .....        | 140 |
| <b>Table 4.1</b>  | Selected classes of carbon skeletons produced by <i>Laurencia</i> species.....                                                            | 165 |
| <b>Table 4.2</b>  | <sup>1</sup> H NMR (400 MHz) and <sup>13</sup> C NMR (CDCl <sub>3</sub> , 100 MHz) data of 7-hydroxylaurene (compound <b>4.9</b> ).....   | 172 |
| <b>Table 4.3</b>  | <sup>13</sup> C NMR (100 MHz) data for compound <b>4.11</b> , as well as compound <b>4.1</b> .....                                        | 174 |
| <b>Table 4.4</b>  | <sup>1</sup> H NMR (400 MHz), <sup>13</sup> C NMR (100 MHz), COSY and HMBC data for compound <b>4.11</b> .....                            | 176 |



|                                                                                                                                                                                                                    |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Table 4.5</b> $^1\text{H}$ NMR (400 MHz) and $^{13}\text{C}$ NMR (100 MHz) data (in $\text{CDCl}_3$ ) comparing <i>cis</i> -neolaurencenyne from <i>Laurencia glomerata</i> and <i>Laurencia okamurai</i> ..... | 178 |
| <b>Table 4.6</b> $^1\text{H}$ NMR (400 MHz), and $^{13}\text{C}$ NMR data comparing the NMR data of 3 <i>Z</i> -bromofucin that was recently isolated, with the results obtained by Mann (2008).....               | 181 |
| <b>Table 5.1</b> Wet weight, dry weight and total extract weight of different <i>Laurencia</i> species.....                                                                                                        | 194 |
| <b>Table 5.2</b> GC parameters (Agilent Technologies 7890A, 7693 Auto-sampler).....                                                                                                                                | 211 |
| <b>Table 5.3</b> Identification number and total extract weight of different <i>Laurencia</i> species tested on the 600 MHz Bruker NMR.....                                                                        | 213 |

## List of Abbreviations

|                                 |                                                                     |
|---------------------------------|---------------------------------------------------------------------|
| °C                              | degrees Celsius                                                     |
| CH <sub>2</sub> Cl <sub>2</sub> | Dichloromethane                                                     |
| COSY                            | <sup>1</sup> H- <sup>1</sup> H Homonuclear Correlation Spectroscopy |
| d                               | doublet                                                             |
| dd                              | double doublet                                                      |
| DCM                             | Dichloromethane                                                     |
| DEPT                            | Distortionless Enhancement of Polarisation Transfer                 |
| DNA                             | Deoxyribose Nucleic Acid                                            |
| EIMS                            | Electron Impact Mass Spectrometry                                   |
| EtOAc                           | Ethyl Acetate                                                       |
| eV                              | electron Volt                                                       |
| FABMS                           | Fast Atom Bombardment Mass Spectrometry                             |
| Fn                              | Fraction                                                            |
| GC-MS                           | Gas Chromatography-Mass Spectrometry                                |
| HMBC                            | Heteronuclear Multiple Bond Correlation                             |
| HSQC                            | Heteronuclear Single Quantum Correlation                            |
| HPLC                            | High Performance Liquid Chromatography                              |
| HREIMS                          | High Resolution Electron Impact Mass Spectrometry                   |
| HR-GCTOFMS                      | High Resolution Gas Chromatography Time of Flight Mass Spectrometry |
| Hz                              | Hertz                                                               |
| IC <sub>50</sub>                | Inhibitory Concentration 50%                                        |

|          |                                                 |
|----------|-------------------------------------------------|
| IR       | Infrared                                        |
| <i>J</i> | Spin-Spin coupling constant (Hz)                |
| lr       | long range                                      |
| LRMS     | Low Resolution Mass Spectrometry                |
| LR-TOFMS | Low Resolution Time of Flight Mass Spectrometry |
| m        | multiplet                                       |
| MeOH     | Methanol                                        |
| MIC      | Minimum Inhibitory Concentration                |
| m/z      | mass to charge ratio                            |
| mw       | molecular weight                                |
| NCI      | National Cancer Institute                       |
| NMR      | Nuclear Magnetic Resonance                      |
| NOESY    | Nuclear Overhauser Enhancement Spectroscopy     |
| ppm      | parts per million                               |
| q        | quartet                                         |
| s        | singlet                                         |
| SAR's    | Structure Activity Relationships                |
| t        | triplet                                         |
| TLC      | Thin Layer Chromatography                       |
| UV       | Ultra Violet                                    |

## Chapter One

### Introduction

#### 1.1 Introduction to marine algae

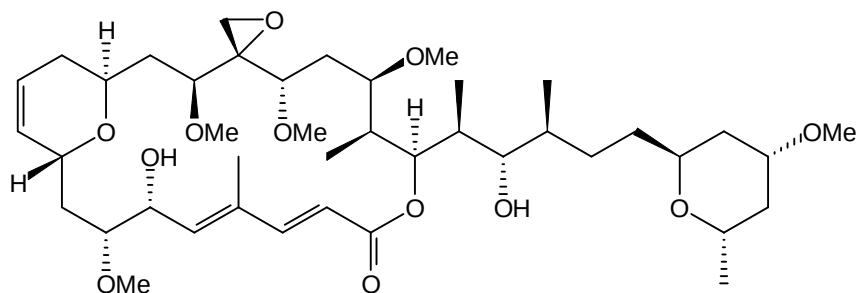
##### *Primary and secondary metabolism*

All living organisms, from the smallest bacterium to the biggest mammal, make use of basic chemical reactions to transform and interconvert organic compounds in order to live, grow and reproduce. Pathways for the metabolism of carbohydrates, proteins, fats and nucleic acids are found to be generally the same in all organisms; these reactions are collectively known as primary metabolism (Dewick, 2000; Rohmer *et al.*, 1993). While primary metabolism is encountered in all organisms, secondary metabolism is only found in specific organisms. Secondary metabolism is an expression of the individuality of the species, and in most cases the function of these compounds (or secondary metabolites) produced *via* this pathway remains unknown. Biologically active secondary metabolites have played a significant part in the development of modern medicine since its inception.

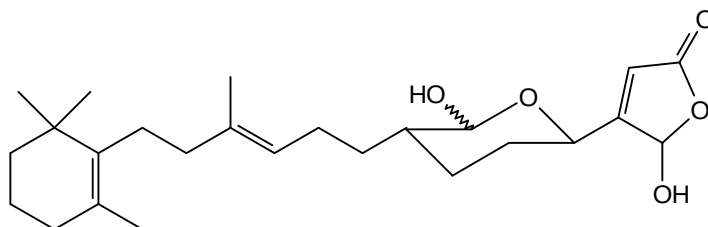
##### *The biological and commercial importance of marine algae*

The biodiversity of the planet is heavily weighted towards the oceans, with the majority of the phyla for the five kingdoms being aquatic. Estimates of the number of aquatic species range from 1.5 to 4.5 million, with the chemistry of the majority of these species being unknown (Munro *et al.*, 1994). With this barely explored biodiversity that is offered by the oceans, the potential for discovering new pharmaceutical compounds from the ocean is enormous. Over the past three decades, a number of natural products (or secondary metabolites) with potent pharmacological activities have been isolated, or derived from lead compounds originating in the ocean. Some of these compounds include antifungal agents [e.g. lobophorolide (**1.1**)] (El Gamal, 2010), anti-inflammatories [e.g. manoalide (**1.2**)] (Munro *et al.*, 1994), antiviral compounds [e.g. Ara-A, which was the lead compound used for the synthesis of acyclovir (**1.3**)] (Munro *et al.*, 1994), antibiotics derived from a marine fungus [e.g. cephalosporin (**1.4**)], insecticides [e.g. nereistoxin (**1.5**)], anthelmintic agents

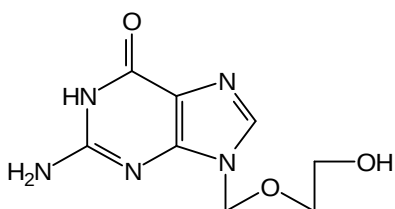
[e.g.  $\alpha$ -kainic acid (**1.6**)] (Trease and Evans, 1983), toxins [e.g. tetrodotoxin (**1.7**)] (Delgado and Remers, 1998) and anti-tumour compounds [e.g. halomon (**1.8**)] (Fuller *et al.*, 1992).



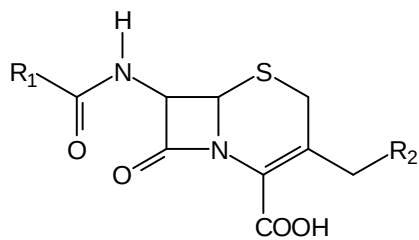
Lobophorolide (**1.1**)



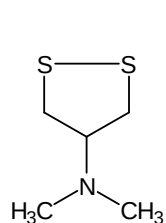
Manoalide (**1.2**)



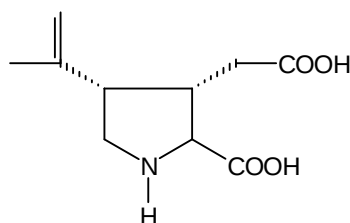
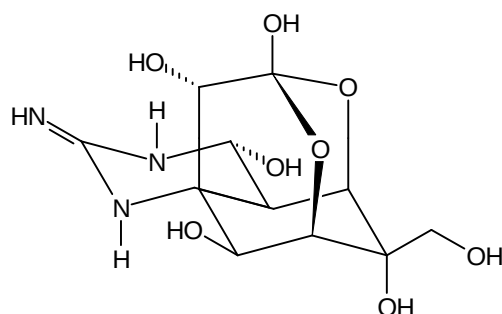
Acyclovir (**1.3**)



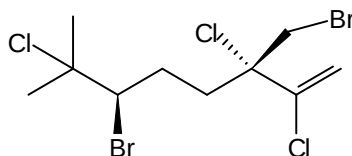
Cephalosporin (**1.4**)



Nereistoxin (1.5)

 $\alpha$ -Kainic acid (1.6)

Tetrodotoxin (1.7)



Halomon (1.8)

Marine algae offer a rich source of biologically active secondary metabolites. These secondary metabolites present a number of unusual compounds which have the potential, as seen above, to become important lead compounds for future drug development. Algae are classified into two main groups, namely, microalgae and macroalgae. Microalgae include blue-green algae, dinoflagellates and diatoms. Macroalgae or seaweed are made up of three groups of marine algae, namely; Chlorophyta (Green algae), Phaeophyta (Brown algae) and Rhodophyta (red algae). The chemistry of both microalgae and macroalgae has provided a fascinating array of interesting and biologically active compounds. Mankind uses seaweed

for a number of different functions, but most often seaweed is used as a rich source of food and nutrition, as well as being an abundant source of agar.

South Africa has a small seaweed industry which is based on the utilisation of several brown and red algae. On the west coast, kelps such as *Ecklonia maxima* and *Laminaria pallida* are collected for alginates. *Ecklonia* is also used for the production of plant-growth stimulant products such as commercial fertilisers, or simply harvested as feed for commercially farmed abalone. *Gracilaria* is collected at Saldanha Bay in the Western Cape and utilised as a rich source of hydrocolloid agar. In the Eastern Cape, *Gelidium* species are harvested from intertidal shores as a good source of agar (Lubke *et al.*, 1998). Worldwide, *Gracilaria*, *Gelidium* and *Pterocladia* species are commonly used in the production of agar.

In China and Japan, diseases due to iodine deficiency are often treated with macroalgae. In addition to this seaweeds have also been used as wound dressings and in many beauty preparations. Seaweeds have also been used to treat various intestinal disorders, vitamin shortages and have even been used as hypoglycaemic agents (Lamela *et al.*, 1989).

Macroalgae have been used as an important source of food in Japan and China for a long time. Several red algae are eaten, the most popular are known as dulse or *Palmaria palmata*, which is a good source of fibre, and carrageen moss (*Chondrus crispus* and *Mastocarpus stellatus*) from which carrageenan is isolated. Carrageenan is a thickener and stabiliser which is commonly used in various milk products such as ice-cream. Globally, the red algae *Kappaphycus* and *Betaphycus* are also important sources of carrageenan (El Gamal, 2010). Nori is the Japanese name for various edible seaweed species which come from the red alga *Porphyra*, including most notably, *P. yezoensis* and *P. tenera*. Of all the red algae, nori is utilised the most. Amongst other things, nori is an indispensable ingredient in the making of sushi. This valuable marine crop is mainly grown by means of aquaculture in China, Japan and South Korea. Total nori production is estimated to be worth over two billion US\$ per year (El Gamal, 2010). Various brown algae are also eaten and are known to be rich in carbohydrates such as laminarin (Deville *et al.*, 2004).

## 1.2 The natural product chemistry of South African marine algae

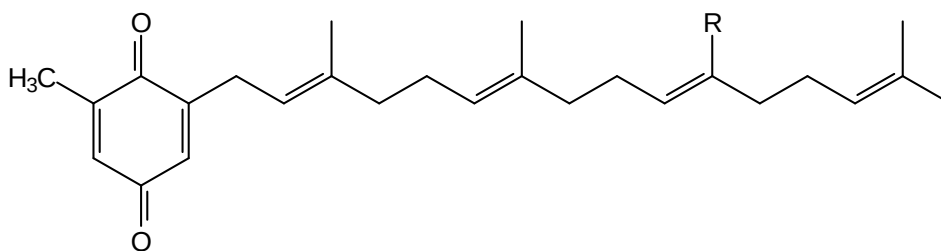
The coastline of South Africa stretches about 3000 km from southern Namibia in the west to southern Mozambique in the east. South Africa has a vast diversity of endemic marine algae, whose chemistry and pharmacology have never been studied. This diversity is due to the unusual mixing of waters from the warm Agulhas and cold Benguela currents, creating an environment unique to this area. This feature causes the coastal area stretching from Port St. Johns to Cape Town to be characterised by a high level of endemic marine species. The coastline of southern Africa (from northern Namibia to southern Mozambique) has over 10 000 species of marine *fauna* and *flora* which amounts to approximately 15% of all the coastal marine species known worldwide. Of this amazing assemblage, approximately 12% of these species are endemic (Branch *et al.*, 1994).

Literature reports on the natural product chemistry of South African marine algae are rather scarce. One of the first publications reported was that of the isolation of four halogenated chamigrenes from the South African red algae *Laurencia glomerata* (Elsworth and Thomson, 1989). These compounds are discussed further in chapter four. Another study later screened 56 South African crude extracts of marine algae for antimicrobial activity; however, this was only a pharmacological study in which characterisation of the compounds responsible for activity were not reported (Vlachos *et al.*, 1997).

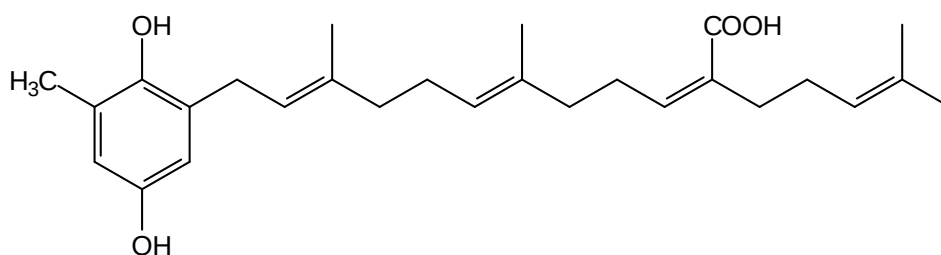
A number of new halogenated compounds were isolated from *Plocamium corallorhiza* (Knott *et al.*, 2005; Mann *et al.*, 2007). These compounds are described under the review of cytotoxic compounds previously isolated from *Plocamium* species which follows on page 12.

Four metabolites were isolated from *Sargassum heterophyllum*, sargaquinoic acid (**1.9**), sargaquinal (**1.10**) sargahydroquinoic acid (**1.11**) and fucoxanthin (**1.12**) (Afolayan *et al.*, 2008). Fucoxanthin and sargaquinal showed good antiplasmodial activity toward a chloroquine-sensitive strain of *Plasmodium falciparum*, while sargaquinoic acid and sargahydroquinoic acid were only moderately active.

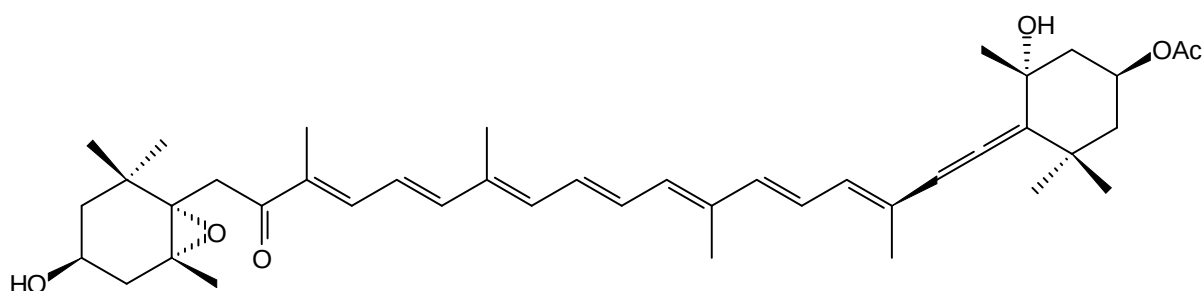




Sargaquinoic acid, R = COOH (**1.9**); Sargaquinal, R = CHO (**1.10**)

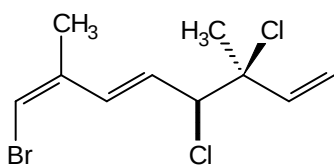


Sargahydroquinoic acid (**1.11**)

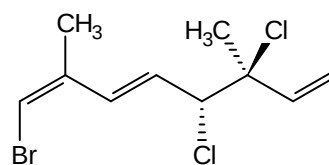


Fucoxanthin (**1.12**)

Two new halogenated monoterpenes (**1.13-1.14**) were later isolated from *Plocamium cornutum*. These compounds also showed good antiplasmodial activity (Afolayan *et al.*, 2009).



**1.13**



**1.14**

Recently, the identification and *in vitro* anti-oesophageal cancer activity of a series of halogenated monoterpenes isolated from *Plocamium suhrii* and *P. cornutum* was published (Antunes *et al.*, 2011). These compounds are described below under the review of cytotoxic compounds isolated from *Plocamium* species.

An article on the antiplasmodial and antimicrobial activities of 78 crude South African marine algal extracts also provided valuable insight into the interesting pharmacological potential of our rich marine *flora* (Lategan *et al.*, 2009).

In a paper written by Davies-Coleman (2010) entitled, '*Natural products research in South Africa: End of an era on land or the beginning of an endless opportunity in the sea?*' it was concluded that marine natural products cannot be ignored as a source of new drugs. The reason for this is that natural products are often required to bind to other proteins to execute the bioactivities for which they were produced by a living organism (Davies-Coleman, 2010). Natural products have privileged structures over random synthetic products as they are designed to be able to bind to specific protein receptors.

Davies-Coleman (2010) further suggests that South Africa's marine *fauna* and *flora* remain a relatively untapped source of novel bio-molecules with unfulfilled pharmaceutical and agrochemical potential, and that endemic South African marine *fauna* and *flora* offer rich rewards for marine natural product chemists in search of novel bioactive secondary metabolites with possible medicinal properties.

### 1.3 Rationale behind the study and project objectives

Cancer is the leading cause of death worldwide and accounted for 7.9 million deaths (around 13% of all deaths) in 2007. For example, breast cancer is responsible for the highest cause of death in women between the ages of 35 and 60 years. With the exception of some geographical differences, breast cancer is also the most common form of cancer that exists amongst women (Pec *et al.*, 2003). In 2008, breast cancer alone caused 460 000 deaths worldwide according to The International Agency for Research on Cancer (2010). Hormone unresponsive breast cancer is still associated with poorer prognosis than hormone receptor expressing malign, mammary tumours. With this in mind, it is obvious that there is a crucial need for new and effective compounds to treat unresponsive breast cancer.

Various anti-cancer agents have been isolated or derived from plants and terrestrial micro-organisms. Four decades ago, C-nucleosides from the Caribbean sponge *Cryptotheca crypta* provided the basis for the synthesis of cytarabine, the first marine-derived anti-cancer agent to be developed for clinical use. Today, cytarabine is used for the treatment of leukaemia and lymphoma. A fluorinated derivate of cytarabine called gemcitabine, has been approved for use in patients with pancreatic, breast, bladder and non-small-cell lung cancer. Several new experimental anti-cancer agents have been isolated from marine sources over the years. Some have even entered clinical and pre-clinical trials (Table 1.1) (Schwartzmann *et al.*, 2001).

**Table 1.1** Marine derived experimental anti-cancer agents (Schwartzmann *et al.*, 2001)

| Organism                            | Group        | Metabolite        | Location           |
|-------------------------------------|--------------|-------------------|--------------------|
| <i>Trididemnum soldium</i>          | Tunicate     | Didemnin B        | Caribbean          |
| <i>Bugula neritina</i>              | Bryozoan     | Bryostatin 1      | Gulf of California |
| <i>Ecteinascidia turbinata</i>      | Tunicate     | Ecteinascidin-743 | Caribbean          |
| <i>Halichondria okadae</i>          | Sponge       | Halichondrin B    | Okinawa            |
| <i>Dolabella auricularia</i>        | Sea hare     | Dolastatin 10     | Indian Ocean       |
| <i>Portieria hornemannii</i>        | Red alga     | Halomon           | Philippines        |
| <i>Apidium albicans</i>             | Tunicate     | Aplidine          | Mediterranean      |
| <i>Aplysia kurodai</i>              | Sea hare     | Aplyronine A      | Japan              |
| <i>Elysia rubefescens</i>           | Mollusc      | Kahalalide F      | Hawaii             |
| <i>Mycale ap.</i>                   | Sponge       | Mycaperoxide B    | Thailand           |
| <i>Micromonospora marina</i>        | Actinomycete | Thiocoraline      | Mozambique Strait  |
| <i>Ascidian didemnum granulatum</i> | Tunicate     | Granulatimide     | Brazil             |

With the idea of finding new cytotoxic compounds from South African marine algae, the following rationale was applied to optimise the chances of successful drug discovery;

A literature review of marine algae belonging to Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae) soon revealed that Rhodophyta is far more prolific in producing cytotoxic secondary metabolites than both Chlorophyta and Phaeophyta. Red algae are generally more pharmacologically active against a wide variety of different *in vitro* cell lines when compared to a variety of other algal classes. The reason for this is that Rhodophyta possess the highest abundance of unique biosynthetic pathways necessary for organohalogen production (Kladi *et al.*, 2004). Further to this, halogenated low molecular weight metabolites have exhibited an impressive range of biological properties; from antimicrobial to insecticidal activity. It is believed that these halogenated compounds are

produced by seaweeds as part of a defence system against micro-organism infections and herbivore grazing (Goodwin *et al.*, 1997). In addition to this, it has been postulated that halogenated compounds are also used to assist in anti-fouling and reduce space competition amongst competing marine algae (Dworjanyn *et al.*, 1999).

A large number of organohalogens have already been isolated from Rhodophyta (Blunt *et al.*, 2011; Faulkner, 2002). The halogenated metabolites that have been isolated to date fall into four major chemical categories, namely; halogenated carbonyls, fatty acid metabolites, phenols and terpenoids. Studies into the natural product chemistry of red seaweed were further stimulated by the discovery of halomon (**1.8**) - an acyclic monoterpene which exhibited selective anti-tumour activity in the National Cancer Institute's (NCI) human tumour and disease orientated *in vitro* screening lines (Fuller *et al.*, 1992).

Red seaweeds from the families Plocamiaceae and Rhizophyllidaceae are known to produce a wide variety of halogenated monoterpenes. Initial pharmacological studies on some of the polyhalogenated monoterpenes warned of the potent mutagenicity or carcinogenicity of natural products from marine algae (Leary *et al.*, 1979). Investigations during the early 1980's indicated that some polyhalogenated monoterpenes had slight cytotoxic and antimicrobial activity (González *et al.*, 1982). This early cytotoxic activity was attributed to the high bromine content of polyhalogenated monoterpenes.

Of the family Plocamiaceae and Rhizophyllidaceae, species from the genera *Plocamium* and *Portieria* are an especially well established source of acyclic and cyclic polyhalogenated monoterpenes (Gunatilaka *et al.*, 1999). Metabolites from these genera are often of interest because of their diverse biological properties which range from ichthyotoxicity to anti-feedant, antimicrobial, cytotoxic and antifungal activity (Naylor *et al.*, 1983). Of all the cytotoxic compounds ever isolated from red marine algae; metabolites isolated from *Plocamium*, *Portieria* and *Laurencia* species make up the majority of those with cytotoxic activity.

A number of halogenated monoterpenes from the genus *Plocamium*, belonging to the Plocamiaceae family, have also demonstrated mutagenic and cytotoxic behaviour. Some of these compounds are known to inhibit oxidative phosphorylation as their mechanism of action (König *et al.*, 1990).

From the family Rhizophyllidaceae, the genus *Portieria hornemannii* is renowned for producing ‘exciting’ chemistry. Ochtodene, which was isolated from *P. hornemannii*, exhibited selective solid tumour activity in cellular *in vitro* assays, but neither toxicity nor anti-tumour activity was observed *in vivo* (Gunatilaka *et al.*, 1999). Halomon was isolated from *P. hornemannii* (Fuller *et al.*, 1992). As a result of its successful selective *in vitro* activity against brain tumours, it was selected for pre-clinical drug development (Wise *et al.*, 2002). Compounds related to halomon, such as isohalomon, also demonstrated a unique differential cytotoxicity profile against several human tumour cell lines.

Another family which is well known for producing pharmacologically active halogenated metabolites from red algae is Rhodomelaceae. *Laurencia* species are probably the most well recognised members of this family, and have been the subject of much investigation since the 1960’s (Naylor *et al.*, 1983). These species are known to produce a wide variety of secondary metabolites, but the most common ones are terpenes (such as sesquiterpenes and diterpenes) and non-terpenoid C<sub>15</sub> acetogenins, some of which are quite complex. Many novel structural groups, unique to the *Laurencia* genus have also been isolated.

The reason for investigating South African *Plocamium*, *Portieria* and *Laurencia* species, is based on the exciting chemistry and pharmacology that compounds isolated from these species have yielded in other countries around the world. Although some work has already been done on the chemistry of South African *Plocamium*, *Portieria* and *Laurencia* species, there are still a number of other South African *Plocamium*, *Portieria* and *Laurencia* species which have not yet been investigated.

Simply put, the discovery of new compounds from South African red marine alga was this study’s primary objective. A vital objective for this study was also to explore the cytotoxic activity of all the new compounds that were isolated, as well as forming a better understanding of the chemotaxonomic usefulness of these compounds.

#### 1.4 Review of cytotoxic compounds isolated from *Plocamium*, *Portieria* and *Laurencia* species

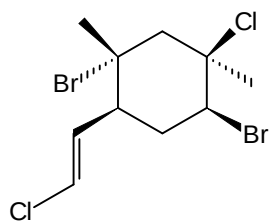
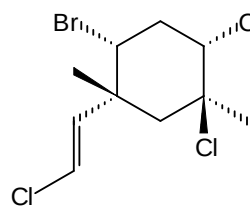
Globally, three families of red algae are well known for producing secondary metabolites with cytotoxic activity, namely, *Plocamium*, *Portieria* and *Laurencia* species (El Gamal, 2010). By focussing on South African species of *Plocamium*, *Portieria* and *Laurencia* species, it was anticipated that further new cytotoxic compounds might be isolated.

A review of cytotoxic secondary metabolites previously isolated from these red algae was compiled. Publications in which pure compounds were isolated and whose chemistry tested for positive for cytotoxic behaviour are recorded below (in alphabetical order).

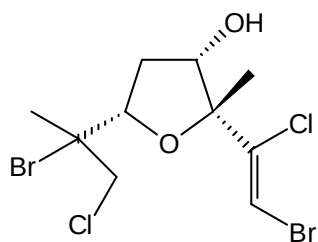
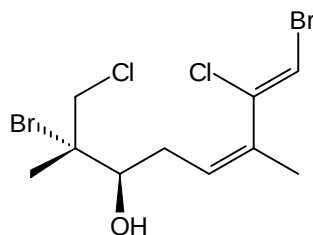
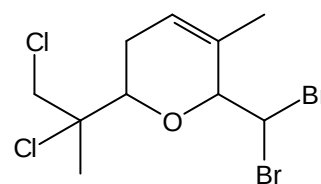
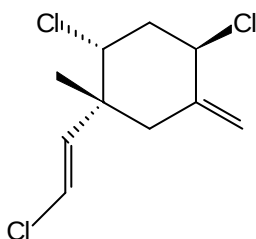
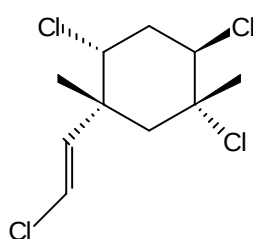
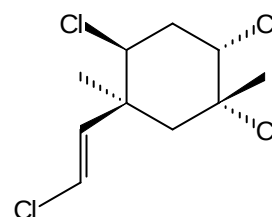
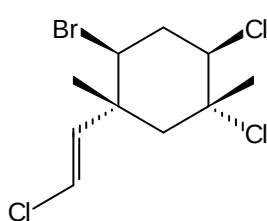
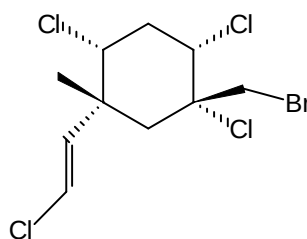
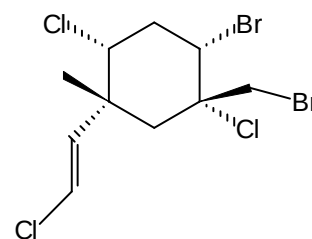
##### *Cytotoxic compounds isolated from Plocamium and Portieria species of red algae*

Secondary metabolites isolated from *Plocamium* and *Portieria* species comprise of a number of diverse and volatile halogenated compounds. These low molecular weight halogenated metabolites have shown an exciting range of biological properties.

*Plocamium cartilagineum* yielded the following two compounds (**1.15-1.16**) which showed potent toxicity against both *Biomphalaria glabrata* and *Artemia salina* (König *et al.*, 1999). No IC<sub>50</sub> data was published.

**1.15****1.16**

The following compounds (**1.17-1.25**) were also isolated from *P. cartilagineum* and showed selective cytotoxic activity against human tumour cell lines (de Inés *et al.*, 2004) (Table 1.2).

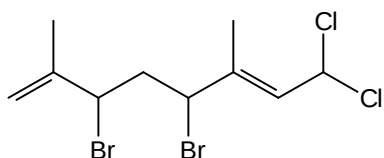
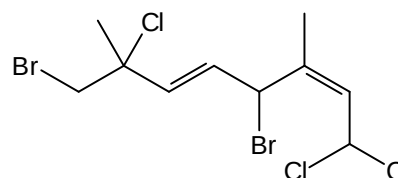
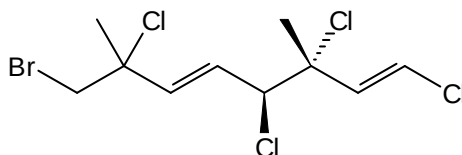
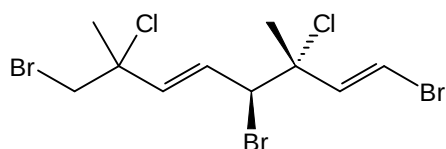
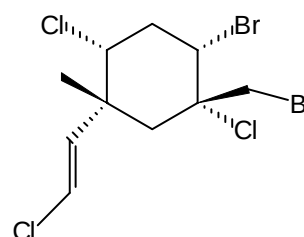
**1.17****1.18****1.19****1.20****1.21****1.22****1.23****1.24****1.25**

**Table 1.2** Minimal inhibitory concentration (MIC) [ $\mu\text{M}$ ] of test compounds **1.17-1.25** on several mammalian cell lines (de Inés *et al.*, 2004)

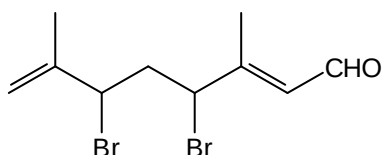
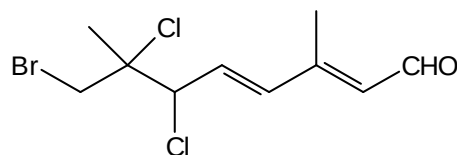
| No.            | CHO  | CT26                                    | SW480                                       | HeLa                                        | SkMe128 |
|----------------|------|-----------------------------------------|---------------------------------------------|---------------------------------------------|---------|
| <b>1.17</b>    | 126  | 63 (IC <sub>50</sub> 30 $\mu\text{M}$ ) | 126                                         | 126                                         | 126     |
| <b>1.18</b>    | 132  | 66 (IC <sub>50</sub> 45 $\mu\text{M}$ ) | 66 (IC <sub>50</sub> 32 $\mu\text{M}$ )     | 132                                         | 132     |
| <b>1.19</b>    | 262  | 262                                     | 131 (IC <sub>50</sub> 73 $\mu\text{M}$ )    | 262                                         | 262     |
| <b>1.20</b>    | 3.30 | 6.52                                    | 3.30                                        | 13.05                                       | 6.52    |
| <b>1.21</b>    | 23   | 181                                     | 5.70 (IC <sub>50</sub> 0.08 $\mu\text{M}$ ) | 5.70 (IC <sub>50</sub> 0.06 $\mu\text{M}$ ) | 23      |
| <b>1.22</b>    | 362  | 362                                     | 362                                         | 362                                         | 362     |
| <b>1.23</b>    | 39   | 78                                      | 78                                          | 312                                         | >312    |
| <b>1.24</b>    | 141  | 141                                     | 141                                         | 282                                         | 282     |
| <b>1.25</b>    | 63   | 125                                     | 125                                         | 125                                         | 250     |
| <b>Lindane</b> | >344 | >344                                    | >344                                        | >344                                        | >344    |



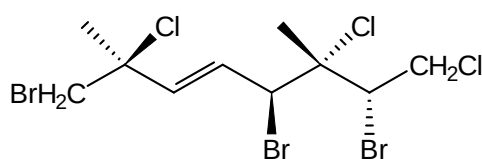
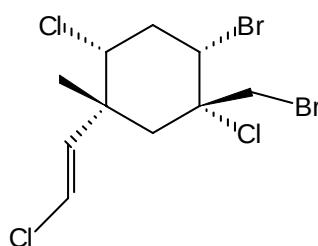
*P. corallorhiza* yielded 5 compounds which demonstrated moderate to good activity towards oesophageal cancer cells (WHCO1) (Knott *et al.*, 2005).  $IC_{50}$  values were 9.3 (**1.26**), 33.8 (**1.27**), 17.2 (**1.28**), 18.1 (**1.29**) and 34.8  $\mu$ M (**1.25**), respectively. For this assay, cisplatin has an  $IC_{50}$  value of 13  $\mu$ M.

**1.26****1.27****1.28****1.29****1.25**

*P. corallorhiza* also yielded four new halogenated monoterpene aldehydes. Two of these compounds were tested for cytotoxic activity. Compound **1.30** demonstrated moderate to good activity towards oesophageal cancer cells (WHCO1) with an  $IC_{50}$  value of 7.5  $\mu$ M. Compound **1.31** was only weakly active and had an  $IC_{50}$  value of 64.8  $\mu$ M (Mann *et al.*, 2007).

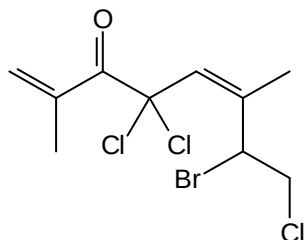
**1.30****1.31**

Two compounds isolated from *Plocamium hamatum* showed moderate cytotoxic activity (König *et al.*, 1999). Compound **1.32** demonstrated the following bioactivity;  $IC_{50}$ : KB 12.4  $\mu$ g/ml and LNCaP 14.8  $\mu$ g/ml.

**1.32****1.25**

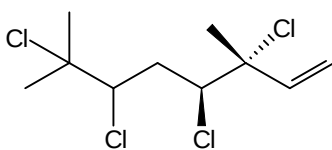
Aside from being anti-algal, the cyclic compound above (**1.25**) also showed moderate cytotoxic behaviour;  $IC_{50}$ : Lu1 12.9  $\mu$ g/ml, KB 13.3  $\mu$ g/ml and ZR-75-1 7.8  $\mu$ g/ml (König *et al.*, 1999).

From *Plocamium oregonum*, plocamenone (**1.33**) was discovered. The close proximity of the dichloromethylene group and the ketone moiety gives this molecule interesting chemical activity. This may explain why compound **1.33** is such a potent mutagen in the Ames reversion assay. At that time, this compound was far more potent than other known *Plocamium* metabolites (Stierle and Sims, 1984). No IC<sub>50</sub> data was published.



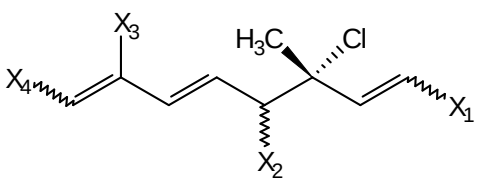
**1.33**

Five known halogenated monoterpenes (**1.34**, **1.35**, **1.37**, **1.39**, **1.40**), together with two new ones (**1.36** and **1.38**) were isolated from the red macroalga *P. suhrii*. During this study five related compounds from *P. cornutum*, as well as the seven compounds from *P. suhrii* were evaluated for their cytotoxic effects on an oesophageal cancer cell line (WHCO1). During this assay compounds **1.34-1.39** showed greater cytotoxicity than the known anti-cancer drug cisplatin (Antunes *et al.*, 2011) (Table 1.3).



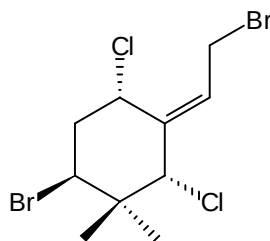
**1.38**

**Table 1.3** Selected structural features and cytotoxic effects of compounds **1.34-1.40** on oesophageal cancer cells (Antunes *et al.*, 2011)



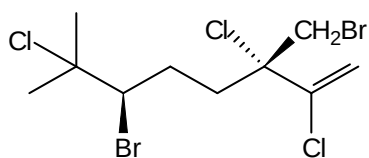
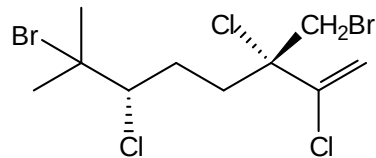
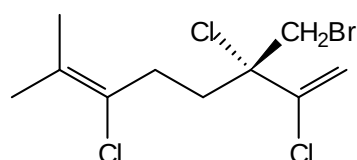
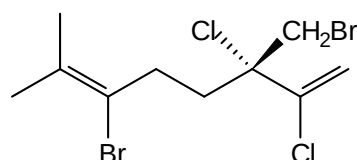
|             | X <sub>1</sub> | X <sub>2</sub>   | X <sub>3</sub>    | X <sub>4</sub> | IC <sub>50</sub><br>(µg/ml) | IC <sub>50</sub> (µM) |
|-------------|----------------|------------------|-------------------|----------------|-----------------------------|-----------------------|
| <b>1.34</b> | 1 <i>E</i> -Br | 4 <i>S</i> * -Cl | CHCl <sub>2</sub> | 7 <i>Z</i> -Cl | 2.5                         | 6.6                   |
| <b>1.35</b> | 1 <i>E</i> -Br | 4 <i>R</i> * -Cl | CHCl <sub>2</sub> | 7 <i>Z</i> -Cl | 3.8                         | 9.9                   |
| <b>1.36</b> | 1 <i>Z</i> -Br | 4 <i>S</i> * -Cl | CHCl <sub>2</sub> | 7 <i>Z</i> -Cl | 3.6                         | 9.3                   |
| <b>1.37</b> | H              | 4 <i>S</i> * -Cl | CHCl <sub>2</sub> | 7 <i>Z</i> -Cl | 2.6                         | 8.5                   |
| <b>1.38</b> |                |                  |                   |                | 2.2                         | 7.9                   |
| <b>1.39</b> | 1 <i>E</i> -Br | 4 <i>R</i> * -Cl | CH <sub>3</sub>   | 7 <i>Z</i> -Br | 3.1                         | 8.4                   |
| <b>1.40</b> | 1 <i>E</i> -Br | 4 <i>S</i> * -Cl | CH <sub>3</sub>   | 7 <i>Z</i> -Br | 5.5                         | 15.1                  |

Ochtodene (**1.41**) from *P. hornemannii* demonstrated selective anti-tumour activity in cellular *in vitro* assays. However, this activity was not seen *in vivo* (Gunatilaka *et al.*, 1999). No IC<sub>50</sub> data was published.

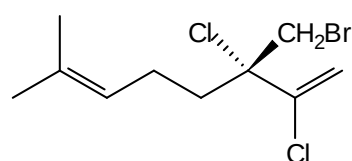
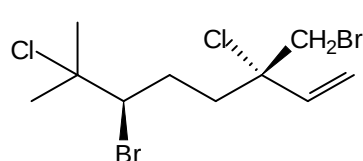
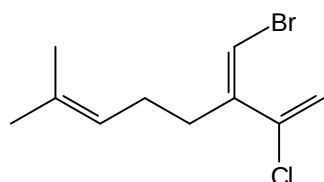
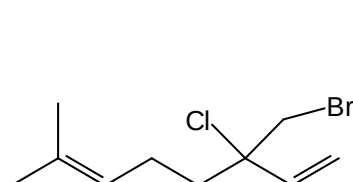


**1.41**

The following four compounds (**1.8**, **1.42-1.44**) were isolated from *P. hornemannii*. These compounds uniformly exhibited the same unique differential cytotoxicity profile as that of halomon against the NCI panel of 60 human tumour cell lines. All four compounds demonstrated comparable potency to each other (Fuller *et al.*, 1994).

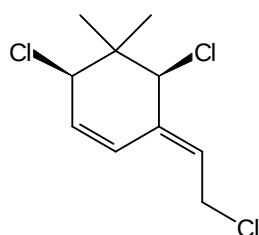
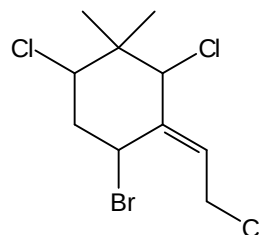
Halomon (**1.8**)Isohalomon (**1.42**)**1.43****1.44**

The cytotoxic profiles of the following four compounds (**1.45-1.48**) are less potent than that of halomon, and they also lack differential cytotoxicity (Fuller *et al.*, 1994).

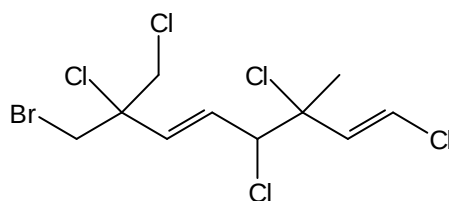
**1.45****1.46****1.47****1.48**

The structural features of compounds **1.8**, **1.42-1.44**; as well as the pharmacological results suggest that the halogen at C-7 is not essential for activity and that hybridization of C-6 and C-7 to  $sp^2$  or  $sp^3$  is also not a critical factor for cytotoxic activity. Fuller (1994) goes on to suggest that a halogen at C-6 is essential for the cytotoxic activity demonstrated by these compounds. In addition to this, a halogen at C-2 is also required for 'halomon-like' activity. During these pharmacological bioassays, carbocyclic compounds demonstrated considerably less cytotoxic activity than compounds **1.8**, **1.42-1.44**. Unlike compounds **1.8**, **1.42-1.44**; carbocyclic compounds also exhibited no differential response on the tumour cell line.

The cyclic compound below (**1.49**) was found to be considerably less potent than halomon, by at least 1 order of magnitude for three different bioassays (Fuller *et al.*, 1992). However, the second cyclic compound (**1.50**) had a potency comparable to that of halomon. Unfortunately, this compound showed little differential response to cell lines when compared to halomon and was consequently not investigated any further (Fuller *et al.*, 1994).

**1.49****1.50**

Compound **1.51** was found to be more potent than compound **1.49**, but it also only exhibited very modest differential activity (Fuller *et al.*, 1992).

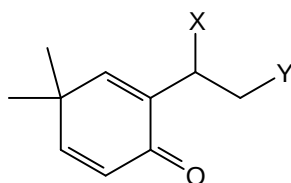
**1.51**

Carbocyclic halogenated monoterpenes from Rhodophyta have been reported to show general toxicity to brine shrimp assays and *in vitro* inhibition of murine leukaemia cells (Fuller *et al.*, 1992). Nonetheless, these results show that carbocyclic halogenated monoterpenes are not particularly effective against solid tumour cell lines. The difference in activity between linear and cyclic halogenated compounds, suggests that linear compounds are not merely acting as electrophiles or alkylating agents (Fuller *et al.*, 1992).

**Table 1.4** Results of comparative testing of compounds **1.8**, **1.42-1.50** in the NCI *in vitro* primary anti-tumour screen (Fuller *et al.*, 1994)

| Mean panel response values ( $\times 10^{-6}\text{M}$ ) |                                               |                                    |                                        |
|---------------------------------------------------------|-----------------------------------------------|------------------------------------|----------------------------------------|
| Compound                                                | GI <sub>50</sub><br>50% net growth inhibition | TGI<br>net total growth inhibition | LC <sub>50</sub><br>50% net cell death |
| <b>1.8</b>                                              | 0.676                                         | 3.02                               | 11.5                                   |
| <b>1.42</b>                                             | 1.32                                          | 4.47                               | 16.2                                   |
| <b>1.43</b>                                             | 0.741                                         | 3.39                               | 17.0                                   |
| <b>1.44</b>                                             | 0.691                                         | 3.02                               | 13.5                                   |
| <b>1.45</b>                                             | 47.0                                          | >100                               | >100                                   |
| <b>1.46</b>                                             | 26.1                                          | 77.0                               | >100                                   |
| <b>1.47</b>                                             | 19.5                                          | 44.7                               | >100                                   |
| <b>1.48</b>                                             | 33.1                                          | >100                               | >100                                   |
| <b>1.49</b>                                             | 20.0                                          | 47.9                               | >100                                   |
| <b>1.50</b>                                             | 1.15                                          | 4.68                               | 20.0                                   |

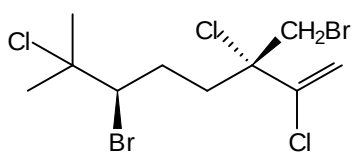
Of the several cyclic monoterpenes that were isolated from *P. hornemannii* in Japan, a mixture of the following two cyclohexadienones (A-B) (**1.52-1.53**) showed significant cytotoxicity against P-388 murine leukaemia cells ( $IC_{50}$  0.5  $\mu$ g/ml), A-549 human lung carcinoma ( $IC_{50}$  5  $\mu$ g/ml) and HCT-8 human colon adenocarcinoma ( $IC_{50}$  5  $\mu$ g/ml) (Kuniyoshi *et al.*, 2003).



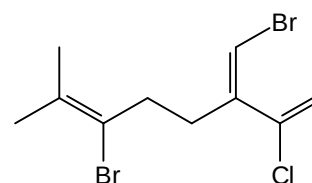
(A) X=Br, Y=Br (**1.52**)

(B) X=Cl, Y=Br (**1.53**)

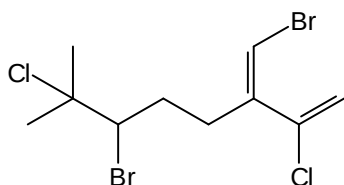
*P. hornemannii* extracted from Madagascar, yielded the following four compounds which were later tested on a Deoxyribose Nucleic Acid Methyltransferase (DNMT) -1 enzyme inhibition assay. This assay is used to identify compounds which may be useful in the reversal of tumour growth. Compounds **1.8** and **1.54** had comparable activities (1.25 and 1.65  $\mu$ M, respectively) and are low micromolar inhibitors of DNA methyl transferase-1; while compounds **1.55** and **1.46** were only weakly active (55.0 and 21.9  $\mu$ M, respectively) (Andrianasolo *et al.*, 2006).



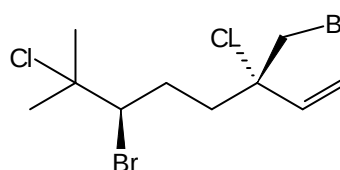
**1.8**



**1.54**



**1.55**



**1.46**

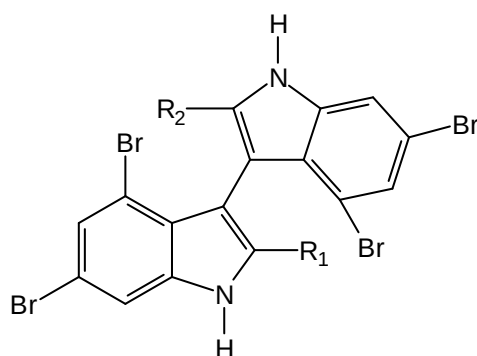


*Review of cytotoxic compounds isolated from the red algae Laurencia*

Although *Laurencia* species are known to produce a wide variety of different secondary metabolites, with different classes of chemical compounds, they are most prolific in producing various sesquiterpene related compounds. Sesquiterpenes from marine organisms comprise of a large group of natural compounds, but only some contain halogens. Most halogenated sesquiterpenes are produced by the genus *Laurencia*. However, sesquiterpene compounds are also found in green algae, soft corals, molluscs and sponges. Many sesquiterpene compounds possess notable biological activity. Sesquiterpenes from algae of the genus *Laurencia* cover 18 structural types, only some of which are halogenated. Chamigrenes or spirane type sesquiterpenes are the most common type of sesquiterpenes found in the genus *Laurencia* (Dembitsky and Tolstikov, 2004).

Publications in which *Laurencia* secondary metabolites were isolated and whose chemistry tested for positive for cytotoxic behaviour are recorded below in alphabetical order.

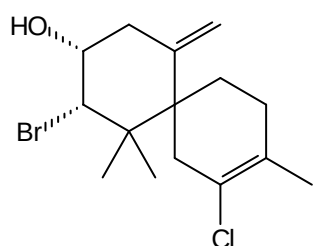
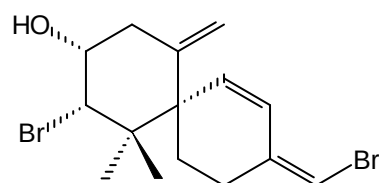
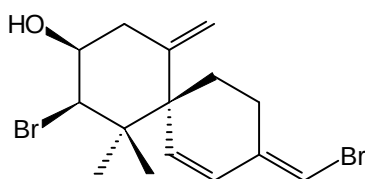
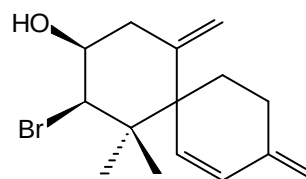
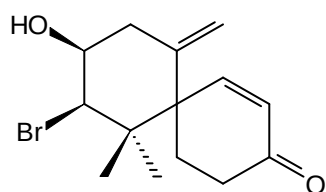
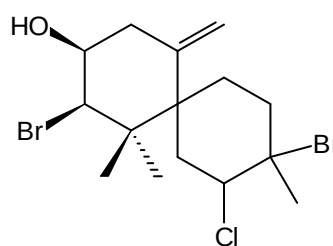
*Laurencia brongniartii* from Taiwan yielded five sulphur-containing polybromoindoles. Two of these compounds were cytotoxic. Compound **1.56** was active against HT-29 (human colon carcinoma) and P-388 (murine leukaemia) cells, while compound **1.57** was active against only P-388 cells (El Gamal, 2010). No IC<sub>50</sub> data was published.



**1.56**    R<sub>1</sub>=SMe    R<sub>2</sub>=SOMe

**1.57**    R<sub>1</sub>=R<sub>2</sub>=SOMe

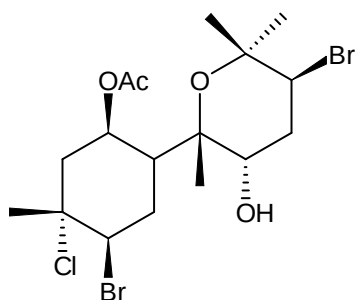
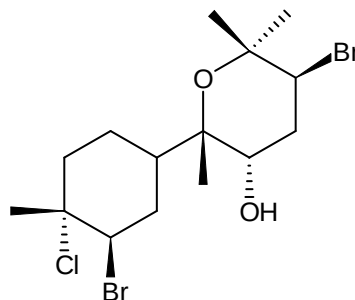
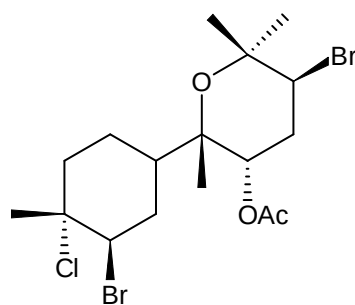
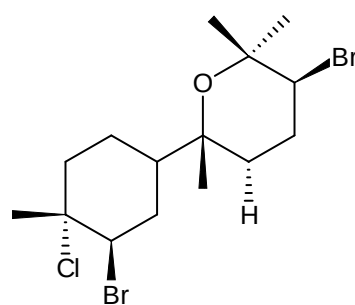
From *Laurencia cartilaginea*, six compounds were extracted (**1.58-1.63**). Compound **1.58**, known as elatol had been previously isolated from *L. elata* and *L. obtusa*, while compounds **1.59-1.61** had been previously isolated from *L. majuscula*. Compounds ma'illione (**1.62**) and *allo*-isoobtusol (**1.63**) were shown to have high anti-cancer activity against HT-29 human carcinoma cells at concentrations of 0.5 and 0.25 µg/ml, respectively (Juagdan *et al.*, 1997). These metabolites showed remarkable results against certain cancer cell lines at low concentrations, especially towards the HT-29 cell line (Table 1.5).

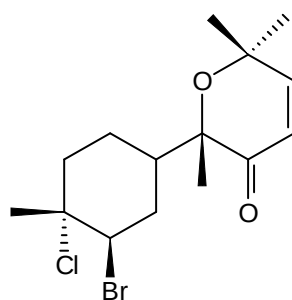
**1.58****1.59****1.60****1.61**Ma'illione (**1.62**)*allo*-isoobtusol (**1.63**)

**Table 1.5** Cytotoxic activity results (IC<sub>50</sub> reported in µg/ml) (Juagdan *et al.*, 1997)

| Cell lines | 1.58 | 1.59  | 1.60  | 1.61 | 1.62 | 1.63 |
|------------|------|-------|-------|------|------|------|
| P-388      | 1.0  | 1.0   | 1.0   | 1.0  | 5.0  | 5.0  |
| A-549      | 0.1  | 1.0   | 1.0   | 1.0  | 5.0  | 1.0  |
| HT-29      | 0.1  | 0.025 | 0.025 | 0.25 | 0.5  | 0.25 |
| MEL-28     | 0.1  | 1.0   | 1.0   | 1.0  | 10.0 | 1.0  |

Five cytotoxic halogenated metabolites (**1.64-1.68**) from the Brazilian red alga, *L. catarinensis* were isolated and evaluated against HT-29, MCF-7 and A-431 cell lines (Lhullier *et al.*, 2010) (Table 1.6).

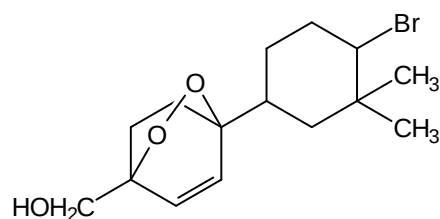
5S-5-Acetoxycaespitol (**1.64**)Caespitol (**1.65**)Acetylcaespitol (**1.66**)Deoxycaespitol (**1.67**)

Caespitenone (**1.68**)

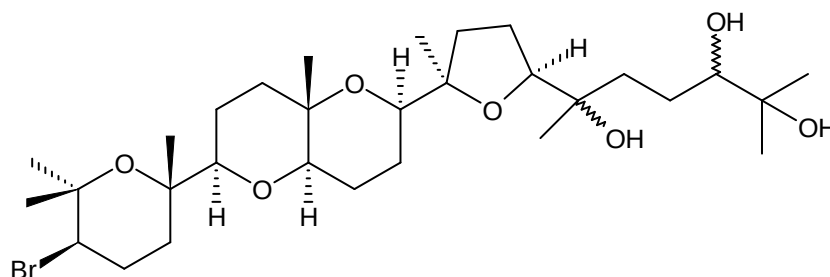
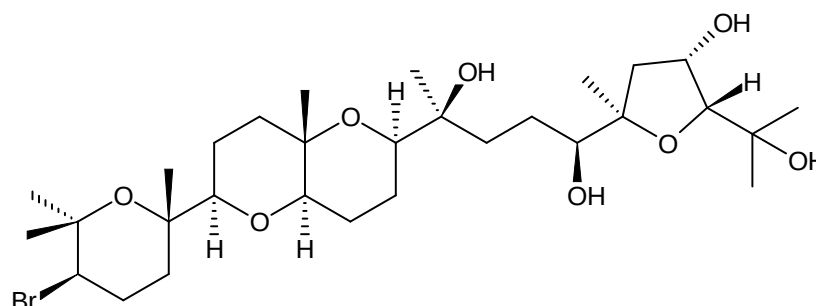
**Table 1.6** *In vitro* cytotoxicity of compounds **1.64-1.68** are expressed as IC<sub>50</sub> in  $\mu\text{M}$  (Lhullier *et al.*, 2010)

| Compound    | HT-29          | MCF-7          | A-431          |
|-------------|----------------|----------------|----------------|
| <b>1.64</b> | 12.4 $\pm$ 0.5 | 11.7 $\pm$ 0.4 | 13.1 $\pm$ 0.7 |
| <b>1.65</b> | 7.6 $\pm$ 0.4  | 9.7 $\pm$ 0.6  | 10.2 $\pm$ 0.8 |
| <b>1.66</b> | 16.1 $\pm$ 0.8 | 17.6 $\pm$ 1.2 | 17.4 $\pm$ 0.9 |
| <b>1.67</b> | 12.5 $\pm$ 0.6 | 11.2 $\pm$ 0.9 | 13.1 $\pm$ 1.2 |
| <b>1.68</b> | 18.9 $\pm$ 1.4 | 19.7 $\pm$ 0.8 | 21.6 $\pm$ 0.6 |
| Miltefosine | 48.6 $\pm$ 4.0 | 42.3 $\pm$ 3.8 | 53.7 $\pm$ 6.2 |

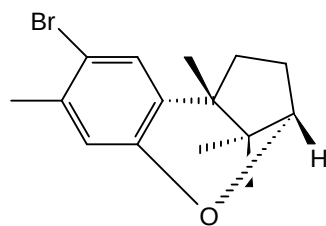
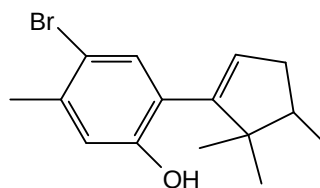
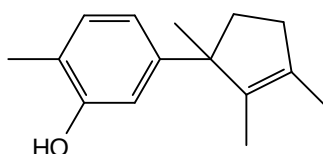
Majapolene A (**1.69**) is a cytotoxic peroxide which was isolated from a Philippine sample of *Laurencia majuscula*. Majapolene A displayed a modest mean response parameter value for all NCI 60-cell lines of 0.4  $\mu\text{M}$  for GI<sub>50</sub>, 0.9  $\mu\text{M}$  for TGI and 2.8  $\mu\text{M}$  for LC<sub>50</sub> (Erickson *et al.*, 1995).

Majapolene A (**1.69**)

Two highly oxygenated squalenoid-derived triterpenoids from the Chinese marine red alga, *Laurencia mariannensis* were isolated and named laurenmariannol (**1.70**) and (21 $\alpha$ )-21-hydroxythysiferol (**1.71**). These compounds displayed significant cytotoxic activity against P-388 tumour cells with IC<sub>50</sub> values of 0.6 and 6.6  $\mu$ g/ml respectively (the positive control was etoposide which had an IC<sub>50</sub> of 0.30  $\mu$ g/ml) (Ji *et al.*, 2008).

**1.70****1.71**

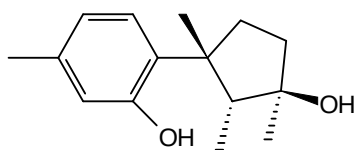
*Laurencia microcladia* yielded three cytotoxic cuparene sesquiterpenes. These compounds exhibited significant activity against two lung cancer cell lines, namely NSCLC-N6 and A-549. Compound **1.72** showed little cytotoxicity, with IC<sub>50</sub> 196.9 and 242.8  $\mu$ M for NSCLC-N6 and A-549 respectively. Compound **1.73** had IC<sub>50</sub> values of 73.4 and 52.4  $\mu$ M for NSCLC-N6 and A-549 respectively, while compound **1.74** had values of 83.7 and 81.0  $\mu$ M for NSCLC-N6 and A-549 respectively. The increased cytotoxicity of metabolites for compounds **1.73** and **1.74** could be attributed to the phenolic hydroxyl or the presence of the double bond in the five-membered ring system. The presence of bromine does not seem to affect cytotoxicity according to these results (Kladi *et al.*, 2005).

**1.72****1.73****1.74**

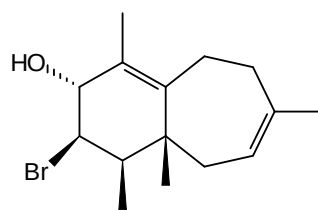
Kladi (2006) then isolated the following compounds (**1.75-1.86**) from *Laurencia obtusa* and *L. microcladia*, of which several showed promising results against human tumour and CHO cell lines. Compounds **1.76**, **1.79**, **1.81** and **1.84** were consistently more cytotoxic than other compounds tested on the cell lines mentioned above. Compounds **1.77**, **1.82** and **1.83** exhibited the weakest activity with  $IC_{50}$  values over 300  $\mu$ M against K-562 and CHO cell lines (Kladi *et al.*, 2006).

Compounds **1.76**, **1.82** and **1.83** are structurally related, and showed that the presence of Br greatly enhanced activity against all cell lines. Metabolites **1.84** and **1.86** showed similar levels of activity, which were higher than those of **1.85**. This suggests that the cyclopropyl ring or the trisubstituted double bond is not essential for the cytotoxicity of the molecule, but that a lack of OH and Br moieties can drastically reduce activity (Kladi *et al.*, 2006).

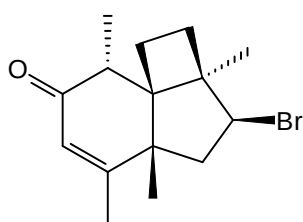
Compounds **1.78**, **1.84**, **1.85** and **1.86** were assayed against NSCLC-N6 and A-549 lung cancer cell lines. Of these compounds, **1.78** and **1.85** demonstrated  $IC_{50}$  values of 153.5  $\mu$ M for A-549 and 150.5  $\mu$ M for NSCLC-N6. Compound **1.84** showed weaker activity than compound **1.85**. This suggests that having Br on the aromatic ring is not that important for cytotoxic activity. However, the trisubstituted double bond on the five-membered ring does enhance the cytotoxicity for this class of compounds against the NSCLC-N6 cell line (Kladi *et al.*, 2006) (Table 1.7).



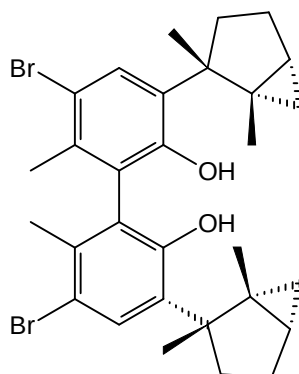
1.75



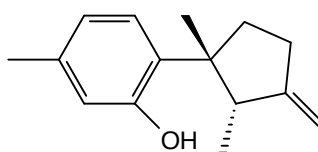
1.76



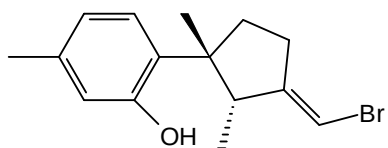
1.77



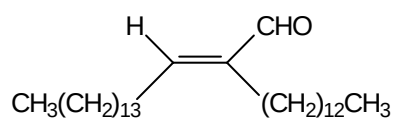
1.78



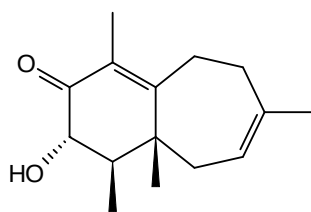
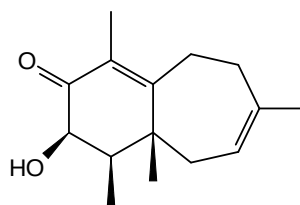
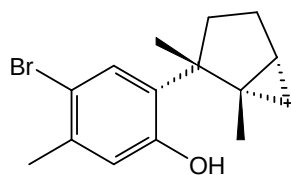
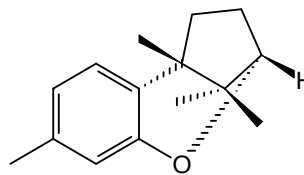
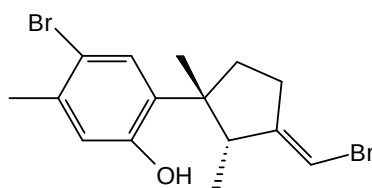
1.79



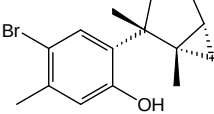
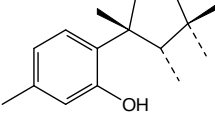
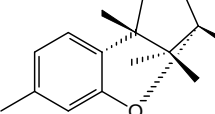
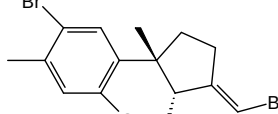
1.80



1.81

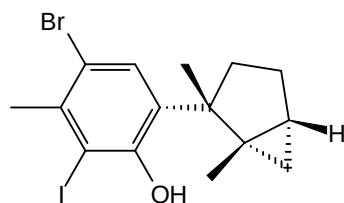
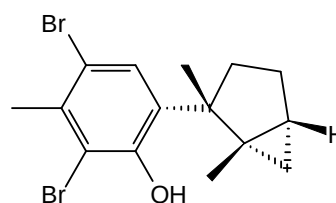
**1.82****1.83****1.84****1.85****1.86**

**Table 1.7** Anti-tumour activity ( $IC_{50}$  in  $\mu M$ ) of selected cuparene sesquiterpenes isolated from *Laurencia* species (Kladi *et al.*, 2006)

| Tumour cell line |  |  |  |  |
|------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| K-562            | 128.3                                                                               | >300                                                                                | >200                                                                                 | 112.7                                                                                 |
| MCF-7            | 67.2                                                                                | 201.7                                                                               | >200                                                                                 | 78.3                                                                                  |
| PC-3             | 76.6                                                                                | 182.3                                                                               | >200                                                                                 | 92.4                                                                                  |
| HeLa             | 83.9                                                                                | 121.3                                                                               | >200                                                                                 | 105.8                                                                                 |
| A-431            | 74.6                                                                                | 176.4                                                                               | >200                                                                                 | 81.6                                                                                  |
| CHO              | 165.8                                                                               | 234.7                                                                               | >200                                                                                 | >200                                                                                  |



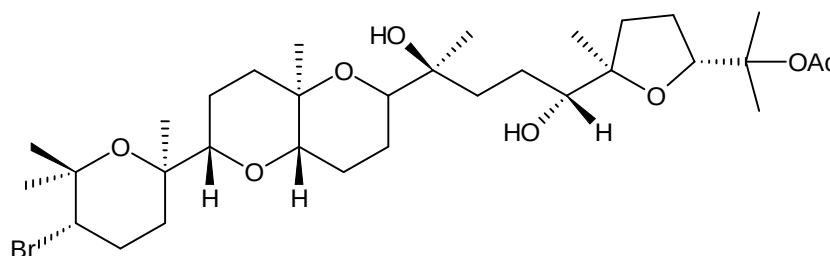
New sesquiterpenes (**1.87-1.88**) from a Greek sample of red algae named *Laurencia microcladia* were also isolated. Two of these compounds exhibited relatively higher cytotoxicity against all the tested cell lines (Table 1.8) (Kladi *et al.*, 2007).

**1.87****1.88**

**Table 1.8** *In vitro* cytotoxic activities of two metabolites (IC<sub>50</sub> μM) (Kladi *et al.*, 2007)

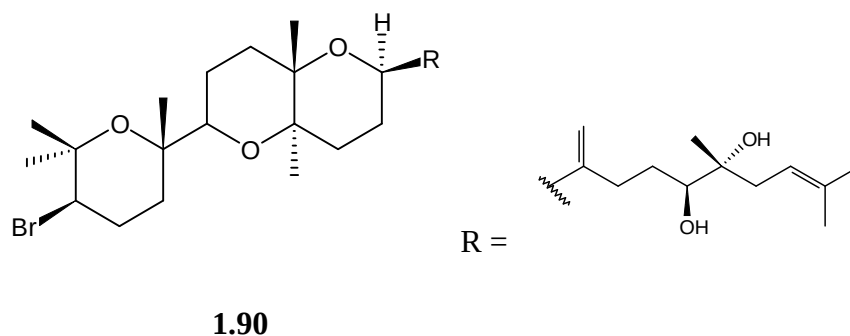
| Compound    | HT-29 | MCF-7 | PC-3 | HeLa  | A-431 |
|-------------|-------|-------|------|-------|-------|
| <b>1.87</b> | 78.4  | 86.3  | 88.5 | 81.4  | 92.7  |
| <b>1.88</b> | 98.7  | 104.1 | 75.2 | 114.6 | 93.4  |

Two new cyclic ethers consisting of squalene carbon skeletons were also isolated from the red alga *L. obtusa*. These compounds were known as teurilene and thyriferyl 23-acetate respectively (**1.89**). The former compound was inactive against the P-388 *in vitro* cell line, while the latter showed remarkable cytotoxic properties (ED<sub>50</sub> of 0.3 ng.ml) (Suzuki *et al.*, 1985).

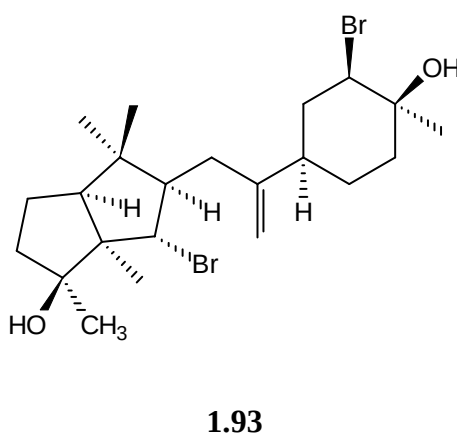
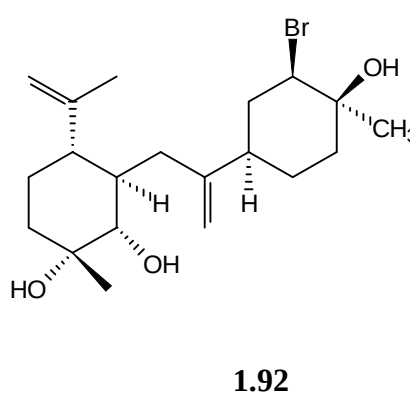
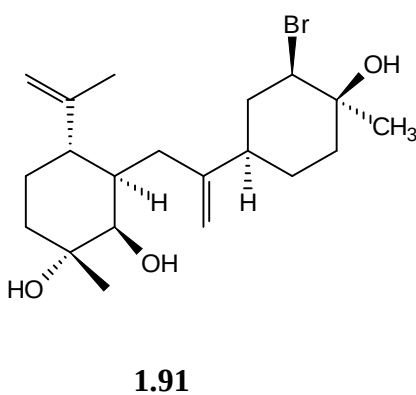


Thyriferyl 23-acetate (**1.89**)

Five new cytotoxic triterpenes were later extracted from another *L. obtusa* sample. These compounds had a similar backbone to thyriferyl 23-acetate (**1.89**) (El Gamal, 2010). The  $IC_{50}$  values were not published. Compound **1.90** represents one of these above mentioned compounds.



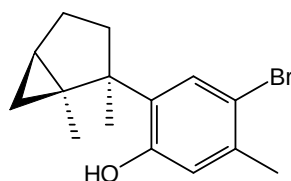
Novel cytotoxic brominated diterpenes (**1.91-1.93**) from the red alga *L. obtusa* were also isolated from a Greek marine algal collection. Compounds **1.92** and **1.93** were the most potent cytotoxic compounds (Table 1.9) (Lliopoulou *et al.*, 2003).



**Table 1.9** *In vitro* anti-tumour activities of compounds **1.91-1.93** against various cell lines (IC<sub>50</sub> in  $\mu\text{M}$ ) (Lliopoulou *et al.*, 2003).

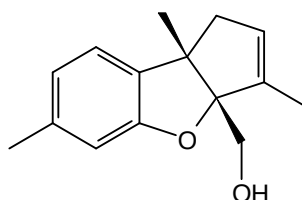
| Cell line | Origin     | 1.91  | 1.92  | 1.93  |
|-----------|------------|-------|-------|-------|
| MCF-7     | Breast     | 140.5 | 135.6 | 172.3 |
| PC-3      | Prostate   | 158.8 | 80.4  | 50.8  |
| HeLa      | Cervix     | 80.5  | 78.0  | 34.4  |
| A-431     | Epidermis  | 78.4  | 65.2  | 65.8  |
| K-562     | Myelogenic | 123.5 | 76.4  | 76.4  |

A crude *Laurencia okamura* extract known to contain laurinterol (**1.94**), has demonstrated the ability to prevent and inhibit melanoma cells by inducing apoptosis, without the adverse effects of synthetic medicines. Later research showed that laurinterol had cytotoxic activity against HeLa cells and demonstrated an IC<sub>50</sub> of 20  $\mu\text{g/ml}$  (Tsukamoto *et al.*, 2005).



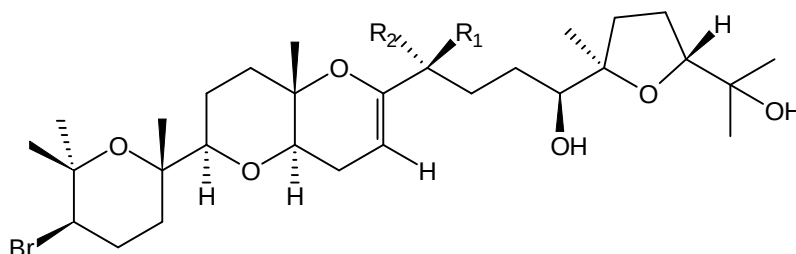
Laurinterol (**1.94**) (Irie *et al.*, 1970)

Debromoepiaplysinol (**1.95**) was isolated from *Laurencia tristicha* and displayed selective cytotoxicity to the HeLa cell line and had an IC<sub>50</sub> of 15.5  $\mu\text{M}$  (Sun *et al.*, 2007).



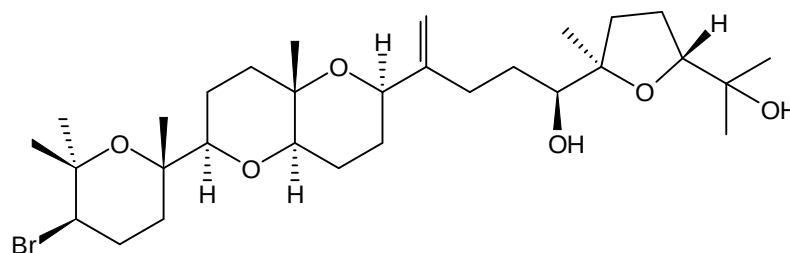
Debromoepiaplysinol (**1.95**)

Two unusual enol-ether moieties were isolated from *Laurencia viridis*. These polyether squalene derivatives, known as thyrсенol A (**1.96**) and thyrсенol B (**1.97**) were isolated together with a previously known compound called dehydrothyrсенiferol (**1.98**), which also had cytotoxic abilities (Table 1.10) (Norte *et al.*, 1997).



Thyrсенol A ( $R_1 = \text{OH}$  ;  $R_2 = \text{CH}_2\text{-OH}$ ) (**1.96**)

Thyrсенol B ( $R_1 = \text{CH}_2\text{-OH}$  ;  $R_2 = \text{OH}$ ) (**1.97**)



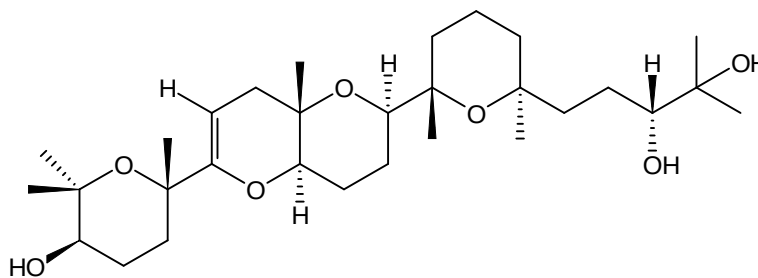
Dehydrothyrсенiferol (**1.98**)

**Table 1.10** *In vitro* anti-tumour activities of thyrсенol A-B and dehydrothyrсенiferol against various cell lines ( $\text{IC}_{50}$  in  $\mu\text{M}$ ) (Norte *et al.*, 1997)

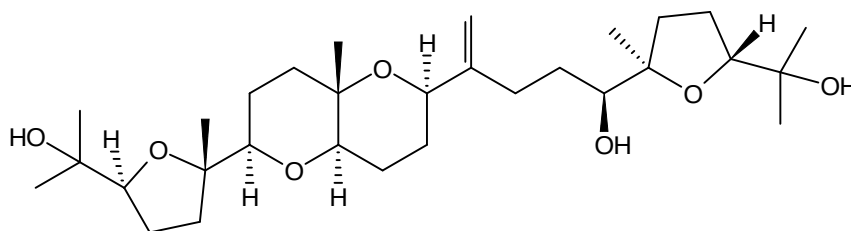
| Compound             | P-388 | A-549 | HT-29 | MEL-28 |
|----------------------|-------|-------|-------|--------|
| Dehydrothyrсенiferol | 0.017 | 4.26  | 4.26  | 8.52   |
| Thyrсенol A          | 0.40  | >1.62 | >1.62 | >1.62  |
| Thyrсенol B          | 0.016 | >1.62 | >1.62 | >1.62  |

Dehydrothysiferol which was first isolated from *Laurencia pinnatifida*, was later shown to induce apoptosis in oestrogen-dependent and independent breast cancer cells (Pec *et al.*, 2003).

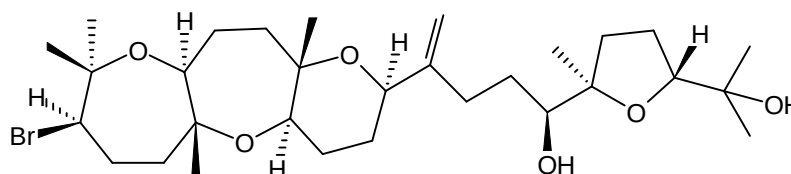
Several new squalene-derived triterpenes (**1.99-1.102**) were isolated from a Spanish sample of *L. viridis*.



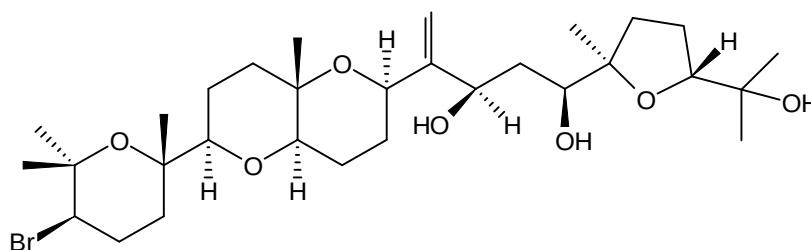
Martiriol (**1.99**)



Pseudodehydrothysiferol (**1.100**)



Dioxepandehydrothysiferol (**1.101**)



16-Epi-hydroxydehydrothysiferol (**1.102**)

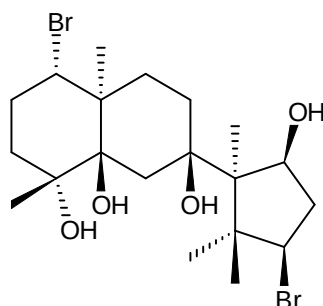
The cytotoxic effects of these compounds were evaluated against a battery of cultured tumour cell lines; P-388 (suspended culture of a lymphoid neoplasm), A-549 (monolayer culture of a human lung carcinoma), HT-29 (monolayer culture of a human colon carcinoma), MEL-28 (monolayer culture of a human melanoma). The results of the screening showed that martiriol was inactive at concentrations lower than 10  $\mu\text{g/ml}$ . Pseudodehydrothysiferol, dioxepandehydrothysiferol, 16-epi-hydroxydehydrothysiferol were inactive at concentrations lower than 1  $\mu\text{g/ml}$  (Manriquez *et al.*, 2001).

The cytotoxic activity of a number of polyethers isolated from *L. viridis* collected off the Canary Islands was evaluated with interesting results (Table 1.11) (Fernández *et al.*, 1998).

**Table 1.11** IC<sub>50</sub> values of biological assays of pure polyether squalene derivatives from *L. viridis* (Fernández *et al.*, 1998)

| Compounds                      | P-388 |       | A-549 |       | HT-29 |       | MEL-28 |       |
|--------------------------------|-------|-------|-------|-------|-------|-------|--------|-------|
|                                | µg/ml | µM    | µg/ml | µM    | µg/ml | µM    | µg/ml  | µM    |
| Thyrsiferol                    | 0.01  | 0.016 | 10.0  | 16.53 | 10.0  | 16.53 |        | 8.52  |
| Dehydrothyrsiferol <b>1.98</b> | 0.01  | 0.017 | 2.5   | 4.26  | 2.5   | 4.26  | 5.0    | 4.26  |
| Dehydrovenustatriol            | 0.01  | 0.017 | 2.5   | 4.26  | 2.5   | 4.26  | 2.5    | 4.26  |
| 15,16-Dehydrovenustatriol      | 0.25  | 0.430 | 2.5   | 4.26  | 2.5   | 4.26  | 2.5    | 4.54  |
| Predehydrovenustatriol         | 1.20  | 2.180 | 2.5   | 4.54  | 5.0   | 9.09  | 2.5    | 4.26  |
| Acetate                        |       |       |       |       |       |       |        |       |
| Isodehydrothyrsiferol          | 0.01  | 0.017 | 2.5   | 4.26  | 2.5   | 4.26  | 2.5    | 1.99  |
| 16-Hydroxyde                   | 0.50  | 0.830 | 1.2   | 1.99  | 1.2   | 1.99  | 1.2    | 4.26  |
| hydrothyrsiferol               |       |       |       |       |       |       |        |       |
| 10-Epi-15,16-                  | 0.50  | 0.850 | 2.5   | 4.26  | 1.2   | 2.04  | 2.5    | 8.52  |
| dehydrothyrsiferol             |       |       |       |       |       |       |        |       |
| 10-Epidehydrothyrsiferol       | 1.00  | 1.700 | 5.0   | 8.52  | 5.0   | 8.52  | 5.0    | >1.62 |
| <b>1.102</b>                   |       |       |       |       |       |       |        |       |
| Thyrsenol A <b>1.96</b>        | 0.25  | 0.400 | >1.0  | >1.62 | >1.0  | >1.62 | >1.0   | >1.62 |
| Thyrsenol B <b>1.97</b>        | 0.01  | 0.016 | >1.0  | >1.62 | >1.0  | >1.62 | >1.0   | >1.62 |

*Laurencia yonaguniensis* from Okinawa yielded neoirietetraol (**1.103**), a brominated diterpene based on a rare neoirieane skeleton. This compound was toxic to brine shrimp *Artemia salina* and had an LC<sub>50</sub> value of 40.1 µM (Takahashi *et al.*, 2002).



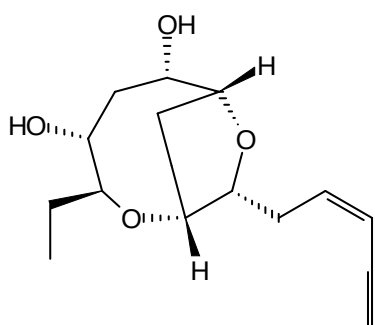
Neoirietetraol (**1.103**)

Laurefurenyne C (**1.104**) and F (**1.105**) were obtained from a *Laurencia* species collected in the Philippines. These compounds showed moderate cytotoxic activity when tested against different tumour cells (Table 1.12) (Abdel-Mageed *et al.*, 2010).

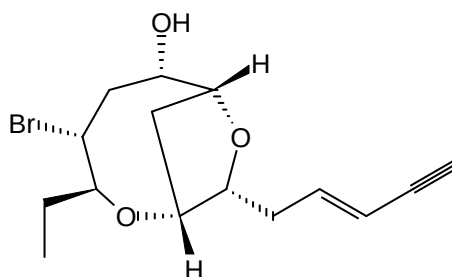
**Table 1.12** Selective cytotoxic activity of Laurefurenynes C and F (Abdel-Mageed *et al.*, 2010)

| Sample number | Murine<br>µg/disk | L1210 | Colon 3 | CFU-GM | H-116 | H-125 | Human<br>CFU-GM |
|---------------|-------------------|-------|---------|--------|-------|-------|-----------------|
| <b>1.104</b>  | 60                | 200   | 200     | -      | 200   | 100   | -               |
| <b>1.105</b>  | 60                | 250   | 450     | 400    | 200   | 100   | -               |

Agents which demonstrate a 250 or greater zone differential against one or more solid tumour cell lines and either the leukaemia or normal cells (CFU-GM) are considered solid tumour selective. Those that show a zone differential of between 150 and 250 units are considered to be moderately solid tumour selective.

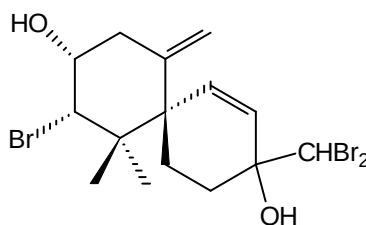


**1.104**



**1.105**

Ma'iliohydrin (**1.106**), a cytotoxic chamigrene dibromohydrin from a Philippine *Laurencia* species also demonstrated cytotoxic activity using the NCI 60-cell line human tumour screen (Francisco *et al.*, 2001), unfortunately the IC<sub>50</sub> values were not recorded.



**1.106**



After reviewing all the major cytotoxic *Plocamium*, *Portieria* and *Laurencia* literature, a need to make sense of all this data soon became apparent. To summarise the bioactivity and structural types of cytotoxic compounds previously isolated from *Plocamium*, *Portieria* and *Laurencia* species, the following had to be done:

- 1) IC<sub>50</sub> values for all the cytotoxic compounds had to be written in standard units, namely,  $\mu\text{M}$  instead of  $\mu\text{g/ml}$ .
- 2) Cytotoxic compounds were grouped in terms of which cell lines they had previously been tested on. For this purpose, only the most commonly used cytotoxic cell lines were investigated. These cell lines include (in alphabetical order); A-431 (skin epithelium - squamous carcinoma) (Table 1.13), A-549 (lung carcinoma) (Table 1.14), CHO (ovary) (Table 1.15), HeLa (cervical cancer) (Table 1.16), HT-29 (colon epithelium – adenocarcinoma) (Table 1.17), MCF-7 (invasive breast carcinoma) (Table 1.18), P-388 (leukaemia cells) (Table 1.19) and WHCO1 (cancerous oesophageal tissue) (Table 1.20).
- 3) Under the heading of each specific cell line, cytotoxic compounds from *Plocamium*, *Portieria* and *Laurencia* were placed in order of increasing IC<sub>50</sub> [ $\mu\text{M}$ ] values. Compounds with IC<sub>50</sub> values greater 100 are essentially not cytotoxic. For this reason, these compounds have not been included below.

Building up summarised databases such as those seen in Tables 1.13-1.20 might below assist future drug developing companies to better understand some of the structure activity relationships (SAR's) that exist between different compounds and selected types of cancer cells. However, it should be noted that there is more to rational drug selection than simply low IC<sub>50</sub> values. For example, comparing the 'drug-likeness' of marine natural products with all other natural products, as measured by an examination of their Lipinski characteristics can also be very useful. Examining Lipinski's 'rule of five' criteria, Lipinski suggested that to be drug-like and orally-bioavailable, a molecule must have a partition coefficient ( $\log P$ ) < 5, a molecular weight < 500 Da, < 5 hydrogen bond donors (HBD) and < 10 hydrogen acceptors (HBA) (Blunt *et al.*, 2011).

From Table 1.14 it can be noted that chamigrenes from *L. cartilaginea* are particularly useful in combating A-549 cell lines. Chamigrenes are far more effective than a variety of other cuparanes isolated from *L. microcladia*.

Halogenated cyclic monoterpenes from *P. cartilagineum* seem to be more effective than cuparene related derivatives (isolated from various *Laurencia* species) against CHO cell lines (Table 1.15).

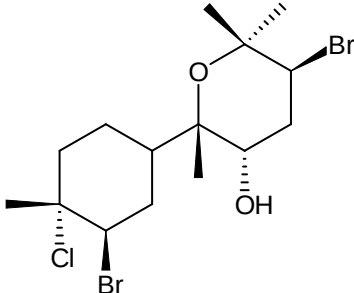
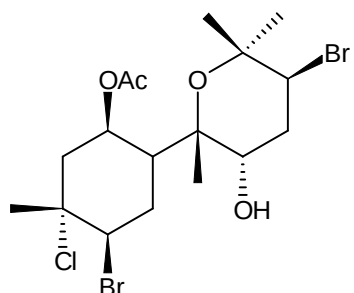
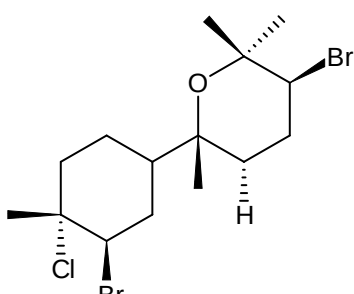
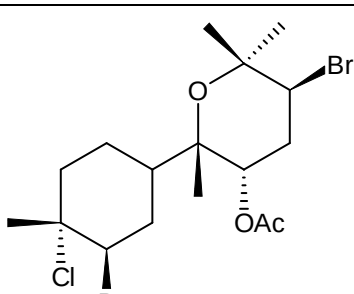
Certain polyhalogenated cyclic monoterpenes from *P. cartilagineum* also demonstrate good activity against HeLa cells (Table 1.16), while diterpenes and cuparanes from *Laurencia* demonstrate less activity.

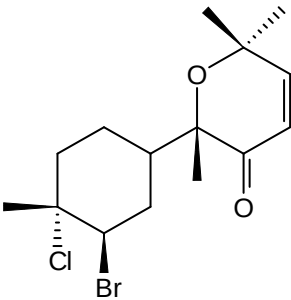
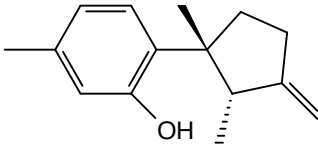
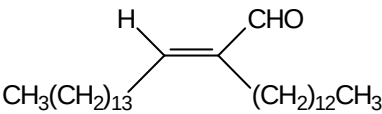
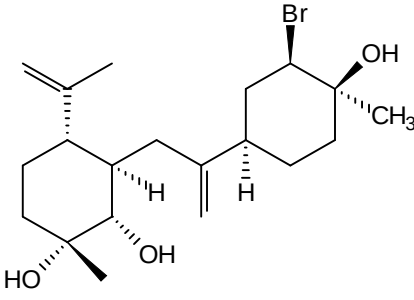
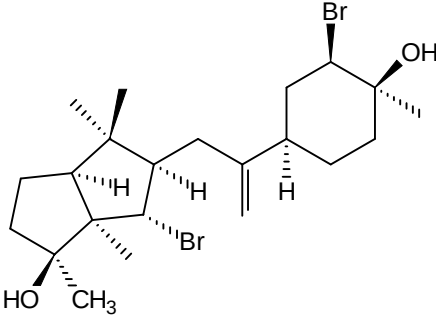
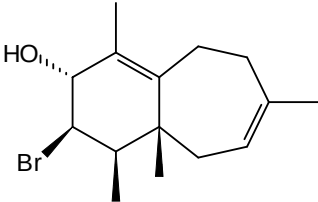
Chamigrenes from *L. cartilaginea* show excellent activity against HT-29 cell lines. This activity is followed by thyrseanol A, thyrseanol B and dehydrothyrseiferol isolated from *L. viridis* (Table 1.17).

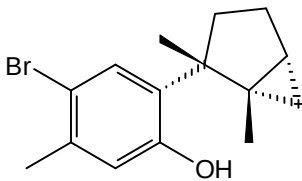
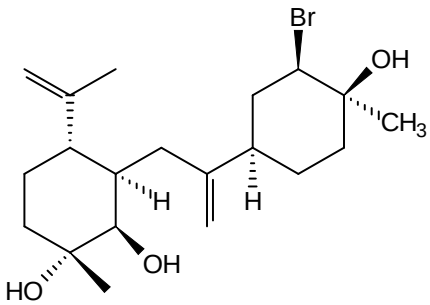
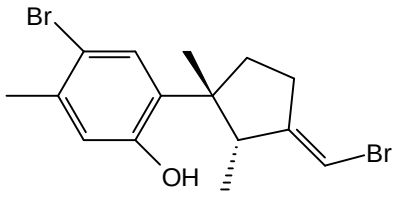
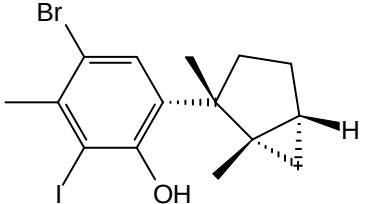
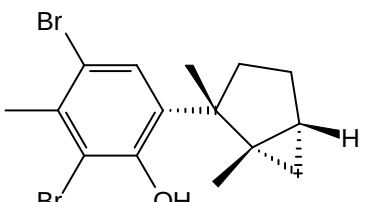
Focussing on MCF-7 breast cancer cell lines (Table 1.18), it becomes immediately obvious that halogenated bisabolene addition products from the Brazilian red alga *L. catarinensis* are the most cytotoxic. This is followed by cuparene derivatives isolated from either *L. microcladia* or *L. obtusa*, and then bi-cyclic cytotoxic brominated diterpenes from the red alga *L. obtusa*.

Polyether squalene derivatives, known as thyrseanol A, thyrseanol B and dehydrothyrseiferol from *L. viridis* (as well as related compounds from *L. obtusa* and *L. mariannensis*) show particularly notable activity against P-388 cell lines (Table 1.19). This activity is unrivalled by other compounds extracted from *Laurencia* species, such as chamigrene related compounds.

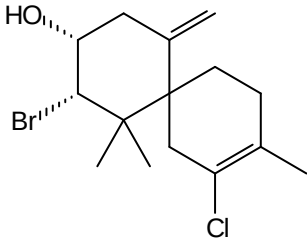
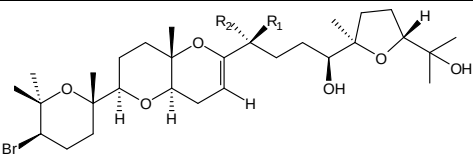
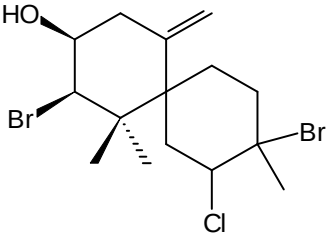
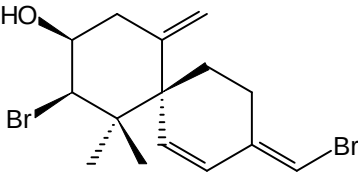
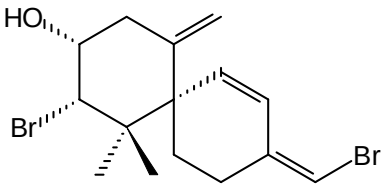
**Table 1.13** IC<sub>50</sub> [μM] data for cytotoxic compounds isolated from *Laurencia* species using A-431 cell lines

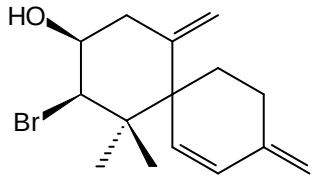
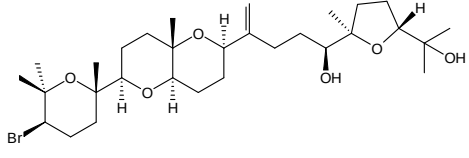
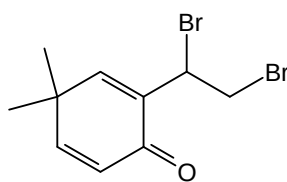
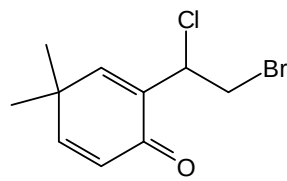
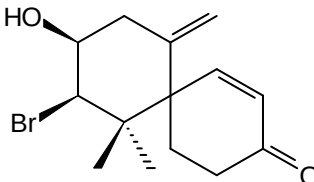
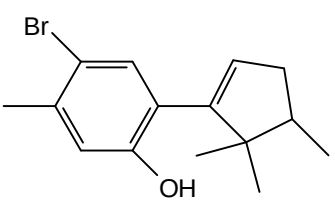
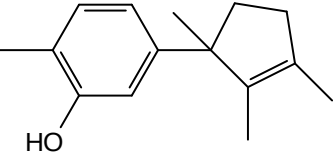
| Species of red algae          | Compound                                                                            | Compound no. | IC <sub>50</sub> - μM | Ref.                            |
|-------------------------------|-------------------------------------------------------------------------------------|--------------|-----------------------|---------------------------------|
| <i>Laurencia catarinensis</i> |    | 1.65         | 10.2                  | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia catarinensis</i> |   | 1.64         | 13.1                  | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia catarinensis</i> |  | 1.67         | 13.1                  | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia catarinensis</i> |  | 1.66         | 17.4                  | (Lhullier <i>et al.</i> , 2010) |

|                               |                                                                                     |             |      |                                   |
|-------------------------------|-------------------------------------------------------------------------------------|-------------|------|-----------------------------------|
| <i>Laurencia catarinensis</i> |    | <b>1.68</b> | 21.6 | (Lhullier <i>et al.</i> , 2010)   |
| <i>Laurencia obtusa</i>       |    | <b>1.79</b> | 23.9 | (Kladi <i>et al.</i> , 2006)      |
| <i>Laurencia microcladia</i>  |    | <b>1.81</b> | 45.8 | (Kladi <i>et al.</i> , 2006)      |
| <i>Laurencia obtusa</i>       |   | <b>1.92</b> | 65.2 | (Lliopoulou <i>et al.</i> , 2003) |
| <i>Laurencia obtusa</i>       |  | <b>1.93</b> | 65.8 | (Lliopoulou <i>et al.</i> , 2003) |
| <i>Laurencia obtusa</i>       |  | <b>1.76</b> | 73.2 | (Kladi <i>et al.</i> , 2006)      |

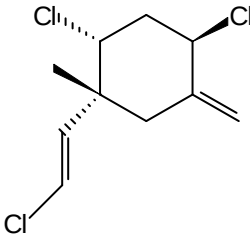
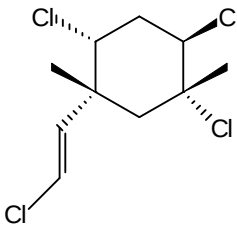
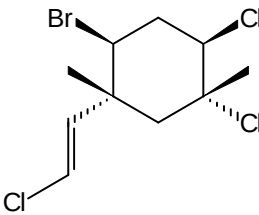
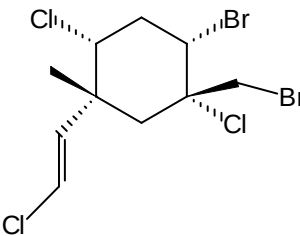
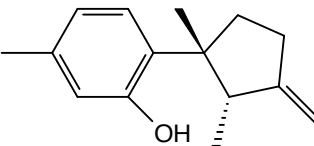
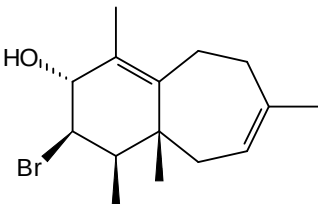
|                              |                                                                                     |             |      |                                   |
|------------------------------|-------------------------------------------------------------------------------------|-------------|------|-----------------------------------|
| <i>Laurencia microcladia</i> |    | <b>1.84</b> | 74.6 | (Kladi <i>et al.</i> , 2006)      |
| <i>Laurencia obtusa</i>      |    | <b>1.91</b> | 78.4 | (Lliopoulou <i>et al.</i> , 2003) |
| <i>Laurencia microcladia</i> |    | <b>1.86</b> | 81.6 | (Kladi <i>et al.</i> , 2006)      |
| <i>Laurencia microcladia</i> |  | <b>1.87</b> | 92.7 | (Kladi <i>et al.</i> , 2007)      |
| <i>Laurencia microcladia</i> |  | <b>1.88</b> | 93.4 | (Kladi <i>et al.</i> , 2007)      |

**Table 1.14** IC<sub>50</sub> [μM] data for cytotoxic compounds isolated from *Portieria* and *Laurencia* species using A-549 cell lines

| Species of red algae          | Compound                                                                                                                                                                                                                           | Compound no. | IC <sub>50</sub> - μM | Ref.                           |
|-------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|-----------------------|--------------------------------|
| <i>Laurencia cartilaginea</i> |                                                                                                                                                   | 1.58         | 0.3                   | (Juagdan <i>et al.</i> , 1997) |
| <i>Laurencia viridis</i>      |  <p>Thyrsenol A (R<sub>1</sub>= OH ; R<sub>2</sub>= CH<sub>2</sub>-OH)<br/>Thyrsenol B (R<sub>1</sub>= CH<sub>2</sub>-OH ; R<sub>2</sub>= OH)</p> | 1.96-1.97    | >1.62                 | (Norte <i>et al.</i> , 1997)   |
| <i>Laurencia cartilaginea</i> |                                                                                                                                                 | 1.63         | 2.4                   | (Juagdan <i>et al.</i> , 1997) |
| <i>Laurencia cartilaginea</i> |                                                                                                                                                 | 1.60         | 2.7                   | (Juagdan <i>et al.</i> , 1997) |
| <i>Laurencia cartilaginea</i> |                                                                                                                                                 | 1.59         | 2.8                   | (Juagdan <i>et al.</i> , 1997) |

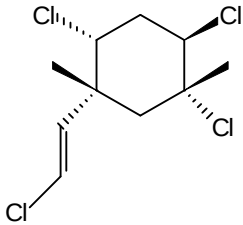
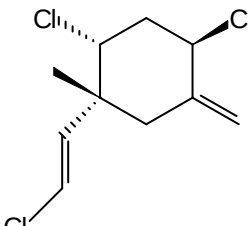
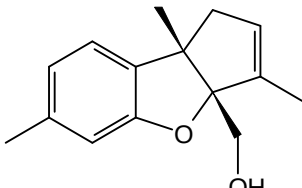
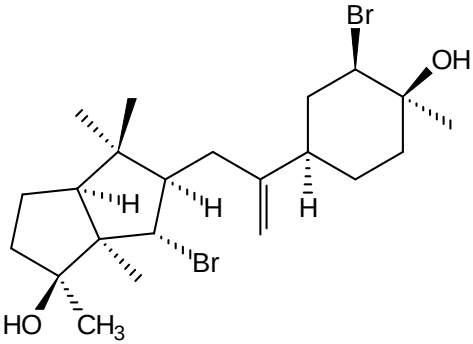
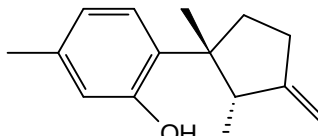
|                               |                                                                                                                                                                                                     |                            |             |                                  |
|-------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|-------------|----------------------------------|
| <i>Laurencia cartilaginea</i> |                                                                                                                    | <b>1.61</b>                | 3.4         | (Juagdan <i>et al.</i> , 1997)   |
| <i>Laurencia viridis</i>      |                                                                                                                    | <b>1.102</b>               | 4.26        | (Norte <i>et al.</i> , 1997).    |
| <i>Portieria hornemannii</i>  | Mixture of two compounds<br><br> | <b>1.52</b><br><b>1.53</b> | 16.2 / 19.0 | (Kuniyoshi <i>et al.</i> , 2003) |
| <i>Laurencia cartilaginea</i> |                                                                                                                  | <b>1.62</b>                | 16.7        | (Juagdan <i>et al.</i> , 1997)   |
| <i>Laurencia microcladia</i>  |                                                                                                                  | <b>1.73</b>                | 52.4        | (Kladi <i>et al.</i> , 2005)     |
| <i>Laurencia microcladia</i>  |                                                                                                                  | <b>1.74</b>                | 81.0        | (Kladi <i>et al.</i> , 2005)     |

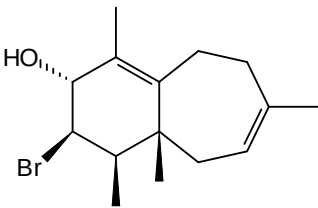
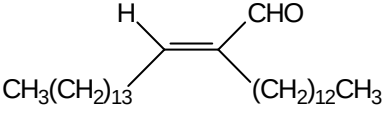
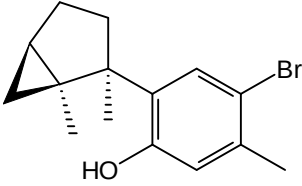
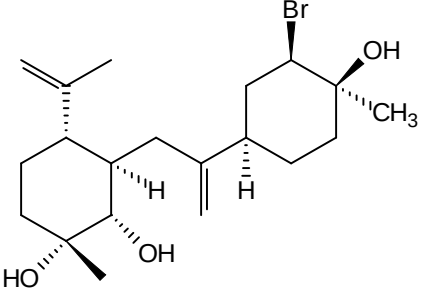
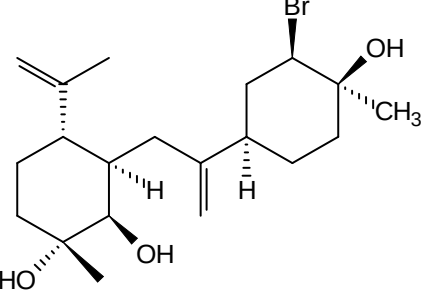
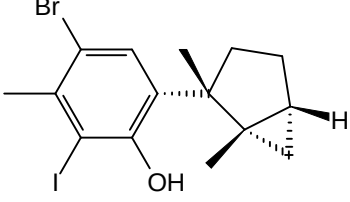
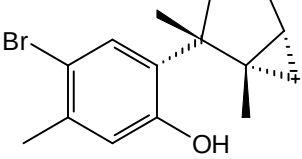
**Table 1.15** IC<sub>50</sub> [μM] data for cytotoxic compounds isolated from *Plocamium* and *Laurencia* species using CHO cell lines

| Species of red algae           | Compound                                                                            | Compound no. | IC <sub>50</sub> - μM | Ref.                   |
|--------------------------------|-------------------------------------------------------------------------------------|--------------|-----------------------|------------------------|
| <i>Plocamium cartilagineum</i> |    | 1.20         | 3.3                   | (de Inés et al., 2004) |
| <i>Plocamium cartilagineum</i> |    | 1.21         | 23.0                  | (de Inés et al., 2004) |
| <i>Plocamium cartilagineum</i> |   | 1.23         | 39.0                  | (de Inés et al., 2004) |
| <i>Plocamium cartilagineum</i> |  | 1.25         | 63.0                  | (de Inés et al., 2004) |
| <i>Laurencia obtusa</i>        |  | 1.79         | 78.2                  | (Kladi et al., 2006)   |
| <i>Laurencia obtusa</i>        |  | 1.76         | 80.2                  | (Kladi et al., 2006)   |

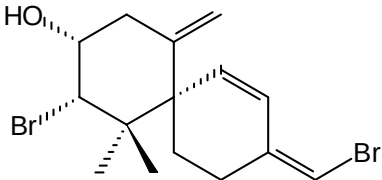
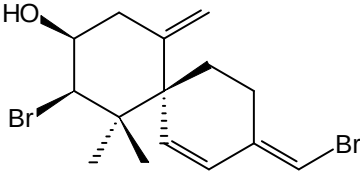
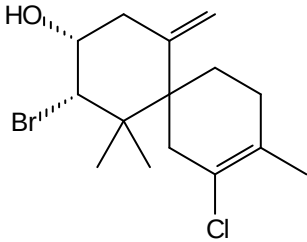
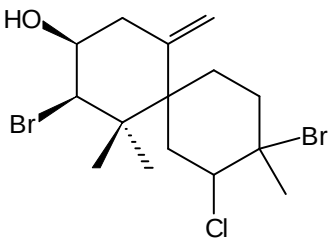
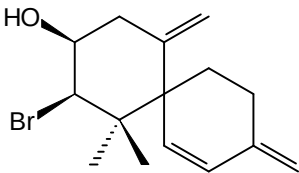
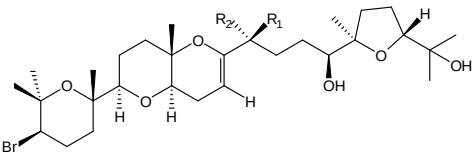


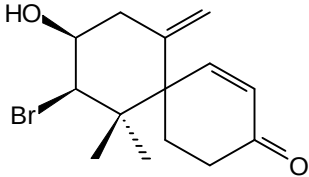
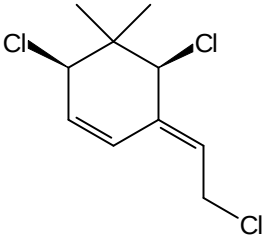
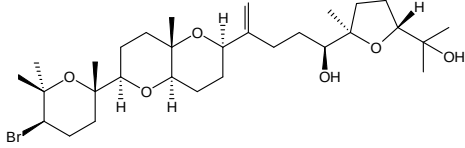
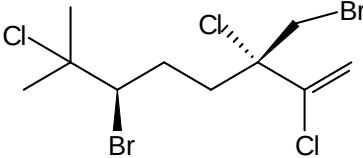
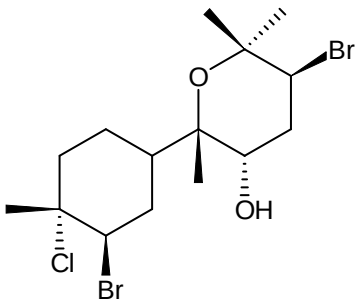
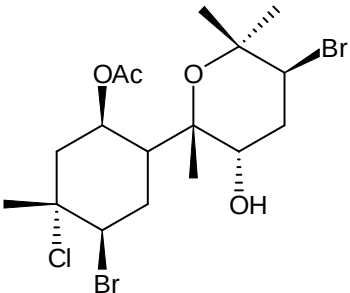
**Table 1.16** IC<sub>50</sub> [μM] data for cytotoxic compounds isolated from *Plocamium* and *Laurencia* species using HeLa cell lines

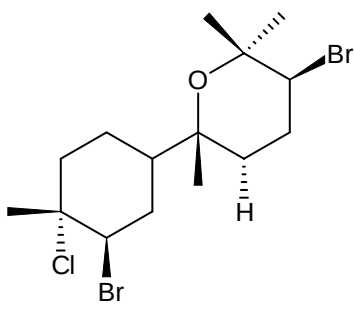
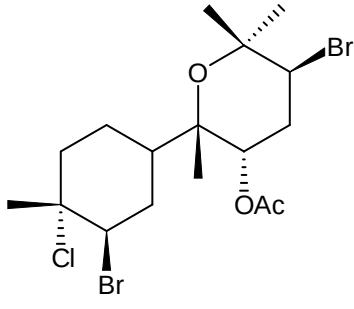
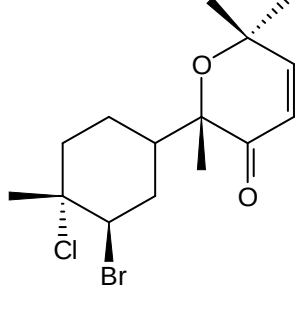
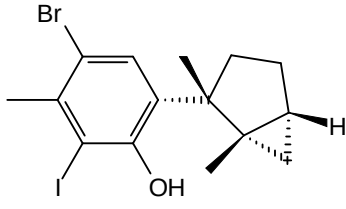
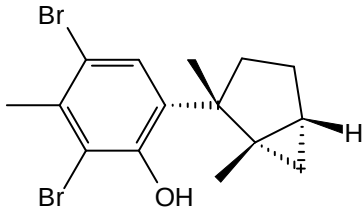
| Species of red algae           | Compound                                                                            | Compound no. | IC <sub>50</sub> - μM | Ref.                              |
|--------------------------------|-------------------------------------------------------------------------------------|--------------|-----------------------|-----------------------------------|
| <i>Plocamium cartilagineum</i> |    | 1.21         | 5.7                   | (de Inés <i>et al.</i> , 2004)    |
| <i>Plocamium cartilagineum</i> |    | 1.20         | 13.1                  | (de Inés <i>et al.</i> , 2004)    |
| <i>Laurencia tristicha</i>     |   | 1.95         | 15.5                  | (Sun <i>et al.</i> , 2007)        |
| <i>Laurencia obtusa</i>        |  | 1.93         | 34.4                  | (Lliopoulou <i>et al.</i> , 2003) |
| <i>Laurencia obtusa</i>        |  | 1.79         | 40.5                  | (Kladi <i>et al.</i> , 2006)      |

|                              |                                                                                     |             |      |                                   |
|------------------------------|-------------------------------------------------------------------------------------|-------------|------|-----------------------------------|
| <i>Laurencia obtusa</i>      |    | <b>1.76</b> | 50.9 | (Kladi <i>et al.</i> , 2006)      |
| <i>Laurencia microcladia</i> |    | <b>1.81</b> | 51.8 | (Kladi <i>et al.</i> , 2006)      |
| <i>Laurencia okamurai</i>    |    | <b>1.94</b> | 71.1 | (Tsukamoto <i>et al.</i> , 2005)  |
| <i>Laurencia obtusa</i>      |   | <b>1.92</b> | 78.0 | (Lliopoulou <i>et al.</i> , 2003) |
| <i>Laurencia obtusa</i>      |  | <b>1.91</b> | 80.5 | (Lliopoulou <i>et al.</i> , 2003) |
| <i>Laurencia microcladia</i> |  | <b>1.87</b> | 81.4 | (Kladi <i>et al.</i> , 2007)      |
| <i>Laurencia microcladia</i> |  | <b>1.84</b> | 83.9 | (Kladi <i>et al.</i> , 2006)      |

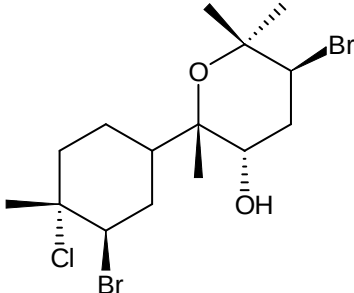
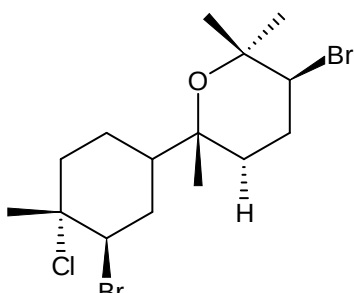
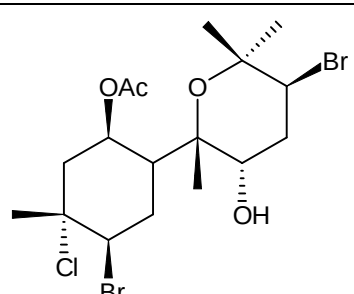
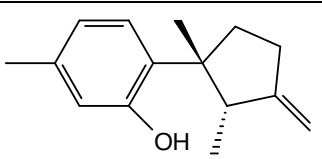
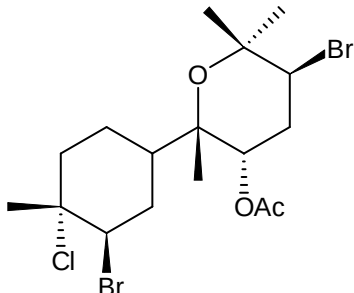
**Table 1.17** IC<sub>50</sub> [μM] data for cytotoxic compounds isolated from *Laurencia* species using HT-29 cell lines

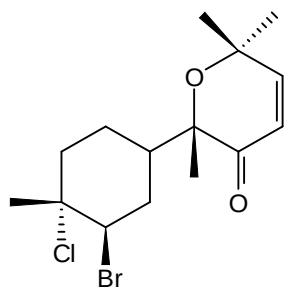
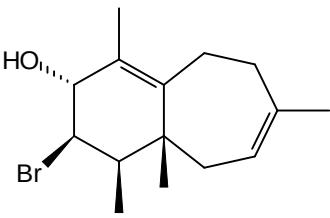
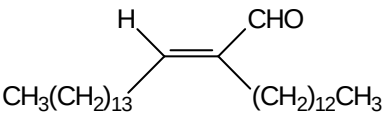
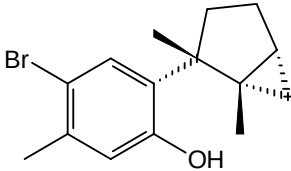
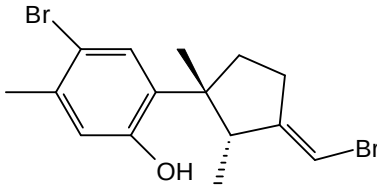
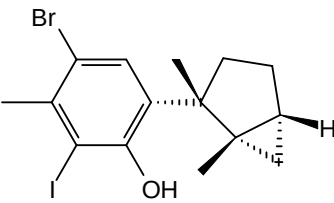
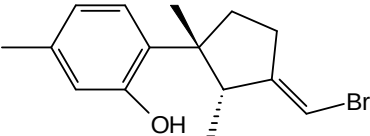
| Species of red algae          | Compound                                                                                                                                                                                                                                    | Compound no. | IC <sub>50</sub> - μM | Ref.                    |
|-------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|-----------------------|-------------------------|
| <i>Laurencia cartilaginea</i> |                                                                                                                                                            | 1.59         | 0.07                  | (Juagdan, et al., 1997) |
| <i>Laurencia cartilaginea</i> |                                                                                                                                                            | 1.60         | 0.07                  | (Juagdan, et al., 1997) |
| <i>Laurencia cartilaginea</i> |                                                                                                                                                           | 1.58         | 0.3                   | (Juagdan, et al., 1997) |
| <i>Laurencia cartilaginea</i> |                                                                                                                                                          | 1.63         | 0.60                  | (Juagdan, et al., 1997) |
| <i>Laurencia cartilaginea</i> |                                                                                                                                                          | 1.61         | 0.84                  | (Juagdan, et al., 1997) |
| <i>Laurencia viridis</i>      | <br>Thyrsenol A (R <sub>1</sub> = OH ; R <sub>2</sub> = CH <sub>2</sub> -OH)<br>Thyrsenol B (R <sub>1</sub> = CH <sub>2</sub> -OH ; R <sub>2</sub> = OH) | 1.96-1.97    | >1.62                 | (Norte et al., 1997)    |

|                               |                                                                                     |              |       |                                 |
|-------------------------------|-------------------------------------------------------------------------------------|--------------|-------|---------------------------------|
| <i>Laurencia cartilaginea</i> |    | <b>1.62</b>  | 1.7   | (Juagdan, <i>et al.</i> , 1997) |
| <i>Portieria hornemannii</i>  |    | <b>1.49</b>  | ~4.20 | (Fuller <i>et al.</i> , 1992)   |
| <i>Laurencia viridis</i>      |    | <b>1.102</b> | 4.26  | (Norte <i>et al.</i> , 1997)    |
| <i>Portieria hornemannii</i>  |   | <b>1.8</b>   | ~5.50 | (Fuller <i>et al.</i> , 1992)   |
| <i>Laurencia catarinensis</i> |  | <b>1.65</b>  | 7.6   | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia catarinensis</i> |  | <b>1.64</b>  | 12.4  | (Lhullier <i>et al.</i> , 2010) |

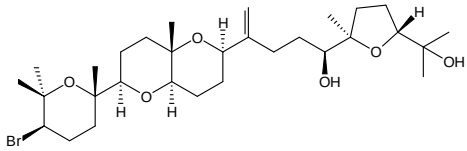
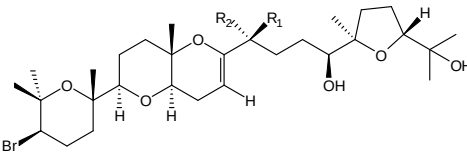
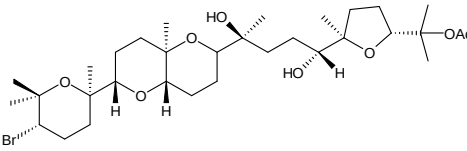
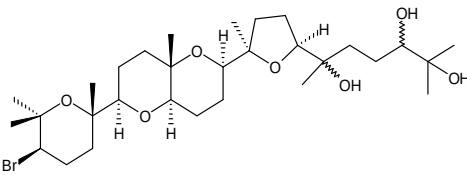
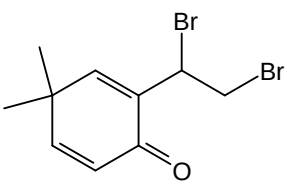
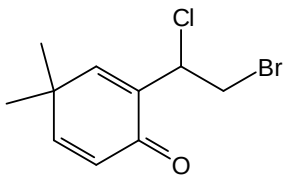
|                               |                                                                                     |             |      |                                 |
|-------------------------------|-------------------------------------------------------------------------------------|-------------|------|---------------------------------|
| <i>Laurencia catarinensis</i> |    | <b>1.67</b> | 12.5 | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia catarinensis</i> |    | <b>1.66</b> | 16.1 | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia catarinensis</i> |   | <b>1.68</b> | 18.9 | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia microcladia</i>  |  | <b>1.87</b> | 78.4 | (Kladi <i>et al.</i> , 2007)    |
| <i>Laurencia microcladia</i>  |  | <b>1.88</b> | 98.7 | (Kladi <i>et al.</i> , 2007)    |

**Table 1.18** IC<sub>50</sub> [μM] data for cytotoxic compounds isolated from *Laurencia* species using MCF-7 cell lines

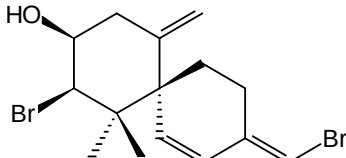
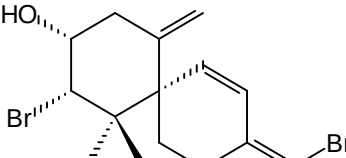
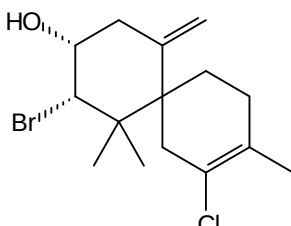
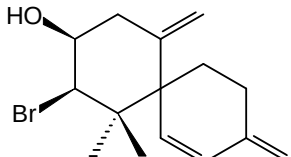
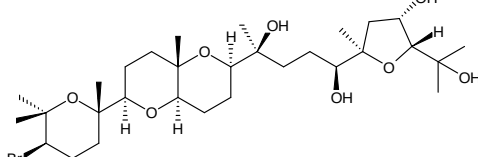
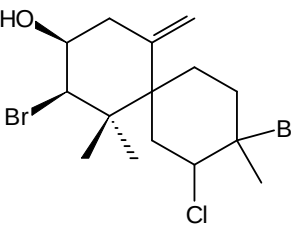
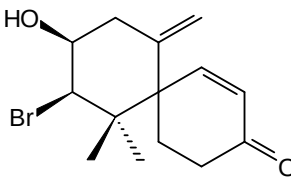
| Species of red algae          | Compound                                                                            | Compound no. | IC <sub>50</sub> - μM | Ref.                            |
|-------------------------------|-------------------------------------------------------------------------------------|--------------|-----------------------|---------------------------------|
| <i>Laurencia catarinensis</i> |    | 1.65         | 9.7                   | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia catarinensis</i> |   | 1.67         | 11.2                  | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia catarinensis</i> |  | 1.64         | 11.7                  | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia obtusa</i>       |  | 1.79         | 15.8                  | (Kladi <i>et al.</i> , 2006)    |
| <i>Laurencia catarinensis</i> |  | 1.66         | 17.6                  | (Lhullier <i>et al.</i> , 2010) |

|                               |                                                                                     |             |      |                                 |
|-------------------------------|-------------------------------------------------------------------------------------|-------------|------|---------------------------------|
| <i>Laurencia catarinensis</i> |    | <b>1.68</b> | 19.7 | (Lhullier <i>et al.</i> , 2010) |
| <i>Laurencia obtusa</i>       |    | <b>1.76</b> | 28.2 | (Kladi <i>et al.</i> , 2006)    |
| <i>Laurencia microcladia</i>  |    | <b>1.81</b> | 51.4 | (Kladi <i>et al.</i> , 2006)    |
| <i>Laurencia microcladia</i>  |   | <b>1.84</b> | 67.2 | (Kladi <i>et al.</i> , 2006)    |
| <i>Laurencia microcladia</i>  |  | <b>1.86</b> | 78.3 | (Kladi <i>et al.</i> , 2006)    |
| <i>Laurencia microcladia</i>  |  | <b>1.87</b> | 86.3 | (Kladi <i>et al.</i> , 2007)    |
| <i>Laurencia obtusa</i>       |  | <b>1.80</b> | 95.5 | (Kladi <i>et al.</i> , 2006)    |

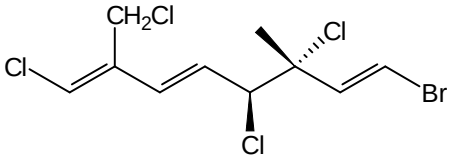
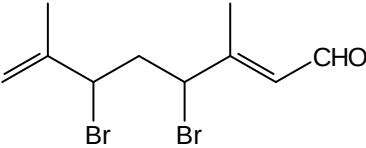
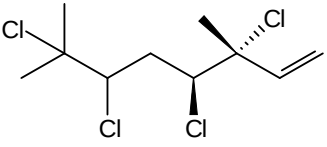
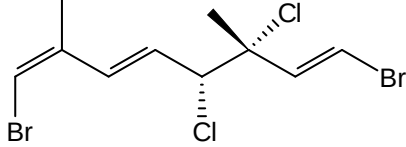
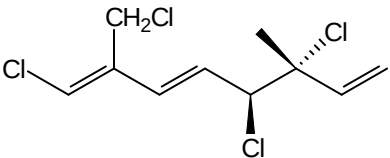
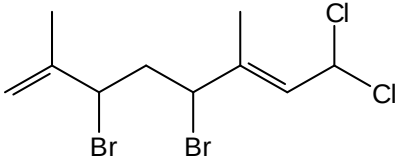
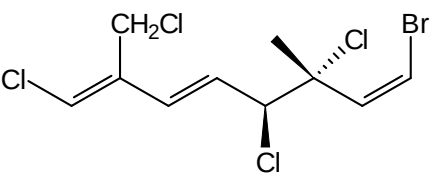
**Table 1.19** IC<sub>50</sub> [μM] data for cytotoxic compounds isolated from *Portieria* and *Laurencia* species using P-388 cell lines

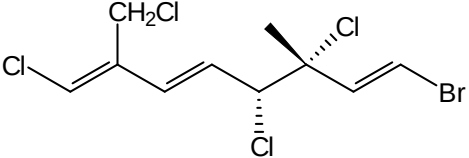
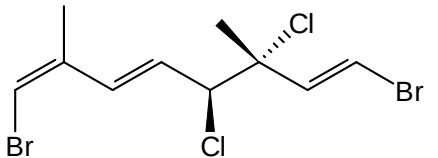
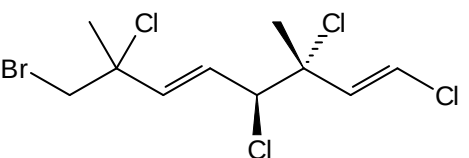
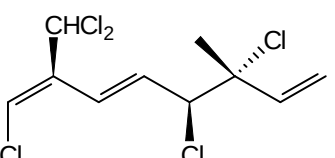
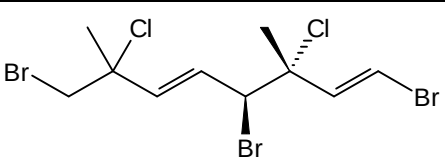
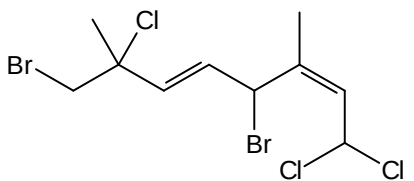
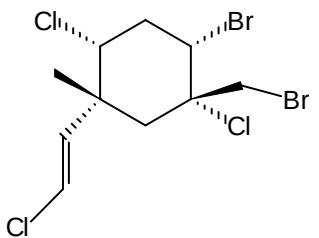
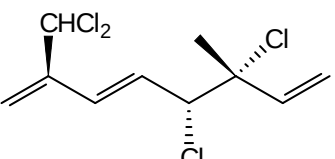
| Species of red algae          | Compound                                                                                                                                                                                                                                  | Compound no.                   | IC <sub>50</sub> - μM | Ref.                             |
|-------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|-----------------------|----------------------------------|
| <i>Laurencia viridis</i>      |                                                                                                                                                          | <b>1.98</b>                    | 0.017                 | (Norte <i>et al.</i> , 1997)     |
| <i>Laurencia viridis</i>      | <br>Thyrsenol A (R <sub>1</sub> = OH ; R <sub>2</sub> = CH <sub>2</sub> -OH)<br>Thyrsenol B (R <sub>1</sub> = CH <sub>2</sub> -OH ; R <sub>2</sub> = OH) | <b>1.96-1.97</b>               | 0.40 / 0.016          | (Norte <i>et al.</i> , 1997)     |
| <i>Laurencia obtusa</i>       |                                                                                                                                                         | <b>1.89</b>                    | 0.30                  | (Suzuki <i>et al.</i> , 1985)    |
| <i>Laurencia mariannensis</i> |                                                                                                                                                        | <b>1.70</b>                    | 1.01                  | (Ji <i>et al.</i> , 2008)        |
| <i>Portieria hornemannii</i>  | Mixture of two compounds<br><br>                                    | <b>1.52</b><br><br><b>1.53</b> | 1.6 / 1.9             | (Kuniyoshi <i>et al.</i> , 2003) |

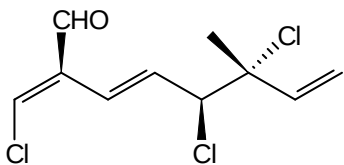
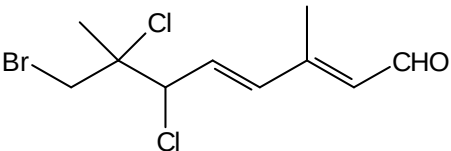
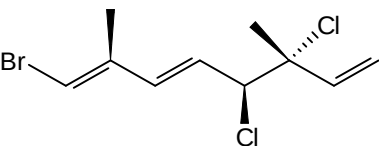
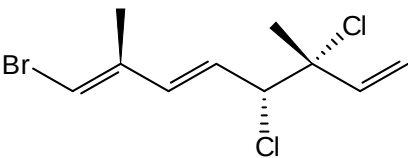


|                               |                                                                                     |             |      |                                 |
|-------------------------------|-------------------------------------------------------------------------------------|-------------|------|---------------------------------|
| <i>Laurencia cartilaginea</i> |    | <b>1.60</b> | 2.7  | (Juagdan, <i>et al.</i> , 1997) |
| <i>Laurencia cartilaginea</i> |    | <b>1.59</b> | 2.8  | (Juagdan, <i>et al.</i> , 1997) |
| <i>Laurencia cartilaginea</i> |    | <b>1.58</b> | 3.0  | (Juagdan, <i>et al.</i> , 1997) |
| <i>Laurencia cartilaginea</i> |   | <b>1.61</b> | 3.4  | (Juagdan, <i>et al.</i> , 1997) |
| <i>Laurencia mariannensis</i> |  | <b>1.71</b> | 10.6 | (Ji <i>et al.</i> , 2008)       |
| <i>Laurencia cartilaginea</i> |  | <b>1.63</b> | 12.1 | (Juagdan, <i>et al.</i> , 1997) |
| <i>Laurencia cartilaginea</i> |  | <b>1.62</b> | 16.7 | (Juagdan, <i>et al.</i> , 1997) |

**Table 1.20** IC<sub>50</sub> [μM] data for cytotoxic compounds isolated from *Plocamium* species using WHCO1 cell lines

| Species of red algae          | Compound                                                                            | Compound no. | IC <sub>50</sub> - μM | Ref.                           |
|-------------------------------|-------------------------------------------------------------------------------------|--------------|-----------------------|--------------------------------|
| <i>Plocamium suhrii</i>       |    | 1.34         | 6.6                   | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium corallorhiza</i> |    | 1.30         | 7.5                   | (Mann <i>et al.</i> , 2007)    |
| <i>Plocamium suhrii</i>       |    | 1.38         | 7.9                   | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium suhrii</i>       |  | 1.39         | 8.4                   | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium suhrii</i>       |  | 1.37         | 8.5                   | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium corallorhiza</i> |  | 1.26         | 9.3                   | (Knott <i>et al.</i> , 2005)   |
| <i>Plocamium suhrii</i>       |  | 1.36         | 9.3                   | (Antunes <i>et al.</i> , 2011) |

|                               |                                                                                     |              |      |                                |
|-------------------------------|-------------------------------------------------------------------------------------|--------------|------|--------------------------------|
| <i>Plocamium suhrii</i>       |    | <b>1.35</b>  | 9.9  | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium suhrii</i>       |    | <b>1.40</b>  | 15.1 | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium corallorhiza</i> |    | <b>1.28</b>  | 17.2 | (Knott <i>et al.</i> , 2005)   |
| <i>Plocamium cornutum</i>     |    | <b>1.107</b> | 17.8 | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium corallorhiza</i> |  | <b>1.29</b>  | 18.1 | (Knott <i>et al.</i> , 2005)   |
| <i>Plocamium corallorhiza</i> |  | <b>1.27</b>  | 33.8 | (Knott <i>et al.</i> , 2005)   |
| <i>Plocamium corallorhiza</i> |  | <b>1.25</b>  | 34.8 | (Knott <i>et al.</i> , 2005)   |
| <i>Plocamium cornutum</i>     |  | <b>1.108</b> | 40.2 | (Antunes <i>et al.</i> , 2011) |

|                               |                                                                                   |              |      |                                |
|-------------------------------|-----------------------------------------------------------------------------------|--------------|------|--------------------------------|
| <i>Plocamium cornutum</i>     |  | <b>1.109</b> | 47.3 | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium corallorhiza</i> |  | <b>1.31</b>  | 64.8 | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium cornutum</i>     |  | <b>1.110</b> | 87.6 | (Antunes <i>et al.</i> , 2011) |
| <i>Plocamium cornutum</i>     |  | <b>1.111</b> | 87.6 | (Antunes <i>et al.</i> , 2011) |

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## Chapter Two

# Polyhalogenated cyclic monoterpenes from the red alga *Plocamium maxillosum* and *Plocamium robertiae*<sup>1</sup>

## 2.1 Abstract

Two new cyclic polyhalogenated monoterpenes (**2.1-2.2**) have been isolated from an organic extract of the red alga *Plocamium maxillosum* (Poiret) Lamouroux. The structures of the new compounds were characterised as 6*E*-chloromethylene-4-(2*E*-chlorovinyl)-4-methylcyclohex-2-enone (**2.1**) and 6*Z*-chloromethylene-4-(2*E*-chlorovinyl)-4-methylcyclohex-2-enone (**2.2**) on the basis of one and two dimensional NMR spectroscopic data and MS analysis.

Compounds **2.1-2.2** showed good cytotoxicity towards breast cancer cell lines.

One new cyclic polyhalogenated monoterpene and one known cyclic polyhalogenated monoterpene were also isolated from an organic extract of *Plocamium robertiae* (F. Schmitz ex Mazza). These compounds are known as 4,5-dibromo-5-chloromethyl-1-chlorovinyl-2-chloro-methylcyclohexane (**2.6**) and 2,4-dichloro-1-chlorovinyl-1-methylcyclohexane-5-ene (**2.9**) or Plocamene D, and were also characterised using one and two dimensional NMR spectroscopic data and MS analysis.

**Keywords:** *Cyclic halogenated monoterpenes; Plocamium maxillosum; Plocamium robertiae*

## 2.2 Introduction

Red algae (Rhodophyta) of the family Plocamiaceae and Rhizophyllidaceae produce a number of different biologically active linear and cyclic polyhalogenated monoterpenes (Kladi *et al.*, 2004). Of the 47 different species of *Plocamium* that occur around the world, at least 10 species occur on South Africa's coast (Guiry, 1998). Of these species, the following are endemic to South Africa; *Plocamium beckeri*, *Plocamium corallorhiza*, *Plocamium cornutum*, *Plocamium glomeratum*, *Plocamium maxillosum*, *Plocamium rigidum* and *Plocamium suhrii*. (For an overview of the secondary metabolites isolated from *Plocamium* species worldwide see 2.8 Appendix 1.)

<sup>1</sup> Initial work on *Plocamium robertiae* was done by Mr Maynard Chiwakata who extracted the alga, fractionated the crude extract by column chromatography and profiled fractions by <sup>1</sup>H NMR spectroscopy.

In the continuing investigation of biologically active metabolites from South African *Plocamium* species (Afolayan *et al.*, 2009; Knott *et al.*, 2005; Mann *et al.*, 2007) the natural product chemistry of the algae *P. maxillosum* and *P. robertiae* has finally been resolved.

*P. maxillosum* grows up to 20 cm long, with fronds up to 3 mm broad. A main characteristic is that groups of laterals consist of one simple and two or sometimes three compound branches (Stegenga *et al.*, 1997). *P. maxillosum* is endemic to South Africa and occurs mostly in the subtidal area. *P. maxillosum* is unique to the west and northeast coasts of South Africa (Silva *et al.*, 1996; Stegenga *et al.*, 1997), having been recorded from Hondeklip Bay to Cape Agulhas. Pocock (1958) recorded it from Mozambique, but this record is considered doubtful, as it is in a very different bio-geographical region from the typical range of this species. *P. robertiae* was considered a narrower form of *P. corallorhiza* by Simons (1964) but several morphological features, such as the narrow main axes width, linear, triangulate simple laterals, reduced and spaced teeth-like structures distinguish it from the latter species (Francis, 2011). Interestingly, *P. robertiae* has only ever been collected from Port Alfred in the Eastern Cape.

Officially, current literature states that *P. robertiae* and *P. corallorhiza* are the same species (Simons, 1964), however, it has been suggested that they are in fact two different species (Bolton, 2011). Unfortunately, DNA sequencing has not yet resolved this issue.

## 2.3 Chapter aims

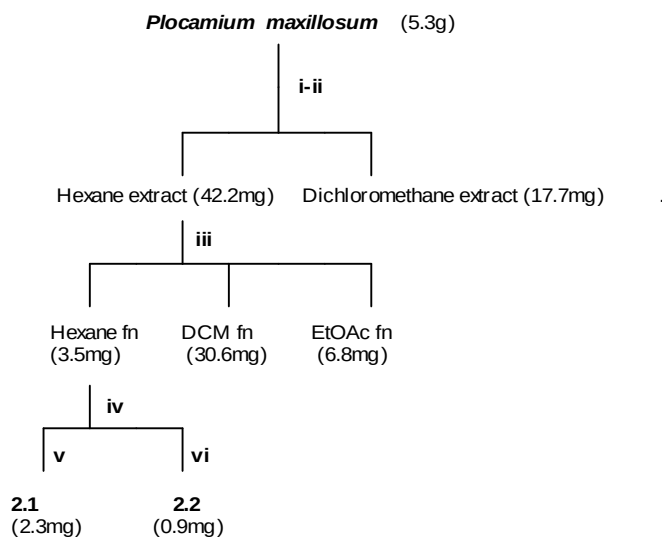
The aim of the study was to isolate and characterise novel compounds from South African marine algae and screen these new compounds for *in vitro* cytotoxic activity.

## 2.4 Results and discussion

In a previous study on *P. maxillosum* using gas chromatography-mass spectrometry (GC-MS) the presence of two main compounds and numerous minor ones in a crude extract of *P. maxillosum* (Knott, 2003) was noted. Due to a paucity of material it was not possible to fully characterise the compounds at that time. Fortunately, after several years the author was given a small sample of this alga for further studies.

### 2.4.1 Collection, extraction and isolation

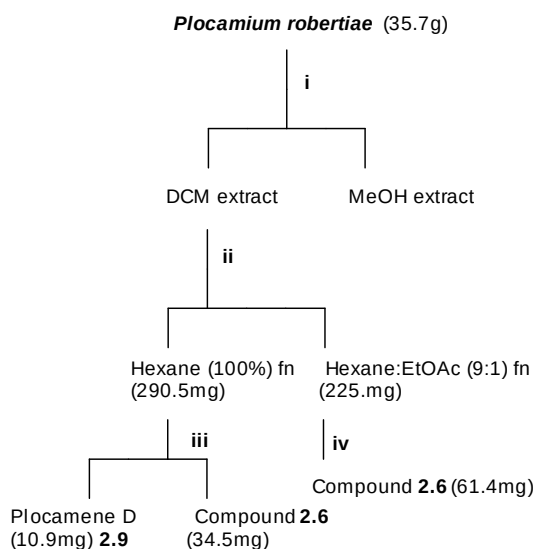
See the experimental section; paragraphs 2.5.2 and 2.5.3 for a full explanation of the collection, extraction and isolation procedure illustrated in Scheme 2.1 below.



**Scheme 2.1** Extraction and isolation of metabolites from *P. maxillosum*

Conditions: i) Extracted three times using 100 ml of  $\text{CH}_2\text{Cl}_2$ -MeOH (2:1) at 30 °C for 30 minutes per extraction ii) The crude extract was partitioned (three times) first using 100 ml hexane (per extraction) followed by 100 ml  $\text{CH}_2\text{Cl}_2$  per extraction (both hexane and  $\text{CH}_2\text{Cl}_2$   $^1\text{H}$  NMR spectra looked the same) iii) The hexane extract was chromatographed on a silica column and eluted with hexane,  $\text{CH}_2\text{Cl}_2$  and EtOAc iv) The hexane fraction was dried and then dissolved in  $\text{CH}_2\text{Cl}_2$  and placed on a silica column v) The hexane-EtOAc (9:1) fraction yielded compound 2.1 vi) Compound 2.2 was the 100% EtOAc fraction

See the experimental section; paragraphs 2.5.2 and 2.5.3 for a full explanation of the collection, extraction and isolation procedure illustrated in Scheme 2.2 below.



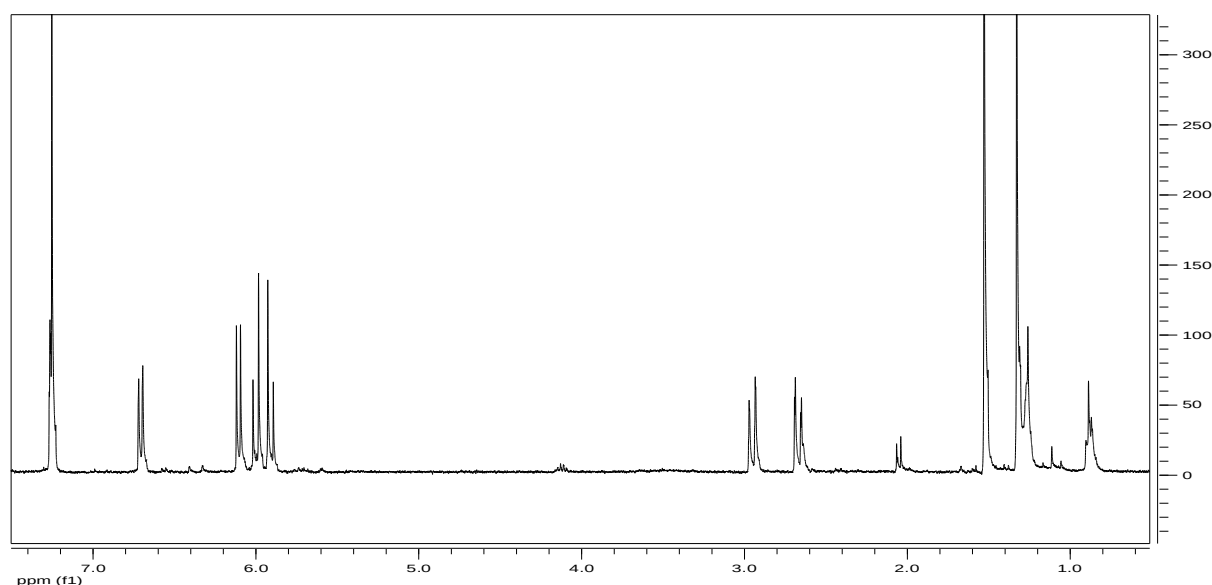
**Scheme 2.2** Extraction and isolation of metabolites from *P. robertiae*

Conditions: i) The frozen algae (35.7 g dry weight) was first extracted with MeOH. The MeOH was drained off and separated. Then the algae was placed in CH<sub>2</sub>Cl<sub>2</sub>-MeOH (2:1) x 3, at 35 °C for 30 minutes per extraction. The CH<sub>2</sub>Cl<sub>2</sub>-MeOH extracts were pooled and H<sub>2</sub>O was added until the CH<sub>2</sub>Cl<sub>2</sub> and aqueous MeOH layers separated. The CH<sub>2</sub>Cl<sub>2</sub> extracts were then combined and dried ii) The CH<sub>2</sub>Cl<sub>2</sub> extract was chromatographed on a silica column and eluted with hexane 100% hexane and hexane-EtOAc (9:1) as eluents iii) 145.5 mg of the 100% hexane fraction was then dissolved in CH<sub>2</sub>Cl<sub>2</sub> chromatographed on a silica column which consisted of 0.59 g silica gel. 100% hexane was used as an eluent. From this compound **2.9** was obtained. Following this, the 100% hexane fraction was separated further using hexane-EtOAc (98:2) using a silica column as mentioned above. This fraction was compound **2.6** iv) 112.9 mg of the hexane-EtOAc (9:1) fraction was also chromatographed on a silica column which consisted of 0.59 g silica gel. This fraction was separated using hexane-EtOAc (9:1) as an eluent and from this compound **2.6** was isolated.

### 2.4.2 Characterisation of compounds from *P. maxillosum* and *P. robertiae*

The isolation and characterisation, as well as the cytotoxic activity of two new halogenated monoterpene cyclohexanone derivatives from organic extracts of *P. maxillosum* are described below. In addition to this, one new cyclic halogenated monoterpene and one known cyclic halogenated monoterpene from *P. robertiae* are also described.

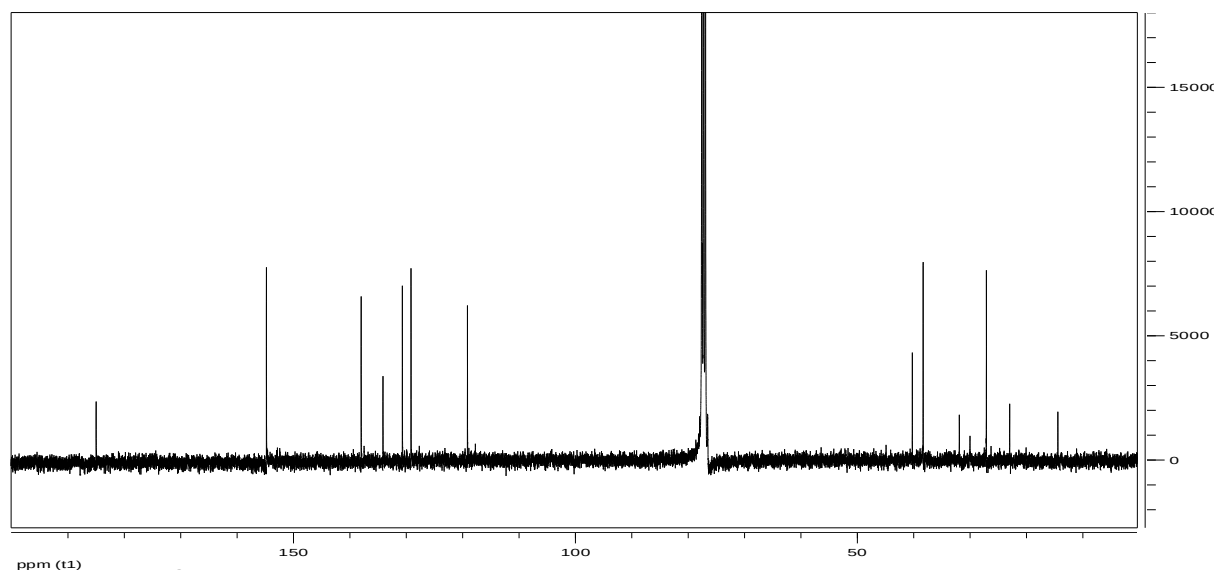
#### Structural elucidation of compound **2.1** from *P. maxillosum*



**Figure 2.1**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **2.1**

The major compound (**2.1**) was isolated as a yellow oil with an optical rotation of  $-250^\circ$  and gave a molecular ion peak at  $m/z$  216.0094, which corresponds to a molecular formula of  $\text{C}_{10}\text{H}_{10}\text{OCl}_2$ . The  $^1\text{H}$  NMR spectrum of **2.1** (Figure 2.1; Table 2.1) indicated the presence of two pairs of mutually coupled, deshielded methines at  $\delta$  6.00 ( $J = 13.5$  Hz) and 5.91 ( $J = 13.5$  Hz), and at  $\delta$  6.10 ( $J = 10.1$  Hz) and 6.70 ( $J = 10.1$  Hz). In addition, a methine singlet at  $\delta$  7.26, a two proton doublet at  $\delta$  2.96 and 2.66 as well as a single methyl group at  $\delta$  1.32 was also observed. The  $^{13}\text{C}$  NMR and DEPT-135 spectra of **2.1** confirmed the presence of ten carbons corresponding to three double bonds ( $\delta$  154.7, 137.8, 134.0, 130.5, 129.0 and 119.0), an ketone ( $\delta$  184.9), a methyl group ( $\delta$  26.8), a methylene ( $\delta$  38.0) and an  $\text{sp}^3$  hybridised quaternary carbon ( $\delta$  40.0) (Figure 2.2). The presence of three double bonds and a ketone group, suggested that compound **2.1** had to be cyclic to account for the five degrees of unsaturation implied by its molecular formula.

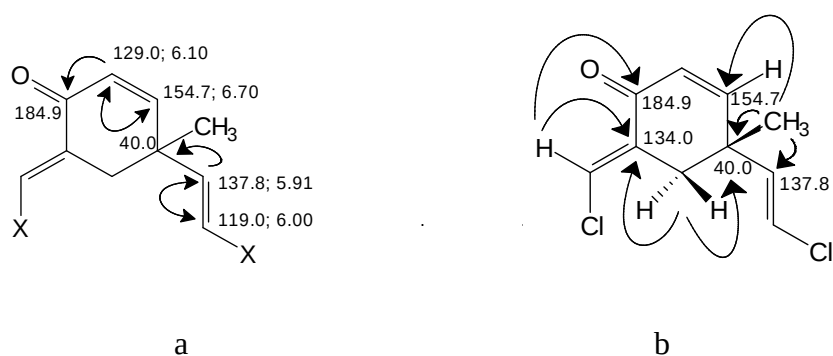




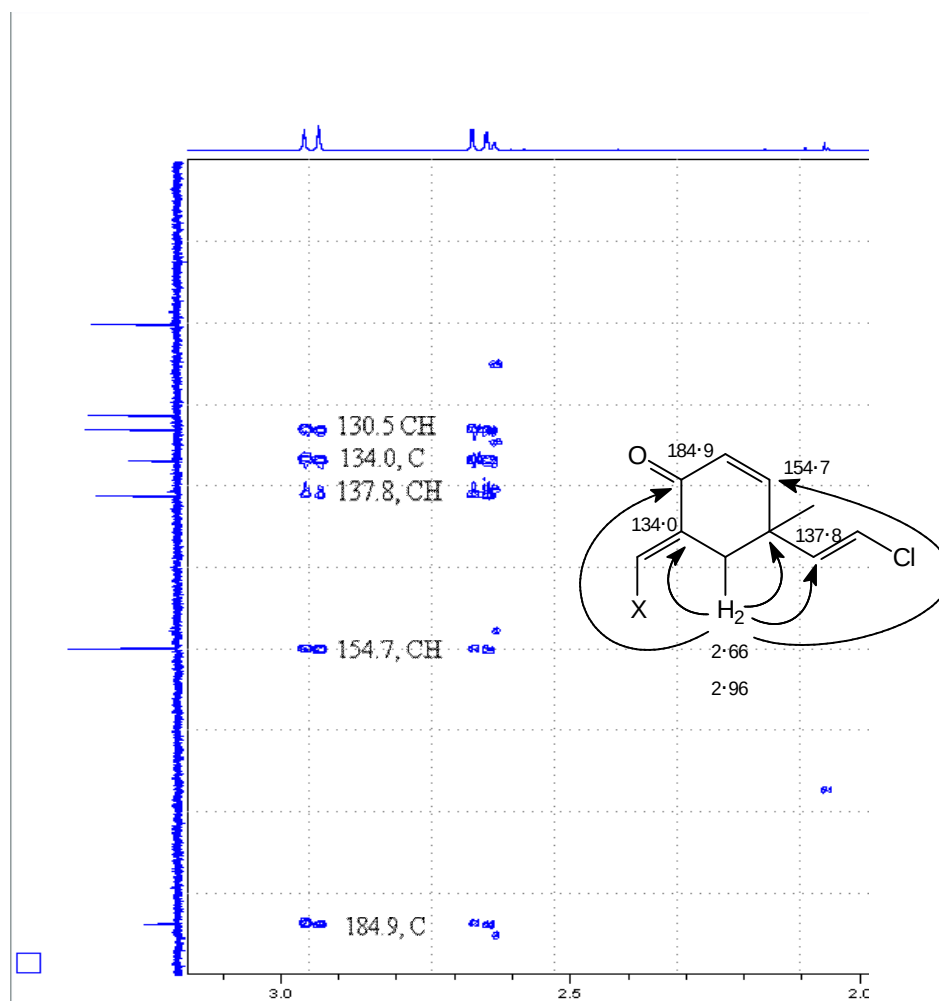
**Figure 2.2**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of compound **2.1**

Furthermore, an infrared band of  $1674\text{ cm}^{-1}$  together with a  $\lambda_{\text{max}}$  of 245 nm supported the presence of an  $\alpha,\beta$ -unsaturated cyclohexenone ring system. Careful analysis of the  $^1\text{H}$ - $^1\text{H}$  COSY in combination with HSQC and HMBC data allowed the assignment of two discrete spin systems, namely;  $\text{C}-\text{CH}=\text{CH}-\text{C}=\text{O}$  ( $\delta$  6.10, d,  $J = 10.1\text{ Hz}$ , 6.70, d,  $J = 10.1\text{ Hz}$ ) and  $\text{C}-\text{CH}=\text{CH}-\text{X}$  ( $\delta$  6.00, d,  $J = 13.5\text{ Hz}$  and 5.91, d,  $J = 13.5\text{ Hz}$ ) (Figure 2.3a). Key HMBC correlations that allowed the assignment of the structure are summarised in Figure 2.3b. The methyl group at  $\delta$  1.32 showed correlations to the quaternary carbon signal at  $\delta$  40.0 and the olefinic methines at  $\delta$  137.8 and 154.7, thus connecting the two fragments.

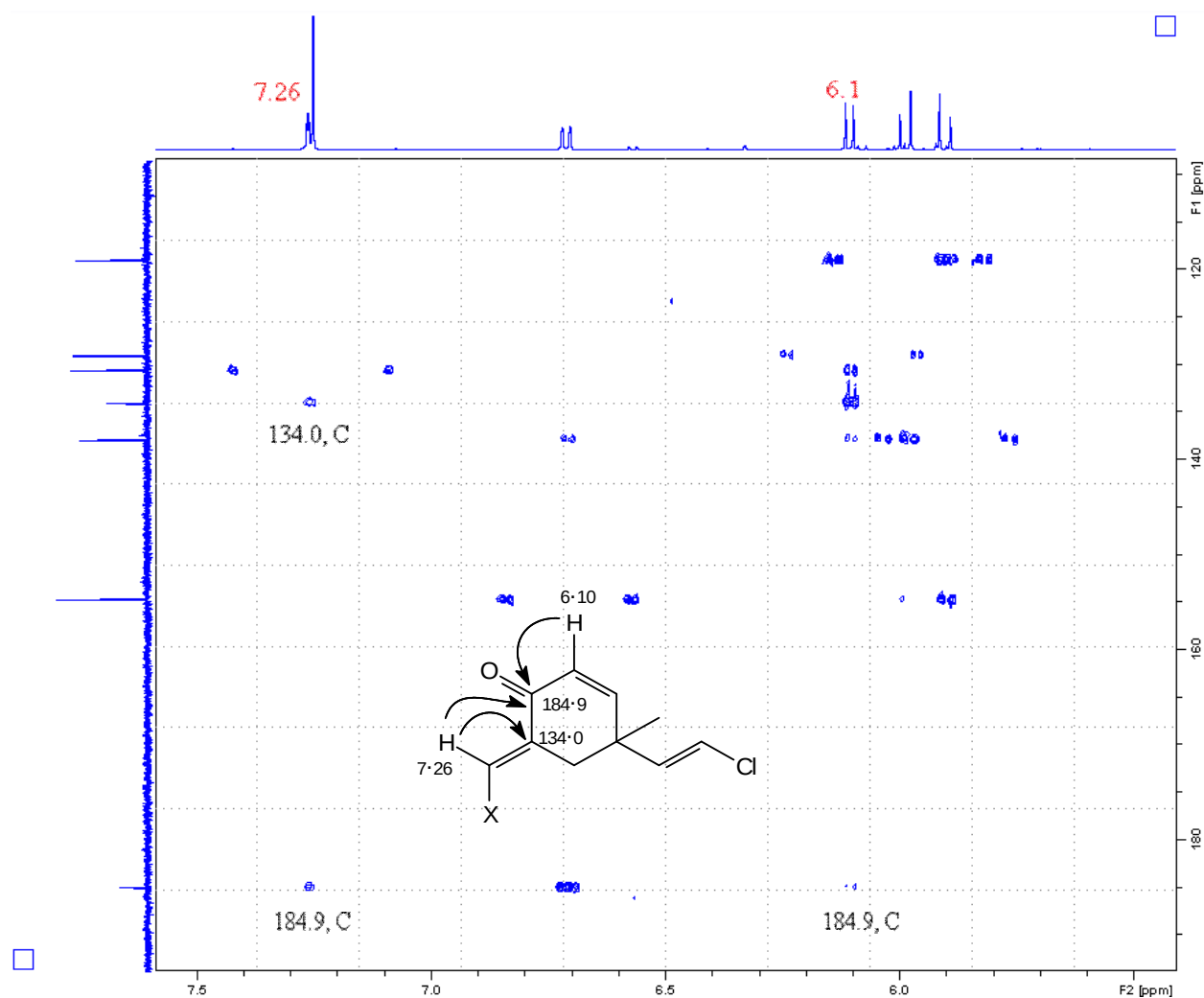
Further HMBC correlations from the diastereotopic methylene signals at  $\delta$  2.96 and 2.66 to the carbon signal at  $\delta$  40.0 and the olefinic carbon at  $\delta$  134.0 (Figure 2.4) and from the olefinic methine at  $\delta$  7.26 to the carbon signals at  $\delta$  134.0 and 184.9 (Figure 2.5) completed the assignment of the planar structure of the cyclohexenone ring system.



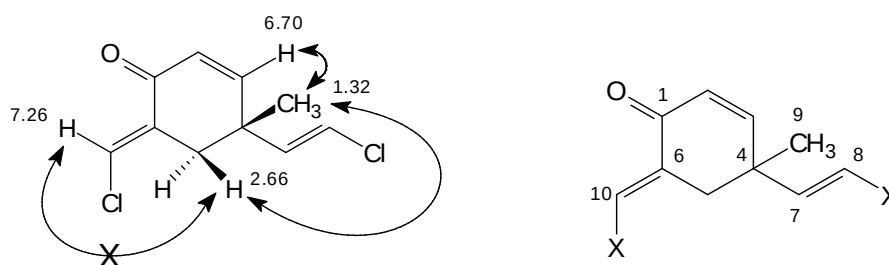
**Figure 2.3** Selected COSY, HSQC and HMBC correlations (a); HMBC correlations (b)



**Figure 2.4** HMBC correlations from the diastereotopic methylene signals at  $\delta$  2.96 and 2.66 to the olefinic carbon at  $\delta$  134.0 (amongst others)



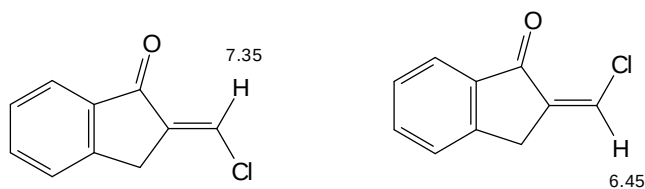
**Figure 2.5** HMBC correlations from the olefinic methine at  $\delta$  7.26 to carbon signals at  $\delta$  134.0 and 184.9. On the other side, one can note how the methine at  $\delta$  6.10 correlates to the carbon signal at  $\delta$  184.9



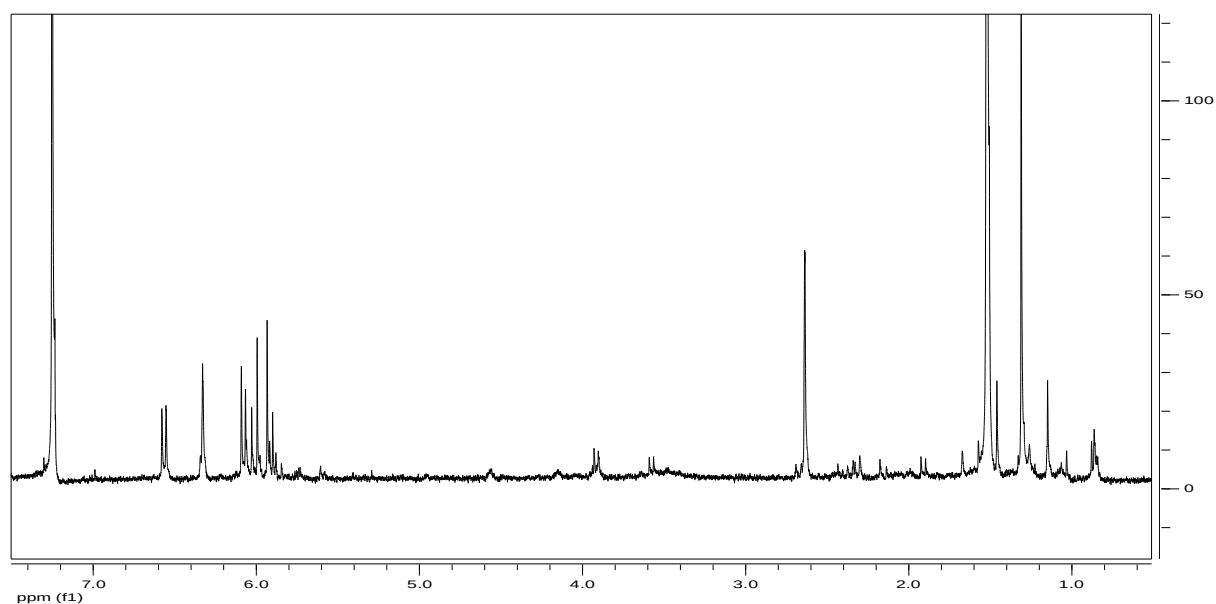
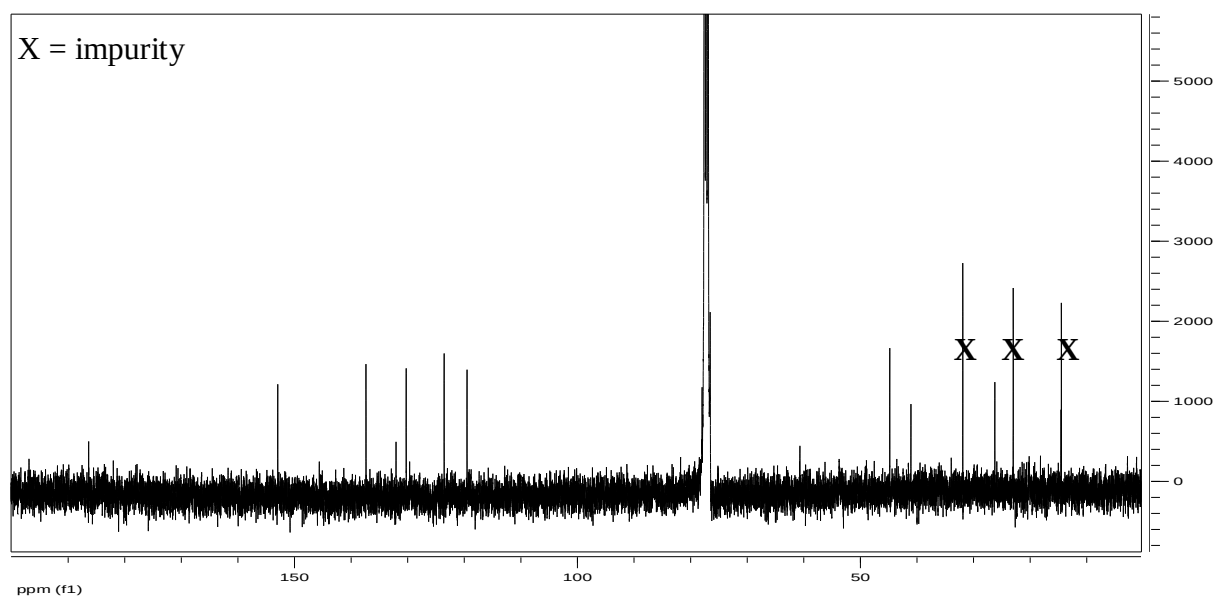
**Figure 2.6** Selected NOESY correlations and proposed numbering system for compound 2.1

The molecular formula proposed from the HR-GCTOFMS data shows that only two chlorines are present. The presence of two chlorine groups was further confirmed by the shape of the isotope cluster. Based on the nature of the carbon shifts these two chlorine groups are most likely placed at  $\delta$  130.5 (H-10) and 119.0 (H-8).

The *trans* geometry of the  $\Delta^{7,8}$  double bond was deduced from the vicinal coupling constant ( $J = 13.5$  Hz). The geometry of the  $\Delta^{6,10}$  double bond (Figure 2.6) was suggested as *trans* after comparison of the  $^1\text{H}$  NMR chemical shift of H-10 to that of known  $\beta$ -chloroenones (Jackman and Sternhall, 1969). The anisotropic effect of the carbonyl deshields the  $\beta$ -vinylic proton to around  $\delta$  7.35 in known *trans*  $\beta$ -chloroenones, while an upfield shift to  $\delta$  6.45 is observed in known *cis*- $\beta$ -chloroenones (Figure 2.7). A chemical shift of  $\delta$  7.26 therefore suggests a *trans*  $\Delta^{6,10}$  double bond in compound **2.1**.

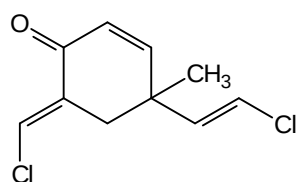
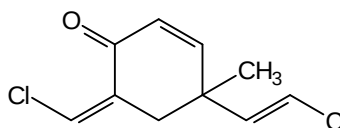


**Figure 2.7** The anisotropic effect of the carbonyl deshields *trans*  $\beta$ -chloroenones, while upfield shift is observed in known *cis*- $\beta$ -chloroenones

Structural elucidation of compound **2.2** from *P. maxillosum***Figure 2.8**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **2.2****Figure 2.9**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of compound **2.2**

Similar to compound **2.1**, the  $^1\text{H}$  NMR spectrum of compound **2.2** indicated two coupled deshielded methines at  $\delta$  6.00 ( $J = 13.2$  Hz, H-8) and 5.91 ( $J = 13.2$  Hz, H-7), as well as another two coupled deshielded methines at  $\delta$  6.08 ( $J = 9.9$  Hz, H-6) and 6.57 ( $J = 9.9$  Hz, H-5) (Figure 2.8; Table 2.1). The most obvious difference between the two compounds was the upfield shift of the olefinic methine from  $\delta$  7.26 in compound **2.1** to  $\delta$  6.28 in compound **2.2** which accounted for the 6Z geometry. In addition to this, the two proton doublet at  $\delta$  2.96 and 2.66 collapsed into a two proton singlet at  $\delta$  2.63 in compound **2.2**. The main difference, with respect to the carbon shifts of both compounds, lies at position 5 ( $\delta$  38.0 versus  $\delta$  44.5) and position 10 ( $\delta$  130.5 versus  $\delta$  123.4) (Figure 2.9). Therefore it was apparent that the change was at the  $\Delta^{6,10}$  double bond, as these differences can be accounted for by the Z-geometry of the exocyclic double bond for compound **2.2**. All other spectroscopic data for compound **2.2** are consistent with this proposed structure.

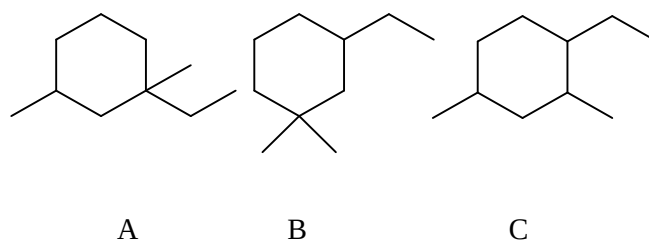
Therefore, compound **2.1** corresponds to 6*E*-chloromethylene-4-(2*E*-chlorovinyl)-4-methylcyclohex-2-enone; and compound **2.2** corresponds to 6*Z*-chloromethylene-4-(2*E*-chlorovinyl)-4-methylcyclohex-2-enone.

**2.1****2.2**

**Table 2.1**  $^1\text{H}$  NMR (400 MHz),  $^{13}\text{C}$  NMR (100 MHz), and HMBC data for compounds **2.1** and **2.2** in  $\text{CDCl}_3$ 

| C no.      | $\delta_{\text{C}}$<br>(100 MHz) | $\delta_{\text{H}}$ , multi, $J$ (Hz)<br>(400 MHz) | HMBC                                   | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$<br>(Hz) | HMBC                                  |
|------------|----------------------------------|----------------------------------------------------|----------------------------------------|---------------------|------------------------------------------|---------------------------------------|
| <b>2.1</b> |                                  |                                                    |                                        | <b>2.2</b>          |                                          |                                       |
| 1          | 184.9, C                         | -                                                  |                                        | 186.3, C            | -                                        |                                       |
| 2          | 129.0, CH                        | 6.10, d, 10.1                                      | C-1, C-6, C-4                          | 130.1, CH           | 6.08, d, 9.9                             | C-6, C-4                              |
| 3          | 154.7, CH                        | 6.70, d, 10.1                                      | C-1, C-4, C-5                          | 152.8, CH           | 6.57, d, 9.9                             | C-1, C-5, C-4, C-9                    |
| 4          | 40.0, C                          | -                                                  |                                        | 40.8, C             | -                                        |                                       |
| 5          | 38.0, $\text{CH}_2$              | 2.96, dd, 14.6, 2.0<br>2.66, dd, 14.6, 2.0         | C-1, C-3, C-6, C-<br>10, C-2, C-4, C-9 | 44.5, $\text{CH}_2$ | 2.63, s                                  | C-1, C-3, C-7, C-6,<br>C-10, C-4, C-9 |
| 6          | 134.0, C                         | -                                                  |                                        | 132.0, C            | -                                        |                                       |
| 7          | 137.8, CH                        | 5.91, d, 13.5                                      | C-3, C-8, C-4, C-5,<br>C-9             | 137.2, CH           | 5.91, d, 13.2                            | C-3, C-8, C-5, C-4,<br>C-9            |
| 8          | 119.0, CH                        | 6.00, d, 13.5                                      | C-7, C-4                               | 119.3, CH           | 6.00, d, 13.2                            | C-7, C-4                              |
| 9          | 26.8, $\text{CH}_3$              | 1.32, s                                            | C-3, C-7, C-8, C-4,<br>C-5             | 26.0, $\text{CH}_3$ | 1.30, s                                  | C-3, C-7, C-8, C-5,<br>C-4            |
| 10         | 130.5, CH                        | 7.26, s                                            | C-1, C-6, C-5                          | 123.4, CH           | 6.28, s                                  | C-1, C-6, C-5                         |

To summarise, although compounds **2.1** and **2.2** are based on a regular monoterpene skeleton, and they represent an uncommon feature not often seen in cyclic halogenated monoterpenes from marine algae. Three different cyclic monoterpene skeletons (A-C) are present in marine algae (Figure 2.10) (Naylor *et al.*, 1983). Cyclohexenone rings with skeleton B are more common in algae from the genus *Ochtodes* (family Rhizophyllidaceae) and *Portieria*, while skeletons A and C are common in the genus *Plocamium*. The vinyl chloride moiety at position 6 of compounds **2.1** and **2.2** is uncommon and new to cyclic marine halogenated monoterpenes.

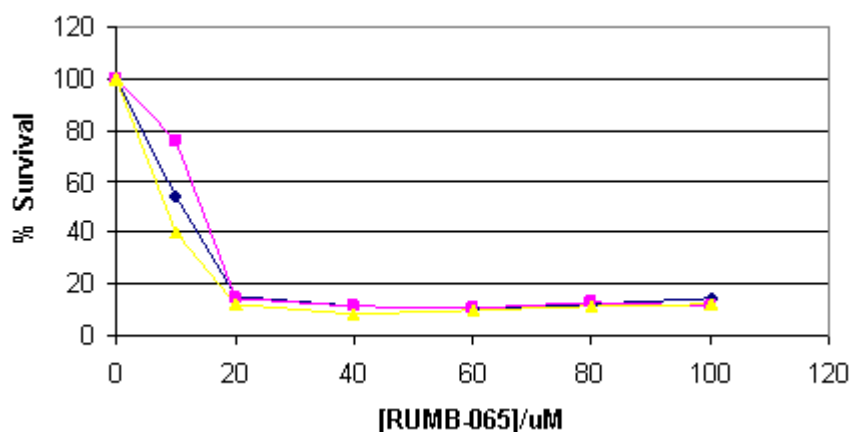
**Figure 2.10** Different cyclic monoterpene skeletons (A-C) present in marine algae



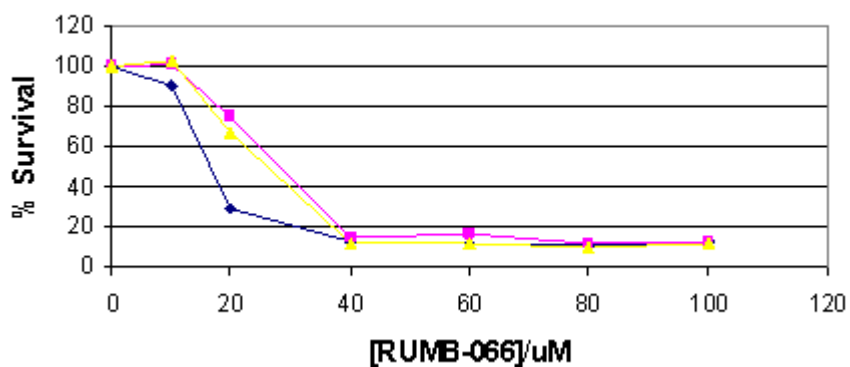


### Bioactivity

Compounds **2.1** and **2.2** were evaluated for activity against the MDA-MB-231 metastatic breast cancer cell line and showed good cytotoxic activity, with  $IC_{50} = 12 \mu M$  for compounds **2.1** and  $IC_{50} = 27 \mu M$  for compound **2.2**. This data is summarised in Figure 2.11. Tamoxifen was used as a standard and had an  $IC_{50}$  of  $0.1 \mu M$  on MDA-MB-231 cell lines.

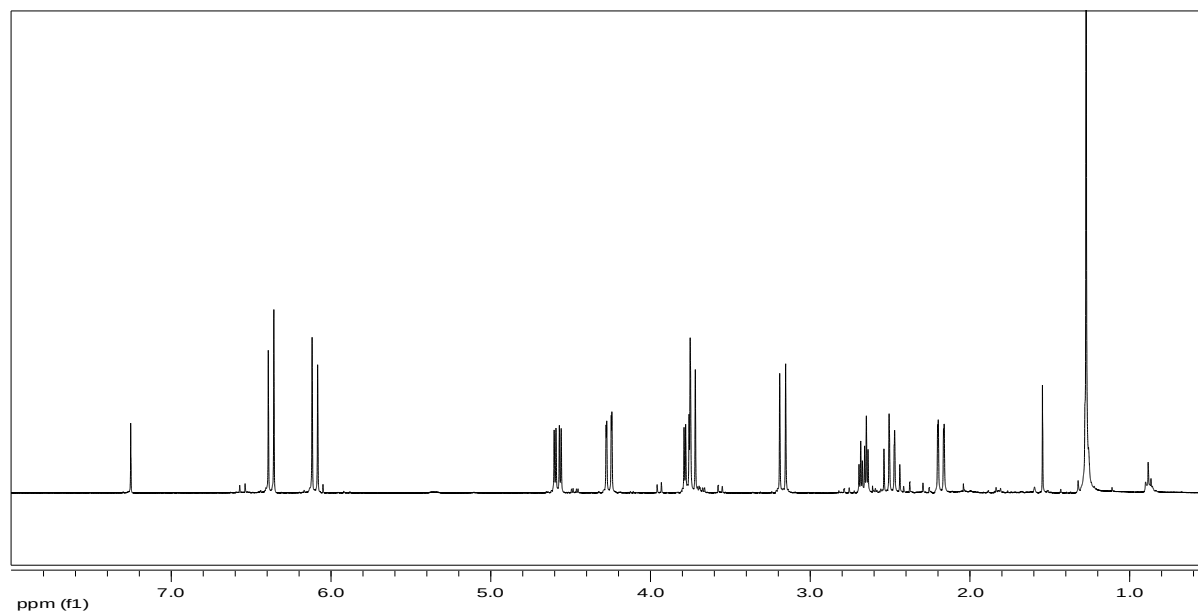
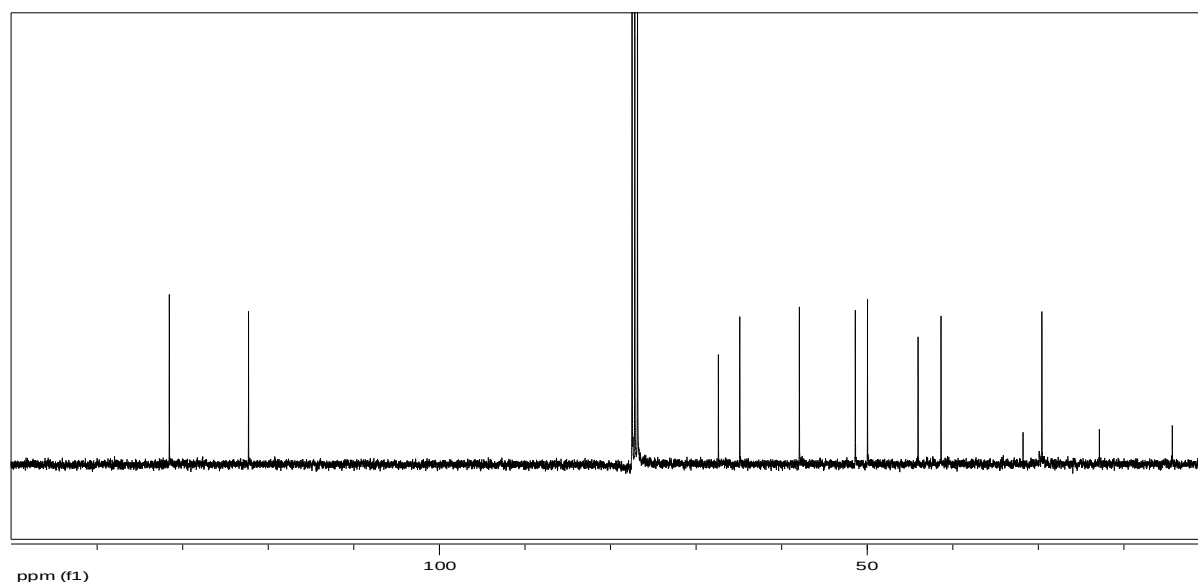


Compound **2.1**



Compound **2.2**

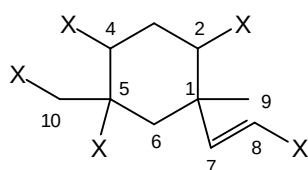
**Figure 2.11** Dose-dependent inhibition of breast cancer cell viability by compounds **2.1** and **2.2** respectively. MDA-MB-231 breast cancer cells were treated with compounds **2.1** and **2.2** in triplicate and cell viability was assessed by using the MTT assay. The half-maximal inhibitory concentrations ( $IC_{50}$ ), as calculated from the linear dose-response region, are indicated for each compound.

Structural elucidation of compound **2.6** and **2.9** from *P. robertiae***Figure 2.12** <sup>1</sup>H NMR spectrum (CDCl<sub>3</sub>, 400 MHz) of compound **2.6****Figure 2.13** <sup>13</sup>C NMR spectrum (CDCl<sub>3</sub>, 100 MHz) of compound **2.6**

HR-GCTOFMS data of compound **2.6** gave a molecular ion peak at  $m/z$  395.8481 corresponding to a molecular formula of  $C_{10}H_{13}Br_2Cl_3$  and two degrees of unsaturation. Several well dispersed resonances were observed on the  $^1H$  NMR spectrum at  $\delta$  6.37 (1H, d,  $J$  = 13.6 Hz), 6.10 (1H, d,  $J$  = 13.6 Hz), 4.57 (1H, dd,  $J$  = 13.0, 4.3 Hz), 3.78 (1H, dd,  $J$  = 13.0, 4.0 Hz), 4.25 (1H, d,  $J$  = 13.2 Hz), 3.73 (1H, d,  $J$  = 13.0 Hz), 3.17 (1H, d,  $J$  = 14.8 Hz), 2.66 (1H, dt,  $J$  = 13.0, 4.0 Hz), 2.48 (1H, m), 2.17 (1H, d,  $J$  = 14.8 Hz) and 1.26 (3H, s) (Figure 2.12).

$^{13}C$  and DEPT-135 NMR analyses showed the expected ten carbon resonances of which two were  $sp^2$  hybridised methines ( $\delta$  131.5 and 122.5), thus indicating the presence of a single double bond. Compound **2.6** had two degrees of unsaturation and only one double bond, which suggested that the structure was cyclic. Further carbon resonances include two  $sp^3$  methines ( $\delta$  64.7 and 57.8), three methylene functions ( $\delta$  51.2, 49.8 and 41.2), one methyl carbon ( $\delta$  29.4) and two quaternary carbons ( $\delta$  67.2 and 43.9) (Figure 2.13; Table 2.2).

Detailed analysis of the COSY (in combination with HSQC data) and HMBC correlation data allowed the assignment of compound **2.6** as follows: The presence of a cyclohexane ring system was implied by means of significant HMBC correlations from the methylene protons at  $\delta$  3.17 and 2.17 (on carbon  $\delta$  49.8) to other carbon resonances at  $\delta$  43.9, 64.7, 57.8 and 67.2 within the ring system. Strong COSY correlations from  $\delta$  2.66 and 2.48 (on carbon  $\delta$  41.2) to methine signals at  $\delta$  3.78 (on carbon  $\delta$  64.7) and 4.57 (on carbon  $\delta$  57.8) was also noted. Further HMBC correlations from the methyl signal at  $\delta$  1.26 to carbons at  $\delta$  43.9, 64.7, 131.5 and 49.8 placed the methyl signal at C-1. In addition, COSY correlation data between  $\delta$  6.10 (d,  $J$  = 13.6 Hz) and 6.37 (d,  $J$  = 13.6 Hz), suggested the presence of a halogenated vinyl group which was most likely placed at C-1. A strong HMBC correlation from the methylene proton at  $\delta$  4.25 and 3.73 (on carbon 51.2) to the signal at  $\delta$  67.2 (C-5), placed the methylene group at position 5. This NMR data was sufficiently similar to that of other known cyclic halogenated compounds, and from this the following carbon-framework and numbering system was proposed (Figure 2.14)(Knott, 2003).

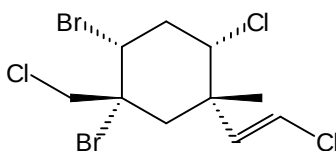


**Figure 2.14** Proposed numbering system for compound **2.6**

$^{13}\text{C}$  shifts at  $\delta$  122.2 (C-8), 64.7 (C-2) and 51.2 (C-10) were consistent with chlorine substituents at these positions. This suggests that the two remaining bromine groups, as implied by the molecular formula must therefore be accommodated at C-4 and C-5. The corresponding carbon shift at C-4, representing a Br, was in agreement with what had already been proposed (Higgs *et al.*, 1977).

The analysis of  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift data to deduce relative stereochemistry is a commonly employed strategy. The expected *trans* geometry around the  $\Delta^{7,8}$  double bond was deduced from the vicinal coupling constant ( $J = 13.6$  Hz), while the relative configuration of the chiral centre at C-1 and C-5 still needs to be confirmed using circular dichroism. An examination of the vicinal coupling constants of the protons at  $\delta$  3.78 (1H, dd,  $J = 13.0, 4.0$ , H-2) and  $\delta$  4.57 (1H, dd,  $J = 13.0, 4.3$ , H-4), together with the chemical shift of the C-9 methyl suggested that both hydrogen moieties must be axial. Based on this information, as well as that of known similar compounds, this implied that the halogens at C-2 and C-4 were equatorial in nature. Unfortunately, a NOESY experiment could not be performed as the compound degraded before a NOESY experiment was possible.

Interestingly, 4,5-dibromo-5-chloromethyl-1-chlorovinyl-2-chloro-methylcyclohexane (**2.6**) was first isolated from *P. corallorhiza* (Knott, 2003), however, during that time the author was unable to fully characterise the compound, due to the complexity of the placing of the correct halogens at C-4 and C-10 respectively. Re-isolating compound **2.6** from *P. robertiae* was a fortuitous event and presented an opportunity to solve the structural aspects of this new compound with more certainty.

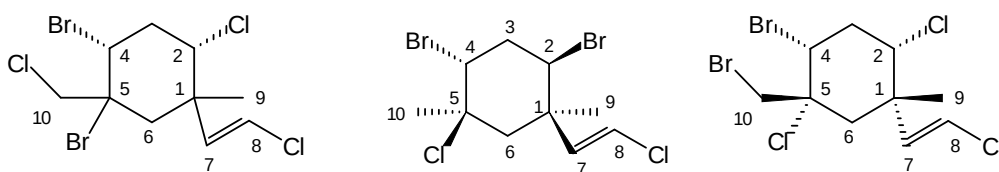


## 2.6

**Table 2.2**  $^1\text{H}$  NMR (400 MHz),  $^{13}\text{C}$  NMR (100 MHz), COSY and HMBC data for compound **2.6** in  $\text{CDCl}_3$  (Knott, 2003)

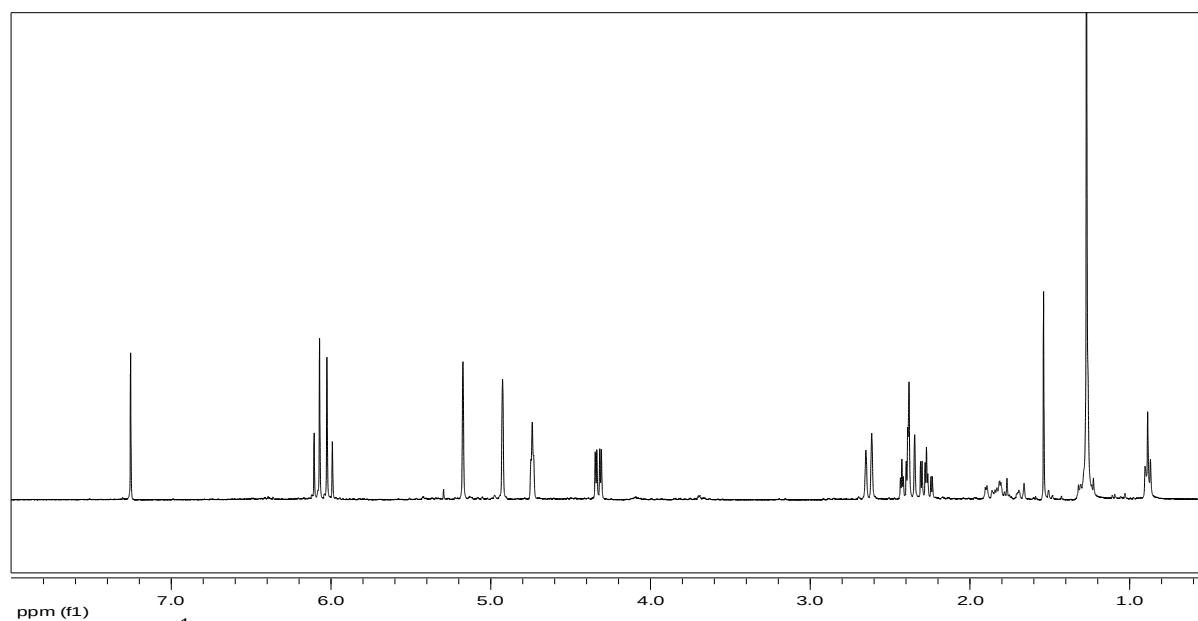
| Carbon no. | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$ (Hz) | $^1\text{H}$ - $^1\text{H}$ COSY | HMBC               |
|------------|---------------------|---------------------------------------|----------------------------------|--------------------|
| 1          | 43.9, C             | -                                     | -                                | -                  |
| 2          | 64.7, CH            | 3.78, dd, 13.0, 4.0                   | H-3a, H-3b                       | -                  |
| 3a         | 41.2, $\text{CH}_2$ | 2.66, dt, 13.0, 4.0                   | -                                | C-4                |
| 3b         |                     | 2.48, q, 13.0                         |                                  |                    |
| 4          | 57.8, CH            | 4.57, dd, 13.0, 4.3                   | H-3a, H-3b                       | C-10               |
| 5          | 67.2, C             | -                                     | -                                | -                  |
| 6a         | 49.8, $\text{CH}_2$ | 3.17, d, 14.8                         | H-6b                             | C-1, C-4, C-2, C-5 |
| 6b         |                     | 2.17, d, 14.8                         |                                  | C-1, C-10, C-5     |
| 7          | 131.5, CH           | 6.10, d, 13.6                         | -                                | C-8                |
| 8          | 122.2, CH           | 6.37, d, 13.6                         | H-7                              | C-7                |
| 9          | 29.4, $\text{CH}_3$ | 1.26, s                               | -                                | C-1, C-6, C-2, C-7 |
| 10a        | 51.2, $\text{CH}_2$ | 4.25, d, 13.0                         | -                                | C-5                |
| 10b        |                     | 3.73, d, 13.0                         |                                  | C-5                |

**Table 2.3**  $^{13}\text{C}$  NMR (100MHz,  $\text{CDCl}_3$ ) data for compound **2.6**, as well as the literature values for compound **2.7** and **2.8** (Higgs *et al.*, 1977) for comparison

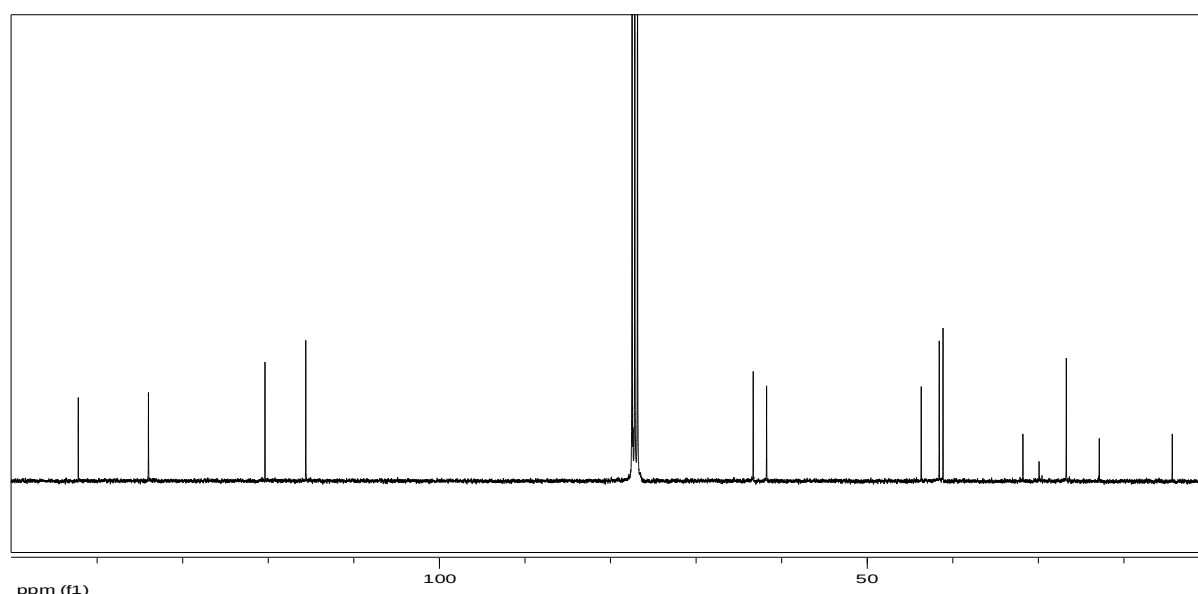


|               | <b>2.6</b>          | <b>2.7</b>          | <b>2.8</b>           |
|---------------|---------------------|---------------------|----------------------|
| <b>Carbon</b> | $\delta_{\text{C}}$ | $\delta_{\text{C}}$ | $\delta_{\text{C}}$  |
| <b>no.</b>    | <b>2.6</b>          | <b>2.7</b>          | <b>2.8</b>           |
| 1             | 43.9, C             | 41.4, C             | 42.0, C              |
| 2             | 64.7, CH            | 56.1, CH            | 64.6, CH             |
| 3             | 41.2, $\text{CH}_2$ | 39.3, $\text{CH}_2$ | 39.1*, $\text{CH}_2$ |
| 4             | 57.8, CH            | 60.5, CH            | 51.1, CH             |
| 5             | 67.2, C             | 70.1, C             | 70.8, C              |
| 6             | 49.8, $\text{CH}_2$ | 47.9, $\text{CH}_2$ | 48.5, $\text{CH}_2$  |
| 7             | 131.5, CH           | 138.2, CH           | 135.3, CH            |
| 8             | 122.2, CH           | 116.9, CH           | 119.1, CH            |
| 9             | 29.4, $\text{CH}_3$ | 30.4, $\text{CH}_3$ | 27.4, $\text{CH}_3$  |
| 10            | 51.2, $\text{CH}_2$ | 33.4, $\text{CH}_3$ | 40.3*, $\text{CH}_2$ |

\*Carbon shifts may be interchangeable

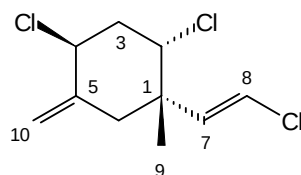


**Figure 2.15**  $^1\text{H}$  NMR spectrum (CDCl<sub>3</sub>, 400 MHz) of compound 2.9



**Figure 2.16**  $^{13}\text{C}$  NMR spectrum (CDCl<sub>3</sub>, 100 MHz) of compound 2.9

2,4-dichloro-1-chlorovinyl-1-methylcyclohexane-5-ene or Plocamene D had already been reported (Crews and Kho-Wiseman, 1976) and had since been re-isolated on a number of occasions by other researches. The  $^{13}\text{C}$  NMR data and  $^1\text{H}$  NMR data below for compound **2.9** is consistent with that reported by Sierle and Sims (1979) and Mann (2008) (Table 2.4).



**2.9**

**Table 2.4**  $^{13}\text{C}$  NMR data and  $^1\text{H}$  NMR data for Plocamene D

| Carbon no. | $\delta_{\text{C}}$  | $\delta_{\text{C}}$<br>Mann (2008) | $\delta_{\text{H}}$  | $\delta_{\text{H}}$<br>Mann (2008) |
|------------|----------------------|------------------------------------|----------------------|------------------------------------|
| 1          | 43.5, C              | 43.6, C                            | -                    | -                                  |
| 2          | 63.2, CH             | 63.3, CH                           | 4.73, t, 3.3         | 4.73, t, 3.4                       |
| 3          | 41.4, $\text{CH}_2$  | 41.5, $\text{CH}_2$                | 2.62-2.42, m         | 2.23-2.43, m                       |
| 4          | 61.6, CH             | 61.7, CH                           | 4.30, dd, 11.2, 3.8  | 4.33, dd, 11.4, 3.9                |
| 5          | 142.1, C             | 142.3, C                           | -                    | -                                  |
| 6          | 41.0, $\text{CH}_2$  | 41.1, $\text{CH}_2$                | 2.30, 2.65, dd, 14.8 | 2.36, 2.64, dd, 14.5               |
| 7          | 133.9, CH            | 134.1, CH                          | 6.00, d, 13.7        | 6.00, d, 13.6                      |
| 8          | 120.3, CH            | 120.4, CH                          | 6.06, d, 13.7        | 6.10, d, 13.6                      |
| 9          | 26.5, $\text{CH}_3$  | 26.7, $\text{CH}_3$                | 1.26, s              | 1.25, s                            |
| 10         | 115.5, $\text{CH}_2$ | 115.7, $\text{CH}_2$               | 4.92, s; 5.17, s     | 4.92, s; 5.17, s                   |

### Bioactivity

Unfortunately, both compounds **2.6** and **2.9** degraded before they could be tested for cytotoxic activity.



## 2.5 Experimental section

### 2.5.1 General experimental procedures

Normal phase TLC was performed on DC Alufolie Kieselgel 60F<sub>254</sub> and plates were viewed under UV light (254 nm). Column chromatography was performed on silica gel 60 (0.04 – 0.063 mm) from Merck, Darmstadt (Germany). All solvents were of HPLC grade (HiPerSolv<sup>TM</sup> and Merck).

The <sup>1</sup>H (400 MHz), <sup>13</sup>C (100 MHz) and DEPT-135 NMR spectra were all recorded on a Bruker 400 NMR spectrometer using standard pulse sequences. HMQC, HMBC, COSY-90 and NOESY NMR spectra were recorded on a Bruker 600 NMR spectrometer using standard pulse sequences. Spectra were referenced to residual protonated solvent resonances (CHCl<sub>3</sub>  $\delta_H$  7.25,  $\delta_C$  77.0). Chemical shifts were reported in ppm, while coupling constants were reported in Hz.

Optical rotations were measured on a Perkin-Elmer 141 polarimeter. UV data was obtained using the Perkin Elmer Precisely Lambda 25 UV/VIS spectrometer. IR spectra were obtained using a Perkin Elmer Spectrum 100 FT-IR spectrometer with a universal ATR sampling accessory.

HR-GCTOFMS was performed at Stellenbosch University (South Africa). The experiments were performed by Fletcher Hiten using a Waters GCT Premier mass spectrometer. HR-GCTOFMS (70 eV) experiments were performed using a HP-5 column (30 m, 0.25 mm ID, 0.25  $\mu$ m film thickness); injector temperature 260 °C; split injection (1:10); the carrier gas was helium 1 ml/min; scanning range from 35 to 600 *m/z* (perfluorotri-*N*-butylamine as mass reference); and temperature ramp 40-300 °C at 20 °C/min over a 5 minute period.

### 2.5.2 Plant material

*P. maxillosum* was collected by snorkelling at Paternoster near Cape Town, South Africa and kept frozen until extraction. A voucher specimen (No. CT02005) is retained at the Faculty of Pharmacy, Rhodes University, South Africa.

*P. robertiae* was collected from the intertidal zone at low tide near Port Alfred in the Eastern Cape, South Africa. It was then frozen until extraction. A voucher specimen (No. PA100730-5a) is retained at the Faculty of Pharmacy, Rhodes University, South Africa.

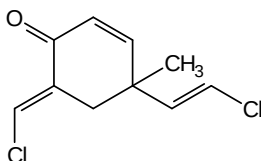
### 2.5.3 Extraction and isolation

A frozen sample of *P. maxillosum* (5.3 g dry weight) was extracted in CH<sub>2</sub>Cl<sub>2</sub>-MeOH (2:1, 3 x at 37 °C for 30 min). The combined CH<sub>2</sub>Cl<sub>2</sub>-MeOH extracts (300 ml) was concentrated and reconstituted in MeOH. The methanolic solution was successively extracted with hexane (3 x 100 ml) and CH<sub>2</sub>Cl<sub>2</sub> (3 x 100 ml) adding sufficient water each time for phase separation. The hexane and CH<sub>2</sub>Cl<sub>2</sub> extracts were concentrated separately to give 42.2 mg hexane extract and 17.7 mg CH<sub>2</sub>Cl<sub>2</sub> extract. The hexane extract was chromatographed on a silica gel column using hexane, CH<sub>2</sub>Cl<sub>2</sub> and EtOAc as eluents. The hexane portion (3.5 mg) was re-chromatographed on silica gel using a (hexane-EtOAc) gradient step of increasing polarity. Compound **2.1** (2.3 mg, 0.04% dry weight) eluted in the hexane-EtOAc (9:1) fraction. Compound **2.2** (0.9 mg, 0.02% dry weight) eluted in the EtOAc fraction.

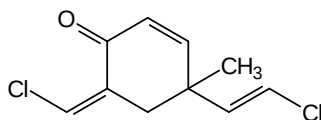
A frozen sample of *P. robertiae* (35.7 g dry weight) was first extracted with MeOH. The MeOH was drained off and separated, and then the alga was placed in CH<sub>2</sub>Cl<sub>2</sub>-MeOH (2:1), at 35 °C for 30 minutes per extraction. This process was repeated three times. The combined CH<sub>2</sub>Cl<sub>2</sub>-MeOH extracts were pooled and H<sub>2</sub>O was added until the CH<sub>2</sub>Cl<sub>2</sub> and aqueous MeOH layers separated. The CH<sub>2</sub>Cl<sub>2</sub> extracts were then combined and evaporated off at 30-35 °C. The CH<sub>2</sub>Cl<sub>2</sub> extract was passed through a silica gel column using 100% hexane as an eluent, from this 290.5 mg of material was extracted. From the hexane-EtOAc (9:1) extraction 225.8 mg was obtained. 112.9 mg of the hexane-EtOAc (9:1) fraction was also placed on a column which consisted of 0.59 g silica gel. This fraction was separated using hexane-EtOAc (9:1) as an eluent. From this compound **2.6** (61.4 mg, 0.172% dry weight) was isolated. 145.5 mg of the 100% hexane fraction was then dissolved in CH<sub>2</sub>Cl<sub>2</sub> and

placed on a column which consisted of 0.59 g silica gel. 100% hexane was used as an eluent. From this compound **2.9** was obtained (10.9 mg, 0.03% dry weight). Following this, the 100% hexane fraction was separated further using hexane-EtOAc (98:2). This fraction was compound **2.6** (34.5 mg, 0.049% dry weight).

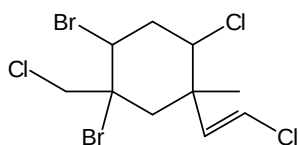
#### 2.5.4 Compounds



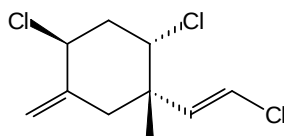
**6E-chloromethylene-4-(2E-chlorovinyl)-4-methylcyclohex-2-enone (2.1):** Yellow oil;  $[\alpha]_D^{28} -250^\circ$  ( $c$  0.001,  $\text{CH}_2\text{Cl}_2$ ); UV ( $\text{CH}_2\text{Cl}_2$ )  $\lambda_{\text{max}}$  245 ( $\epsilon$  348); IR (dry film)  $\nu_{\text{max}}$  3078, 2962, 2921, 1674, 1590, 1454, 1310, 1229, 938, 804  $\text{cm}^{-1}$ ;  $^1\text{H}$  ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  ( $\text{CDCl}_3$ , 100 MHz) NMR data see Table 2.1; HR-GCTOFMS  $m/z$  216.0094 (calculated for formula  $\text{C}_{10}\text{H}_{10}\text{O}_3\text{Cl}_2$  216.0109)



**6Z-chloromethylene-4-(2E-chlorovinyl)-4-methylcyclohex-2-enone (2.2):** Light yellow oil;  $[\alpha]_D^{28} -67^\circ$  ( $c$  0.0006,  $\text{CH}_2\text{Cl}_2$ ); UV ( $\text{CH}_2\text{Cl}_2$ )  $\lambda_{\text{max}}$  245 ( $\epsilon$  610); IR (dry film)  $\nu_{\text{max}}$  3078, 2962, 2921, 1674, 1598, 1454, 1229, 938, 852, 822  $\text{cm}^{-1}$ ;  $^1\text{H}$  ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  ( $\text{CDCl}_3$ , 100 MHz) NMR data see Table 2.1; HR-GCTOFMS  $m/z$  216.0135 (calculated for formula  $\text{C}_{10}\text{H}_{10}\text{O}_3\text{Cl}_2$  216.0109)



**4,5-dibromo-5-chloromethyl-1-chlorovinyl-2-chloro-methylcyclohexane (2.6):** Colourless oil; UV (Hexane)  $\lambda_{\max}$  204 ( $\epsilon$  4393); IR (dry film)  $\nu_{\max}$  (KBr) 3445, 2927, 2856, 1723, 1615, 1456, 1382, 1291, 1125, 1074  $\text{cm}^{-1}$ ;  $^1\text{H}$  ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  ( $\text{CDCl}_3$ , 100 MHz) NMR data see Table 2.2; HR-GCTOFMS  $m/z$  395.8481 (calculated for formula  $\text{C}_{10}\text{H}_{13}\text{Br}_2\text{Cl}_3$  395.8450)



**2,4-dichloro-1-chlorovinyl-1-methylcyclohexane-5-ene (2.9):** As previously reported (Higgs *et al.*, 1977). Bright yellow oil;  $^1\text{H}$  ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  ( $\text{CDCl}_3$ , 100 MHz) NMR data see Table 2.4

### 2.5.5 Cytotoxicity assays

Cell lines and culture conditions – The human breast cancer cell line, MDA-MB-231 (ATCC: HTB-26), was donated by Dr Sharon Prince, University of Cape Town, South Africa. The cells were routinely maintained in culture in Dulbecco's Modified Eagle Medium supplemented with 5 % foetal calf serum, 1 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin at 37 C in a humidified 9% CO<sub>2</sub> incubator.

Cytotoxicity assay – Cell viability upon treatment with the compounds was assessed by means of an MTT assay using the Cell Proliferation Kit I (Roche). Cells were seeded at 6000 cells/well in a 96-well plate and incubated overnight before treatment with either the DMSO vehicle control or a range of concentrations (0, 10, 20, 40, 60, 80, 100 µM) with the compounds and incubated for 96 hours. After treatment, 10 µL of a 10 mg/mL MTT solution was added to each well and incubated for 4 hours before solubilising the resultant precipitate in SDS buffer overnight. The absorbance at 550 nm was recorded and the percentage survival was calculated relative to the vehicle-treated control. The experiment was carried out in triplicate on each of the three plates.

## 2.6 References

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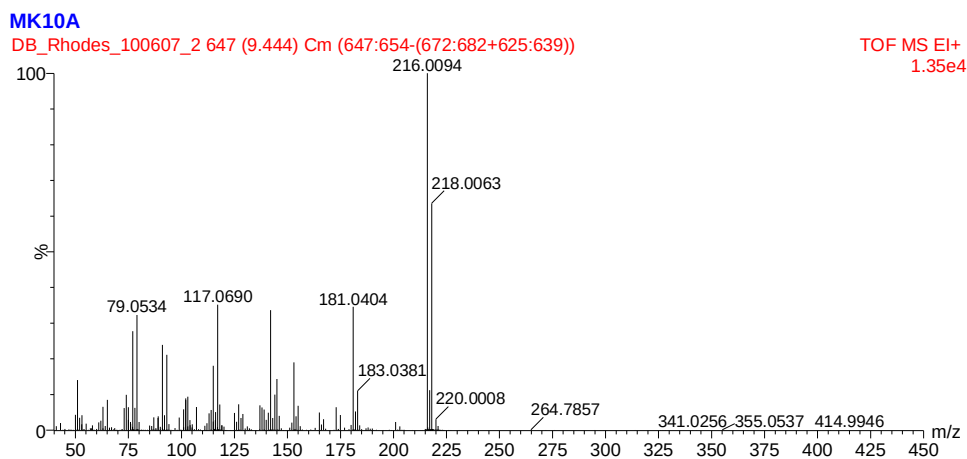
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## 2.7 Supplementary data



Elemental composition:

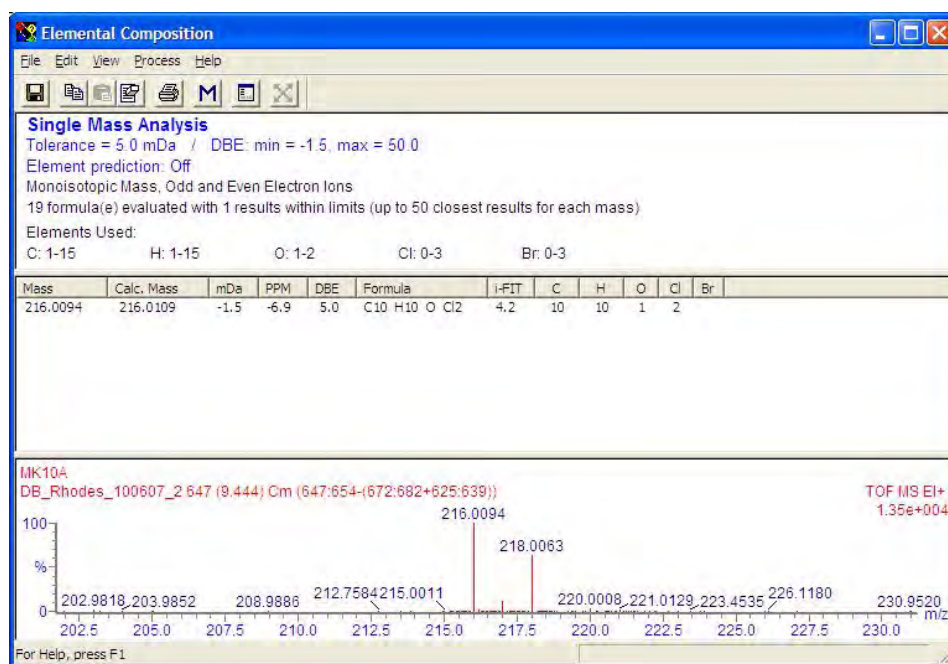
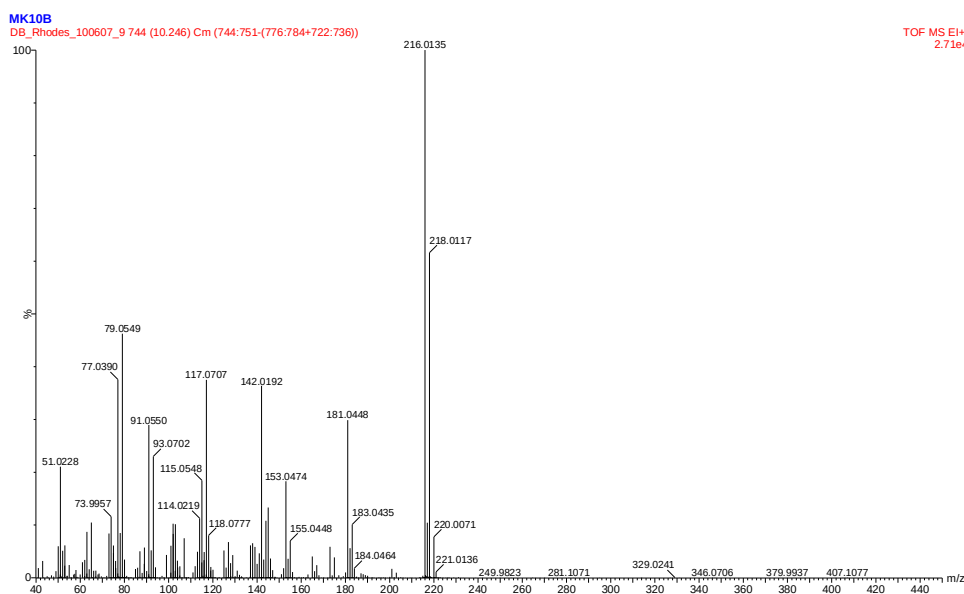


Figure 2.17 HR-GCTOFMS data for compound 2.1





Elemental composition:

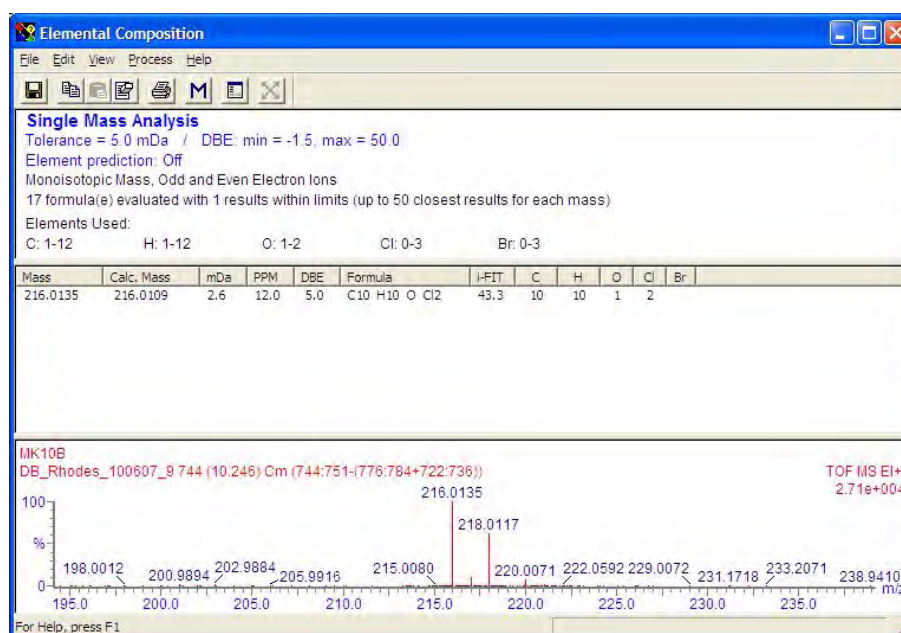
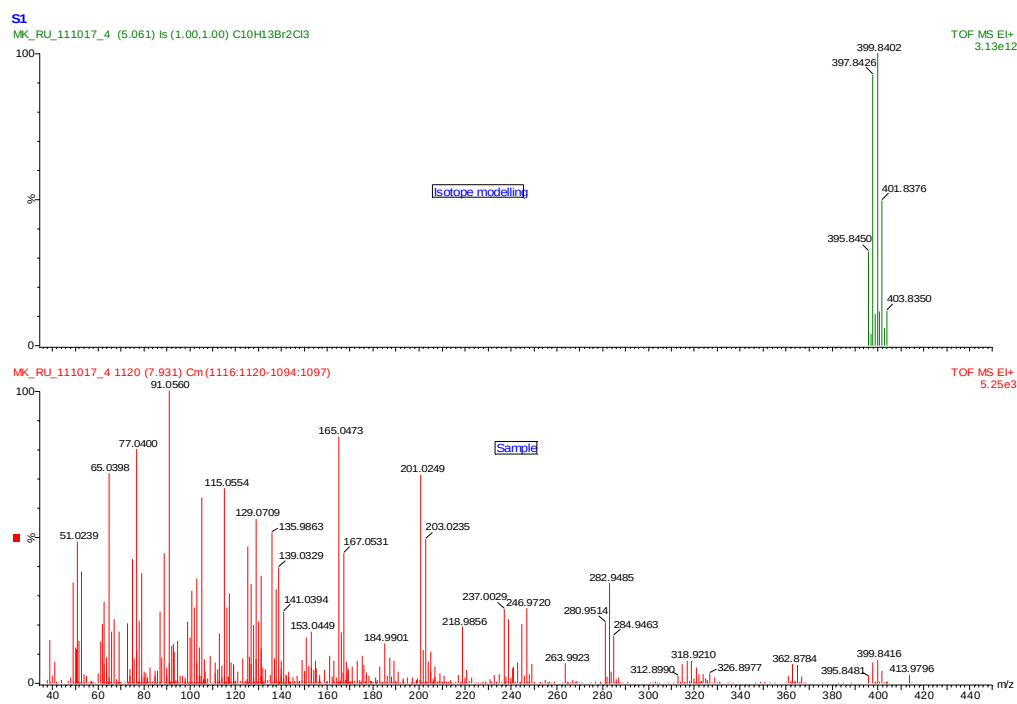
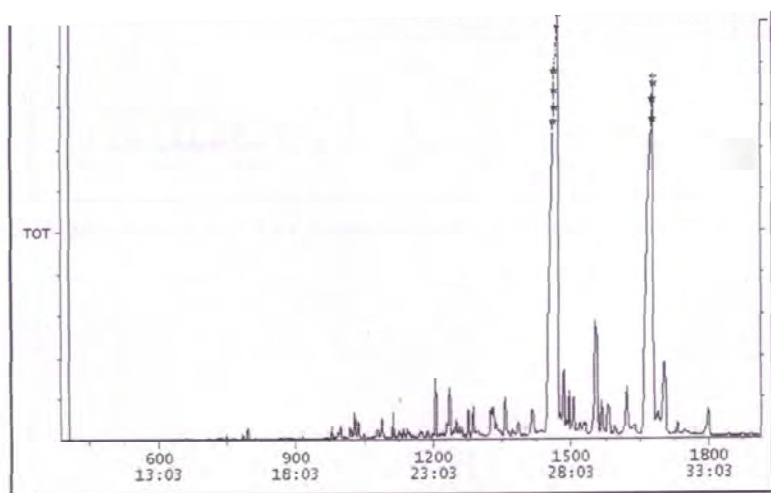


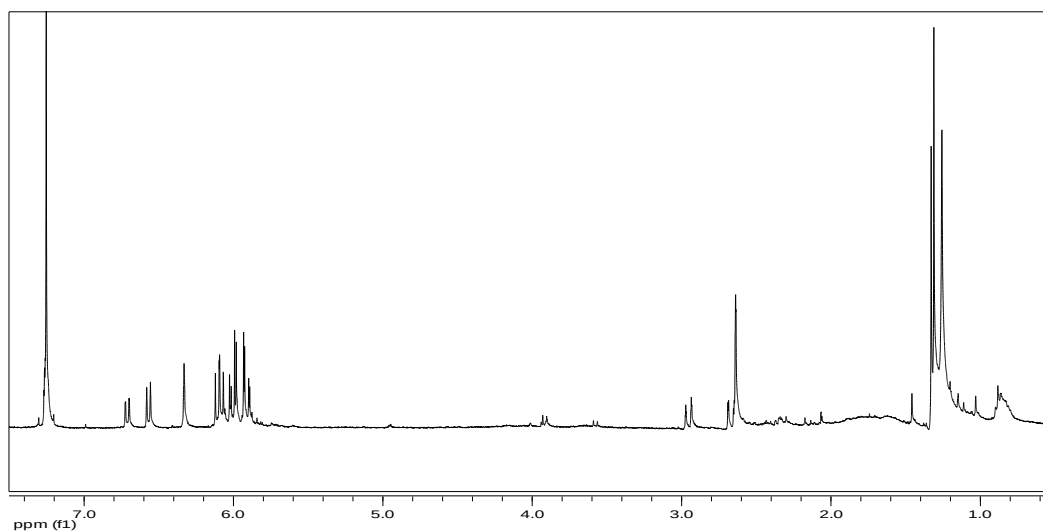
Figure 2.18 HR-GCTOFMS data for compound 2.2



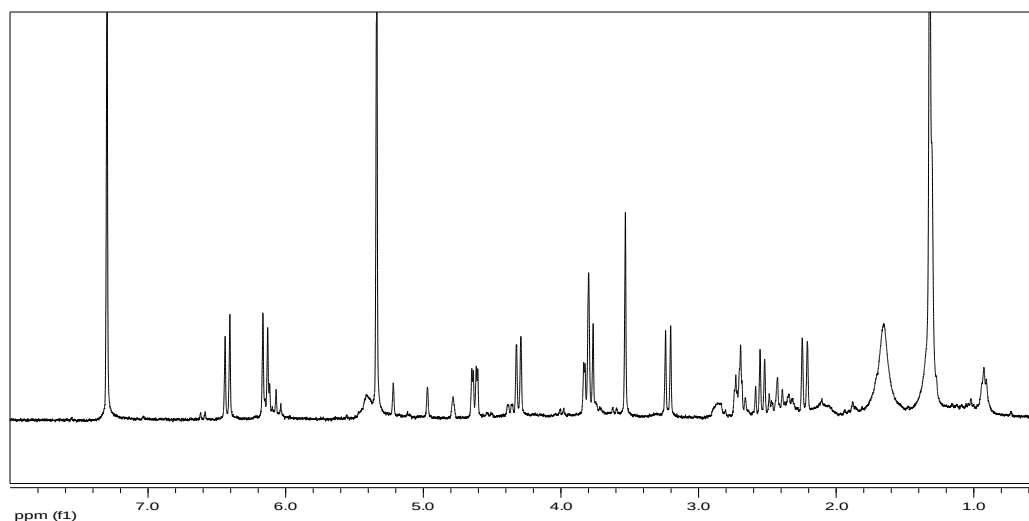
**Figure 2.19** HR-GCTOFMS data for compound **2.6**



**Figure 2.20** A previous study on *P. maxillosum* using gas chromatography-mass spectrometry (GC-MS) observed the presence of two main compounds and numerous minor ones (Knott, 2003)



**Figure 2.21**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of the crude  $\text{CH}_2\text{Cl}_2$  extract of *P. maxillosum*



**Figure 2.22**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of the crude hexane extract of *P. robertiae*

## 2.8 Appendix 1

New compounds isolated from various *Plocamium* species worldwide are tabulated below (Table.2.5-2.19). Structural re-assignments have substituted the original suggestions for some of the references, however some re-assignments may still be present. (Knott, 2003)

**Table 2.5** Metabolites isolated from *Plocamium angustum*

| No.       | Isolated Compounds     | Location                              | Reference                     |
|-----------|------------------------|---------------------------------------|-------------------------------|
| 2.10-2.11 | 2,6-Dimethyloctadienes | Cape Northumberland<br>(W. Australia) | (Dunlop <i>et al.</i> , 1979) |

**Table 2.6** Metabolites isolated from *Plocamium brasiliense*

| No.  | Isolated Compounds      | Location | Reference                          |
|------|-------------------------|----------|------------------------------------|
| 2.12 | 2,6-Dimethyloctatrienes | Brazil   | (Vasconcelos <i>et al.</i> , 2010) |

**Table 2.7** Metabolites isolated from *Plocamium cartilagineum*

| No.       | Isolated Compounds                       | Location                   | Reference                     |
|-----------|------------------------------------------|----------------------------|-------------------------------|
| 2.13      | 2,6-Dimethyloctenes                      | Kaikoura (N. Zealand)      | (Blunt <i>et al.</i> , 1985)  |
| 2.14      |                                          | Figueira de Foz (Portugal) | (Abreu <i>et al.</i> , 1996)  |
| 2.15      | 2,6-Dimethyloctadienes                   | Kaikoura (N. Zealand)      | (Blunt <i>et al.</i> , 1985)  |
| 2.16      | 2,6-Dimethyloctatrienes<br>Cartilagineal | California (USA)           | (Crews and Kho-Wiseman, 1974) |
| 2.17-2.27 |                                          | La Jolla (USA)             | (Mynderse and Faulkner, 1975) |

|           |                                         |                              |                                     |
|-----------|-----------------------------------------|------------------------------|-------------------------------------|
| 2.28      |                                         | L' Estartit (Spain)          | (König <i>et al.</i> , 1990)        |
| 2.29      |                                         | Figueria da Foz (Portugal)   | (Abreu <i>et al.</i> , 1996)        |
| 2.30-2.31 |                                         | Schouten Beach<br>(Tasmania) | (Jongaramruong and Blackman, 2000)  |
| 2.9, 2.32 | <b>1-Ethyl-1,3-dimethylcyclohexanes</b> | Antarctica                   | (Stierle <i>et al.</i> , 1979)      |
| 2.33-2.35 |                                         | USA                          | (Higgs <i>et al.</i> , 1977)        |
| 2.36-2.39 |                                         | Chile                        | (San-Martin <i>et al.</i> , 1991)   |
| 2.40-2.42 | <b>1-Ethyl-2,4-dimethylcyclohexanes</b> | USA                          | (Higgs <i>et al.</i> , 1977)        |
| 2.43-2.48 | <b>Hydoxy bisnor monoterpenes</b>       | Kaikoura (N. Zealand)        | (Blunt <i>et al.</i> , 1985)        |
| 2.49-2.51 | <b>Polyhalodroxyated monoterpenes</b>   | Chile                        | (Diaz-Marrero <i>et al.</i> , 2002) |
| 2.52-2.54 | <b>Furanoid monoterpenes</b>            | Chile                        | (Darias <i>et al.</i> , 2001)       |
| 2.55-2.56 | <b>Tetrahydropyran monoterpenes</b>     | Chile                        | (Cueto <i>et al.</i> , 1998)        |

|           |                                                                                      |                             |                               |
|-----------|--------------------------------------------------------------------------------------|-----------------------------|-------------------------------|
| 2.57-2.60 | <b>Homosesquiterpenic fatty acids</b>                                                | Maltese Islands and Corsica | (Řezanka and Dembitsky, 2001) |
|           | <b>Non-Terpenoid compounds</b><br><br>Floridoside and Poly- $\beta$ -hydroxybutyrate | Figueira da Foz (Portugal)  | (Abreu <i>et al.</i> , 1997)  |

**Table 2.8** Metabolites isolated from *Plocamium coccineum*

| No.       | Isolated Compounds                       | Location                  | Reference                      |
|-----------|------------------------------------------|---------------------------|--------------------------------|
| 2.61      | <b>1-Ethyl-1, 3-dimethylcyclohexanes</b> | Bastiaqueiro (N.W. Spain) | (Castedo <i>et al.</i> , 1984) |
| 2.62-2.63 |                                          | Bastiaqueiro (N.W. Spain) | (Sardina <i>et al.</i> , 1985) |

**Table 2.9** Metabolites isolated from *Plocamium corallorhiza*

| No.       | Isolated Compounds                                                 | Location                     | Reference                    |
|-----------|--------------------------------------------------------------------|------------------------------|------------------------------|
| 2.64-2.66 | <b>2,6-Dimethyloctadienes</b><br><br>Plocoralides A-C              | Kalk Bay (South Africa)      | (Knott <i>et al.</i> , 2005) |
| 2.67-2.70 | <b>2,6-Dimethyloctadienes and 2,6-Dimethyloctatriene aldehydes</b> | Kenton-On-Sea (South Africa) | (Mann <i>et al.</i> , 2007)  |

**Table 2.10** Metabolites isolated from *Plocamium cornutum*

| No.       | Isolated Compounds     | Location                | Reference                       |
|-----------|------------------------|-------------------------|---------------------------------|
| 2.71-2.72 | 2,6-Dimethyloctatriene | Kalk Bay (South Africa) | (Afolayan <i>et al.</i> , 2009) |

**Table 2.11** Metabolites isolated from *Plocamium costatum*

| No.       | Isolated Compounds                             | Location                          | Reference                         |
|-----------|------------------------------------------------|-----------------------------------|-----------------------------------|
| 2.73      | 2,6-Dimethyloctene                             | Eaglehawk Neck<br>(Tasmania)      | (König <i>et al.</i> , 1999)      |
| 2.74-2.75 | 2, 6-Dimethyloctadienes<br>Costatol, Costatone | Port MacDonnell<br>(S. Australia) | (Kazlauskas <i>et al.</i> , 1976) |
| 2.76      |                                                | Robe (S. Australia)               | (Stierle <i>et al.</i> , 1976)    |
|           | Polysaccharides                                | Tauranga (New Zealand)            | (Falshaw <i>et al.</i> , 1999)    |

**Table 2.12** Metabolites isolated from *Plocamium cruciferum*

| No.  | Isolated Compounds                          | Location               | Reference                    |
|------|---------------------------------------------|------------------------|------------------------------|
| 2.77 | 2,6-Dimethyloctene                          | Rosy Morn (N. Zealand) | (Bates <i>et al.</i> , 1979) |
| 2.78 | Degraded or mixed<br>biogenesis monoterpene | Kaikoura (N. Zealand)  | (Blunt <i>et al.</i> , 1978) |
| 2.79 |                                             | Rosy Morn (N. Zealand) | (Bates <i>et al.</i> , 1979) |

**Table 2.13** Metabolites isolated from *Plocamium hamatum*

| No.  | Isolated Compounds              | Location                | Reference                    |
|------|---------------------------------|-------------------------|------------------------------|
| 2.80 | 2,6-Dimethyloctene              | Palm Is. (W. Australia) | (Coll <i>et al.</i> , 1988)  |
| 2.81 | 2,6-Dimethyloctadiene           | Palm Is. (W. Australia) | (König <i>et al.</i> , 1999) |
| 2.82 | 1-Ethyl-1,3-dimethylcyclohexane | Palm Is. (W. Australia) | (Coll <i>et al.</i> , 1988)  |

**Table 2.14** Metabolites isolated from *Plocamium mertensii*

| No.       | Isolated Compounds               | Location                  | Reference                     |
|-----------|----------------------------------|---------------------------|-------------------------------|
| 2.83      | 1-Ethyl-1,3-dimethylcyclohexane  | Australia                 | (Norton <i>et al.</i> , 1977) |
| 2.84      | Mertensene                       | Carnac Is. (W. Australia) | (Capon <i>et al.</i> , 1984)  |
| 2.85-2.86 | 1-Ethyl-2,4-dimethylcyclohexanes | Australia                 | (Norton <i>et al.</i> , 1977) |
| 2.87      |                                  | Carnac Is. (W. Australia) | (Capon <i>et al.</i> , 1984)  |

**Table 2.15** Metabolites isolated from *Plocamium oregonum*

| No.       | Isolated Compounds                           | Location         | Reference     |
|-----------|----------------------------------------------|------------------|---------------|
| 2.88-2.89 | 2,6-Dimethyloctadienes<br>Oregonene A (2.89) | California (USA) | (Crews, 1977) |



**Table 2.16** Metabolites isolated from *Plocamium suhrii*

| No.  | Isolated Compounds | Location                 | Reference                      |
|------|--------------------|--------------------------|--------------------------------|
| 2.90 | 2,6-Dimethyloctene | Noordhoek (South Africa) | (Antunes <i>et al.</i> , 2011) |
| 2.91 | Octatrienes        | Noordhoek (South Africa) | (Antunes <i>et al.</i> , 2011) |

**Table 2.17** Metabolites isolated from *Plocamium telfairae*

| No.  | Isolated Compounds                            | Location      | Reference                       |
|------|-----------------------------------------------|---------------|---------------------------------|
| 2.92 | 1-Ethyl-1,3-dimethylcyclohexane<br>Telfairine | Fakui (Japan) | (Watanabe <i>et al.</i> , 1989) |

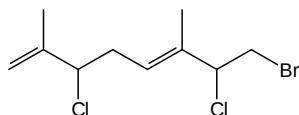
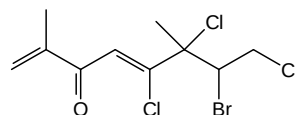
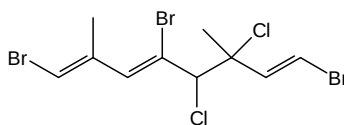
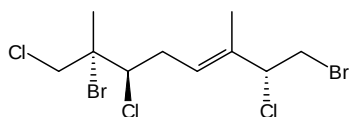
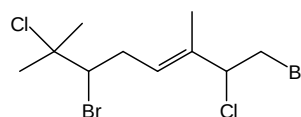
**Table 2.18** Metabolites isolated from *Plocamium violaceum*

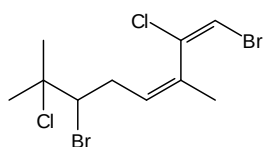
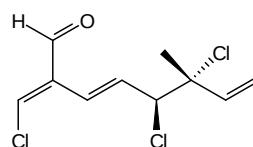
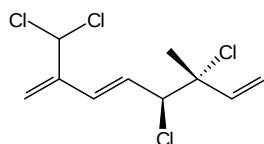
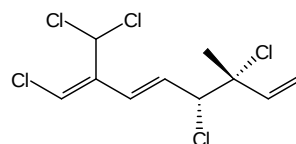
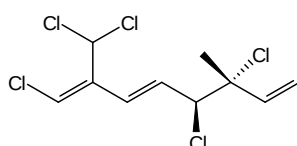
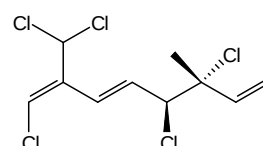
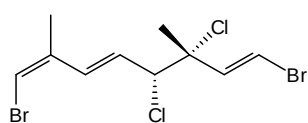
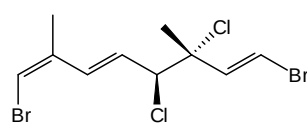
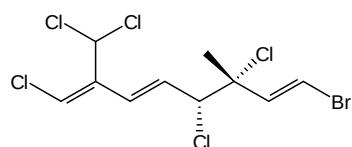
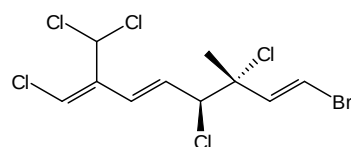
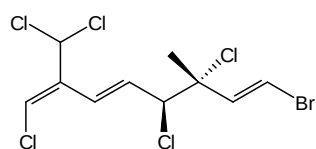
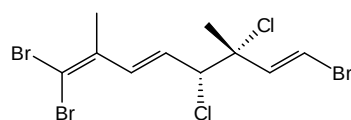
| No.        | Isolated Compounds                                | Location         | Reference                     |
|------------|---------------------------------------------------|------------------|-------------------------------|
| 2.93-2.95  | 2,6-Dimethyloctadienes<br>Preplocamene A, B and C | California (USA) | (Crews and Kho-Wiseman, 1977) |
| 2.96       | 2,6-Dimethyloctatriene                            | California (USA) | (Crews <i>et al.</i> , 1984)  |
| 2.97       | 1-Ethyl-1,3-dimethylcyclohexanes<br>Violacene     | California (USA) | (Mynderse and Faulkner, 1974) |
| 2.98-2.100 | Plocamene D and E                                 | California (USA) | (Crews <i>et al.</i> , 1978)  |

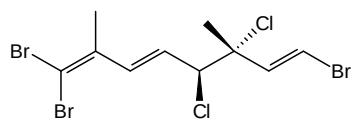
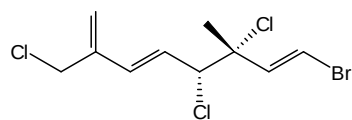
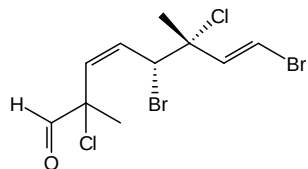
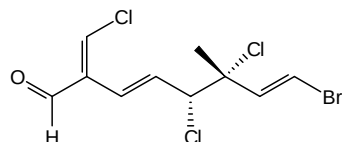
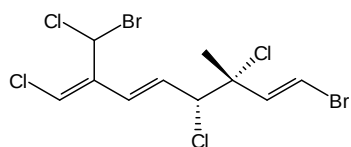
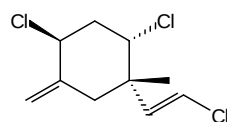
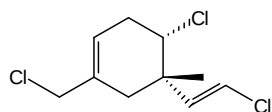
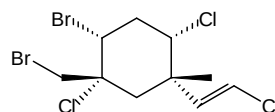
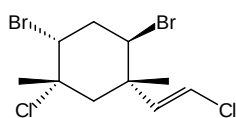
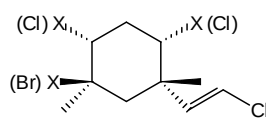
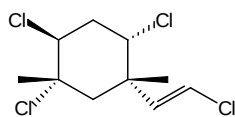
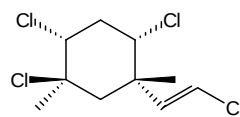
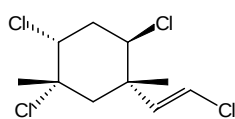
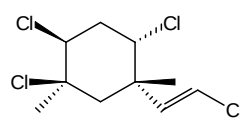
|              |                                                    |                  |                                 |
|--------------|----------------------------------------------------|------------------|---------------------------------|
|              | <b>1-Ethyl-2,4</b><br><b>-dimethylcyclohexanes</b> |                  |                                 |
| <b>2.101</b> | Plocamene-C; Violacene-2                           | California (USA) | (Mynderse <i>et al.</i> , 1975) |
| <b>2.102</b> | Plocamene B                                        | California (USA) | (Crews and Kho-Wiseman, 1975)   |

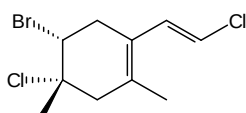
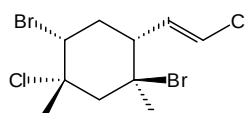
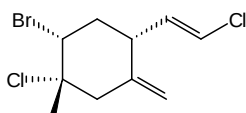
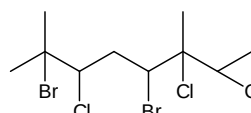
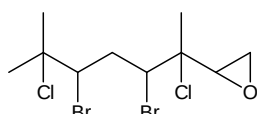
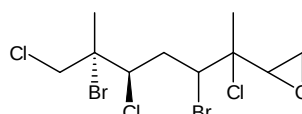
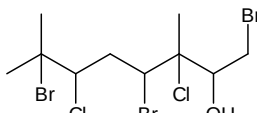
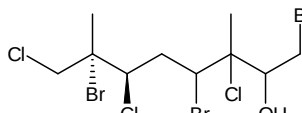
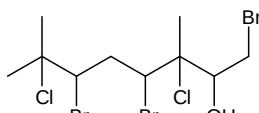
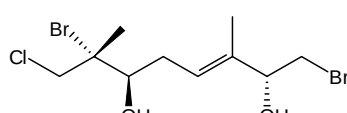
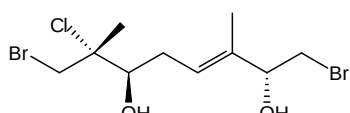
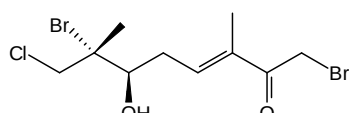
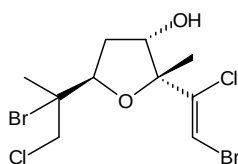
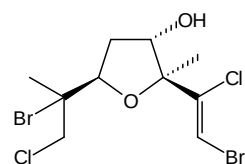
**Table 2.19** Metabolites isolated from *Plocamium* species

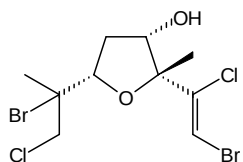
| No.                     | Isolated Compounds             | Location   | Reference                      |
|-------------------------|--------------------------------|------------|--------------------------------|
| <b>2.103-<br/>2.106</b> | <b>2,6-Dimethyloctatrienes</b> | Antarctica | (Stierle <i>et al.</i> , 1979) |

**2.10****2.11****2.12****2.13****2.14**

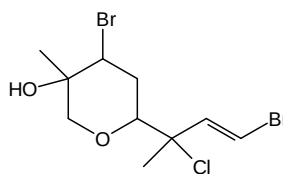
**2.15****2.16****2.17****2.18****2.19****2.20****2.21****2.22****2.23****2.24****2.25****2.26**

**2.27****2.28****2.29****2.30****2.31****2.9****2.32****2.33****2.34****2.35****2.36****2.37****2.38****2.39**

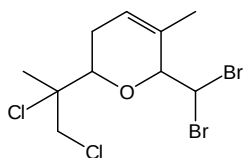
**2.40****2.41****2.42****2.43****2.44****2.45****2.46****2.47****2.48****2.49****2.50****2.51****2.52****2.53**



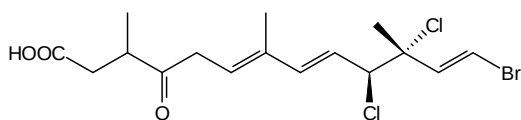
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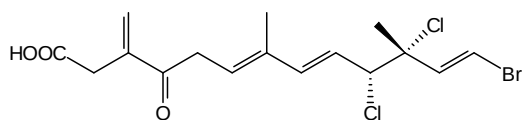
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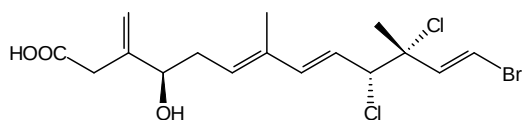
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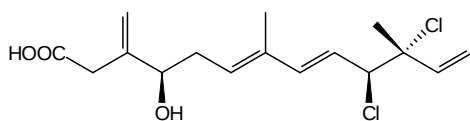
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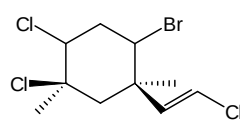
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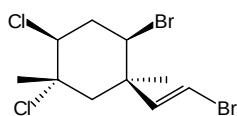
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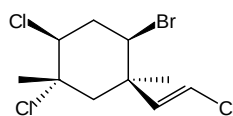
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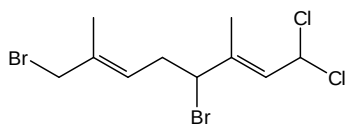
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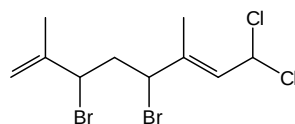
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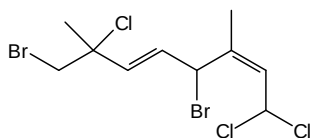
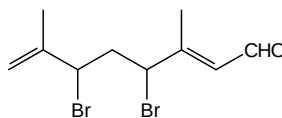
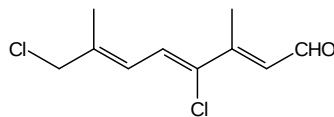
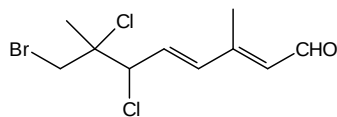
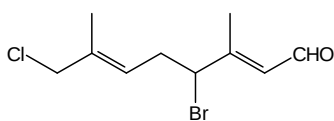
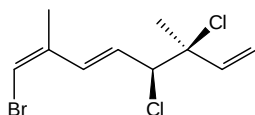
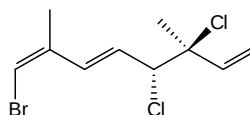
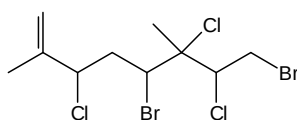
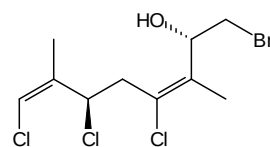
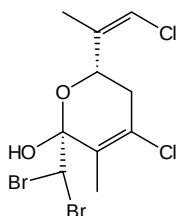
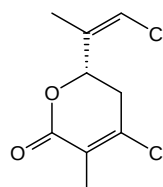
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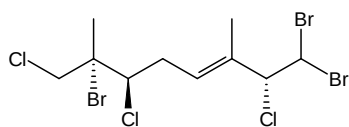
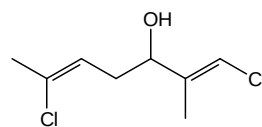
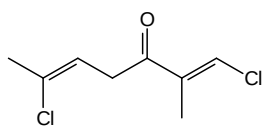
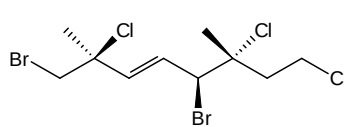
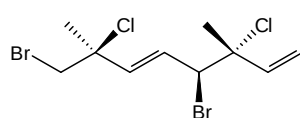
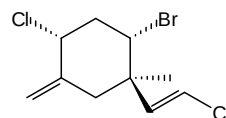
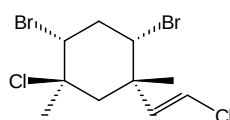
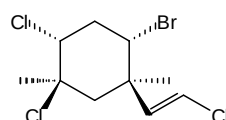
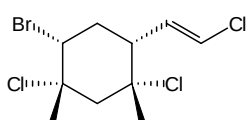
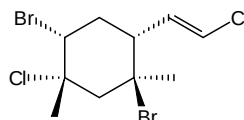
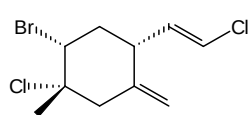
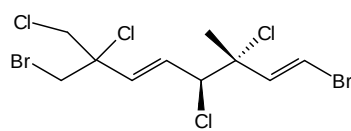
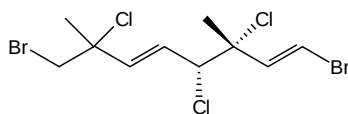


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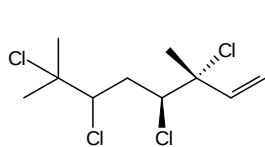
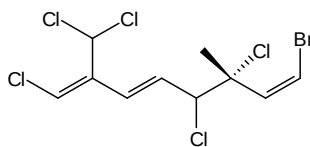
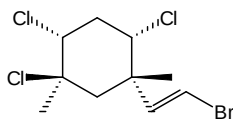
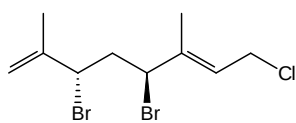
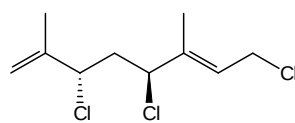
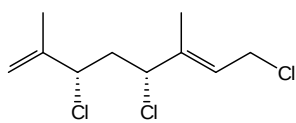
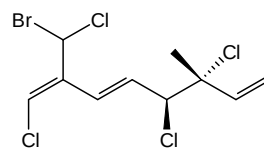
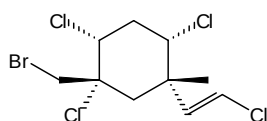
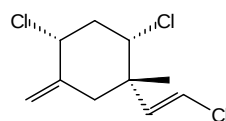
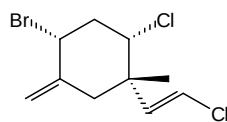
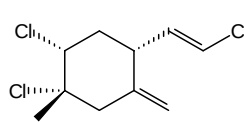
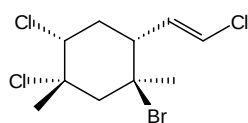
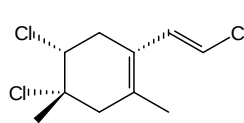


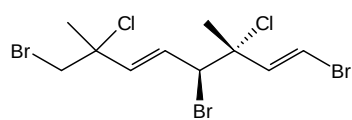
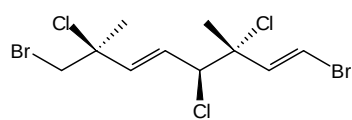
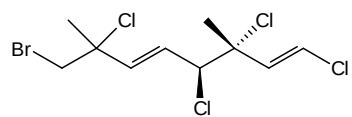
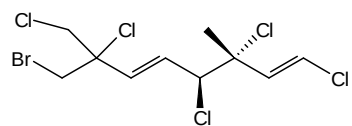
2.65

**2.66****2.67****2.68****2.69****2.70****2.71****2.72****2.73****2.74****2.75****2.76**

**2.77****2.78****2.79****2.80****2.81****2.82****2.83****2.84****2.85****2.86****2.87****2.88****2.89**



**2.90****2.91****2.92****2.93****2.94****2.95****2.96****2.97****2.98****2.99****2.100****2.101****2.102**

**2.103****2.104****2.105****2.106**

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## Chapter Three

# The isolation of halomon related secondary metabolites from the red alga *Portieria hornemannii*<sup>1</sup>

### 3.1 Abstract

Three new halogenated monoterpenes (**3.1-3.3**), as well as one known halogenated monoterpene (**3.4**) were isolated from the South African red marine alga, *Portieria hornemannii* (Lyngbye) (collected from Port Edward). The structures of these new compounds were characterised as 3*Z*-1,6-dibromo-3-(bromomethylidene)-2,7-dichloro-7-methyloctane (**3.1**), 1*E*,3*Z*-1,6-dibromo-3-(bromomethylidene)-7-chloro-7-methyloct-1-ene (**3.2**), and 1*Z*,3*Z*-1,6-dibromo-3-(bromomethylidene)-7-chloro-7-methyloct-1-ene (**3.3**). One known halogenated monoterpene (**3.5**) and a unique carboxylic acid nor-monoterpene, named, 5-bromo-2-(bromomethylidene)-6-chloro-6-methylheptanoic acid (**3.6**) were also isolated from the South African red marine alga, *P. hornemannii* (collected from Noordhoek). The structures of these new compounds were determined using a combination of one- and two-dimensional NMR spectroscopic data, as well as corresponding MS analysis. These halomon related compounds showed no or little activity against MDA-MB-231 breast cancer cell lines (metastatic).

**Keywords:** Halogenated monoterpenes; Halomon related compounds; *Portieria hornemannii*; Portieric acid A

<sup>1</sup> Initial work on the Port Edward collection of *Portieria hornemannii* was done by Ms Monique Lines who extracted, purified and partially characterised compounds **3.1**, **3.2** and **3.3**.

### 3.2 Introduction

A large number of halogenated metabolites have been isolated from many genera belonging to red seaweeds (Rhodophyta) (Blunt *et al.*, 2011; Faulkner, 2002). Red seaweeds from the families Plocamiaceae and Rhizophyllidaceae, in particular, produce a wide variety of halogenated and biologically active monoterpenes (Kladi *et al.*, 2004). It is believed that these compounds are produced by red alga as defensive mechanisms against predators that feed on the fronds of these marine alga (Paul *et al.*, 1987; Paul *et al.*, 2006; Paul and Pohnert, 2011). Previous reports have shown that *P. hornemannii* produces numerous linear and cyclic halogenated monoterpenes (Fuller *et al.*, 1992; Fuller *et al.*, 1994; Gunatilaka *et al.*, 1999; Lines, 2008). (For an overview of secondary metabolites previously isolated from *P. hornemannii* see 3.8 Appendix 1.) The structural elucidation of these compounds is not a trivial task because molecular ions are often not obtained, regardless of the ionisation method that is used. In addition to this, the location of chlorine and bromine groups on the ten carbon chain can be masked by the cumulative effects of numerous substituents on the monoterpene chain or ring (Naylor *et al.*, 1983).

Since the isolation of 6*R*-bromo-3*S*-(bromomethyl)-7-methyl-2,3,7-trichloro-1-octene or otherwise known as halomon (Fuller *et al.*, 1992), a lot of interest has been re-directed towards halogenated monoterpenes. Halomon was flagged by the National Cancer Institute (NCI) when it showed strong differential cytotoxicity towards the brain, colon and renal cell lines of the human *in vitro* tumour cell lines, while leukaemia and melanoma lines were relatively less sensitive (Fuller *et al.*, 1992). For example, compared to some of the less sensitive melanoma and leukaemia lines, several of the more sensitive brain, renal, and colon tumour cell lines were 1000-fold or more sensitive to halomon at the GI<sub>50</sub> response level and 100-fold at the LC<sub>50</sub> level (Fuller *et al.*, 1992). As a result of its unique cytotoxicity profile; halomon was selected for pre-clinical drug development (Fuller *et al.*, 1994). A major rate-limiting step in the further development of this compound, was the lack of reliable natural or synthetic sources from which halomon could be obtained. The reason for this is that *P. hornemannii*, from which halomon was first extracted, demonstrated extreme site-to-site and temporal variations with regards to the concentrations and types of halogenated monoterpenes that have been isolated during relevant extractions (Gunatilaka *et al.*, 1999).



### 3.3 Chapter aims

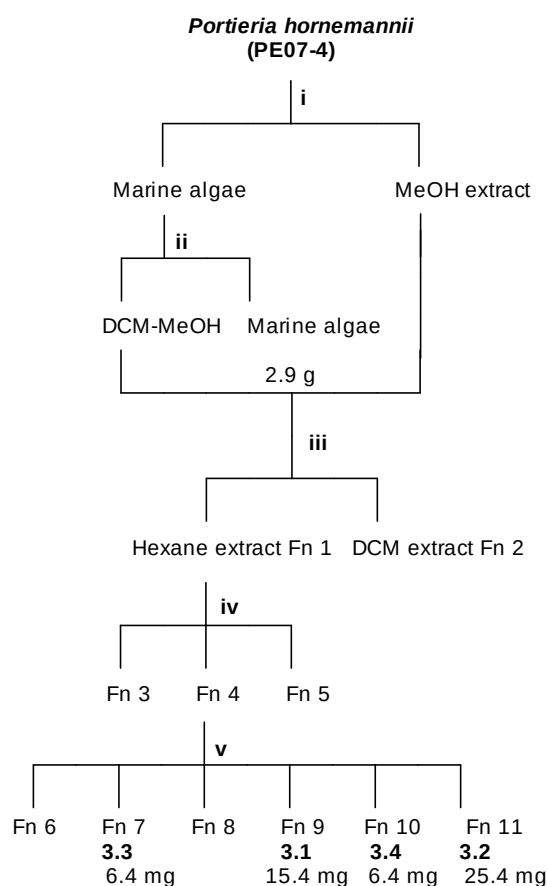
The aim of this study was to investigate the natural product chemistry of South African *P. hornemannii*, as well as screen new halomon related monoterpenes for any potential cytotoxic activity towards *in vitro* cancer cell lines.

As previously mentioned, globally, *P. hornemannii* has demonstrated extreme site-to-site and temporal variations with regards to the concentrations and types of halogenated monoterpenes that have been isolated during relevant extractions. Thus, a second objective was to investigate the possible geographical variation of metabolites from South African *P. hornemannii* species.

### 3.4 Results and discussion

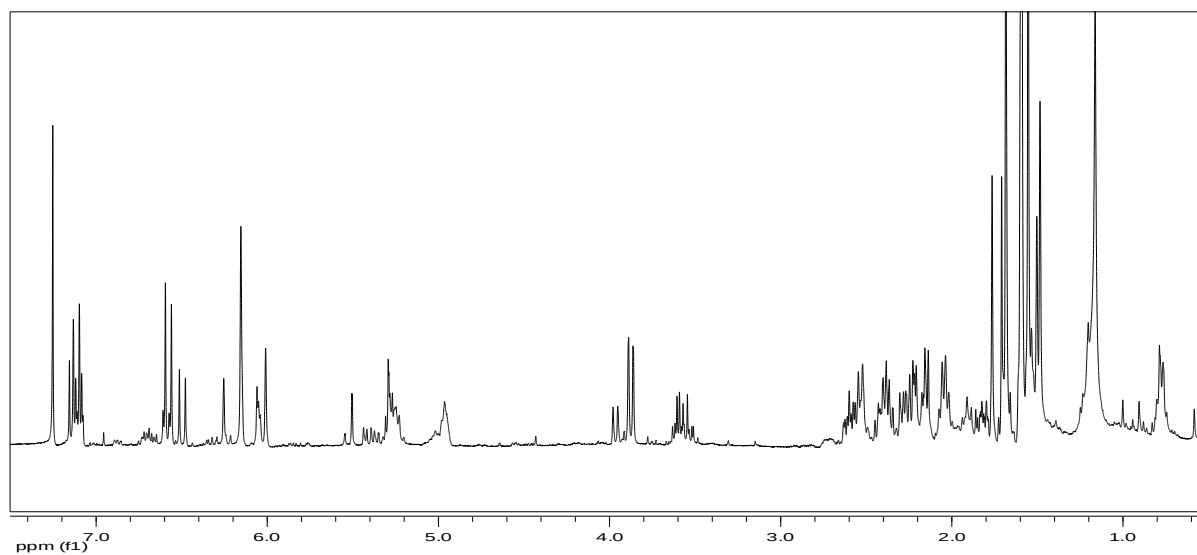
#### 3.4.1 Collection, extraction and isolation

See the experimental section; paragraphs 3.5.2 and 3.5.3 for a full explanation of the collection, extraction and isolation procedure illustrated in Scheme 3.1 below.

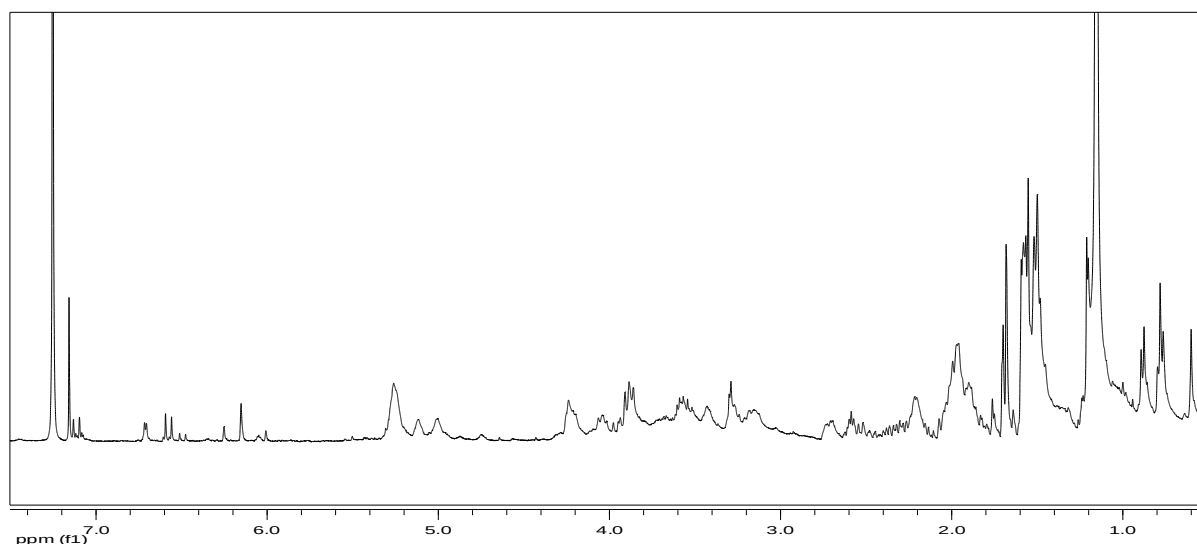


**Scheme 3.1** Extraction and isolation of metabolites from *P. hornemannii* (Port Edward, January 2007)

Conditions: i) MeOH extraction ii) CH<sub>2</sub>Cl<sub>2</sub>-MeOH (2:1) extraction iii) MeOH and CH<sub>2</sub>Cl<sub>2</sub>-MeOH extracts were combined and concentrated (2.9 g). Reconstitution with MeOH, was followed firstly by extraction with hexane, and secondly CH<sub>2</sub>Cl<sub>2</sub> (with the addition of H<sub>2</sub>O until separation occurred) on two separate occasions. The CH<sub>2</sub>Cl<sub>2</sub> extract was not as 'clean' or as concentrated as that of the hexane extract (Figure 3.1 and Figure 3.2) iv) Silica gel (10 g) column chromatography of the hexane extract (776.6 mg) was performed. Fraction 3 is the 100% hexane extract. Fraction 4 was obtained using a solvent system of 9:1 hexane:EtOAc v) Normal phase HPLC using 100% hexane

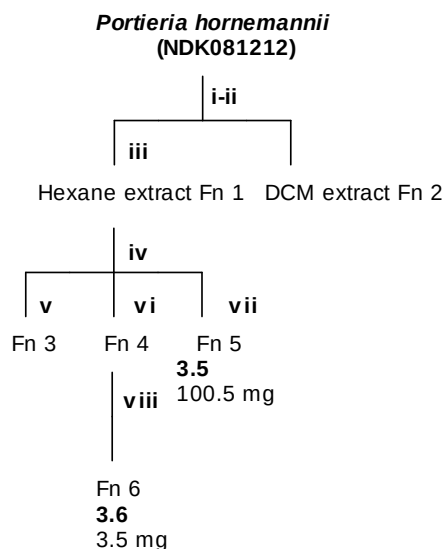


**Figure 3.1** Fraction 1 - Hexane extract of PE07-4A



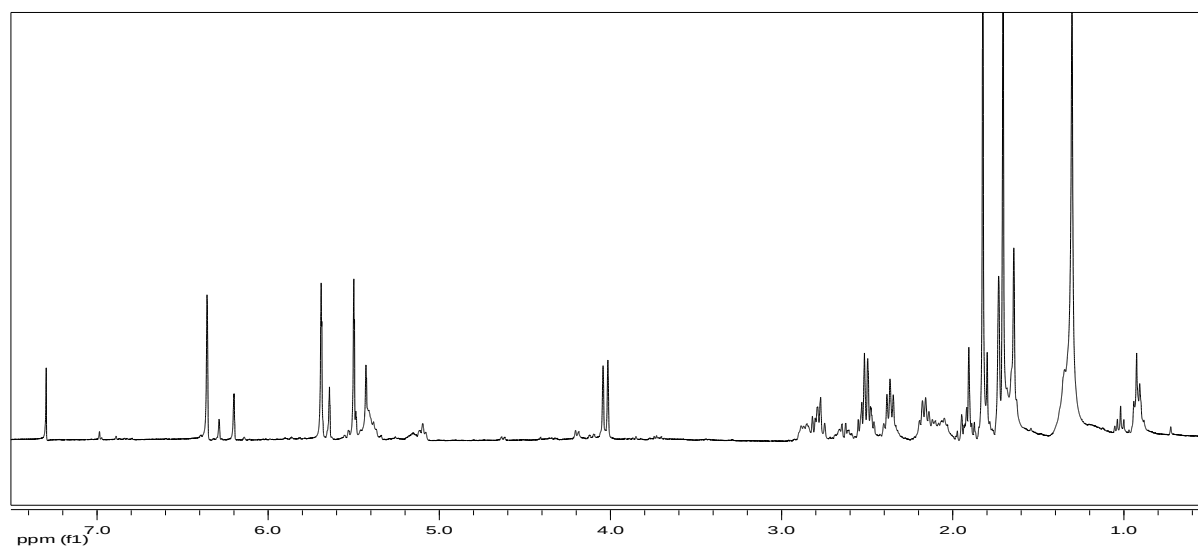
**Figure 3.2** Fraction 2 - CH<sub>2</sub>Cl<sub>2</sub> extract of PE07-4B

See the experimental section; paragraphs 3.5.2 and 3.5.3 for a full explanation of the collection, extraction and isolation procedure illustrated in Scheme 3.2 below.

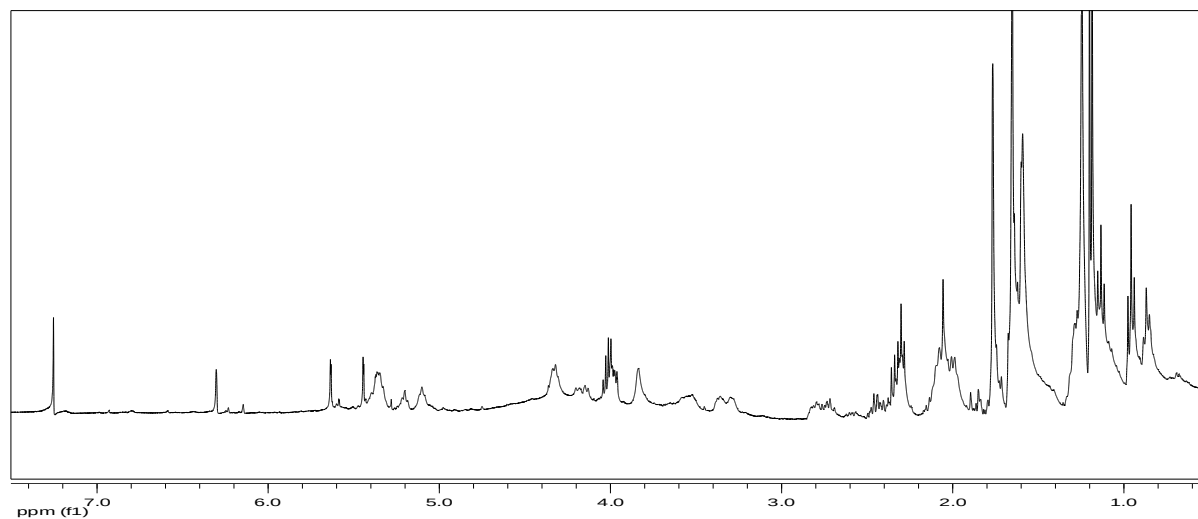


**Scheme 3.2** Extraction and isolation of metabolites from *P. hornemannii* (Noordhoek, December 2008)

Conditions: i)  $\text{CH}_2\text{Cl}_2$ -MeOH (2:1) extraction (100 ml x 3) ii) Solvent partition of the crude  $\text{CH}_2\text{Cl}_2$ -MeOH (2:1) extract with hexane (3.37 g) (Figure 3.3) and  $\text{CH}_2\text{Cl}_2$  (2.72 g) (Figure 3.4) iii) 1.06 g from the crude hexane extract was dissolved in hexane and placed on a 10 g silica column previously equilibrated with hexane. To the silica column, hexane and increasing proportions of EtOAc were used as the eluent. The 100% hexane fraction from this column yielded 741.9 mg iv) The 741.9 mg fraction was then placed on another silica column, and once again hexane and increasing proportions of EtOAc were used as an eluent. From this 12 sub-fractions were separated and grouped on the basis of TLC and  $^1\text{H}$  NMR data v) Fraction 3 (132.3 mg) was obtained from hexane:EtOAc (9:1) vi) Fraction 4 (307.8 mg) was from hexane:EtOAc (8:2) vii) Fraction 5 (100.5 mg) came from hexane:EtOAc (6:4) and was the known pure compound 3Z-6-bromo-3-(bromomethylidene)-2,7-dichloro-7-methyloct-1-ene viii) 307.8 mg was made up with hexane and run through a cotton wool plug. Of this fraction, 100 mg was separated and chromatographed using the HPLC. Fraction 6 or portieric acid A was collected after 25 minutes on the HPLC (using 100% hexane)



**Figure 3.3** Fraction 1 - Hexane extract of NDK081212

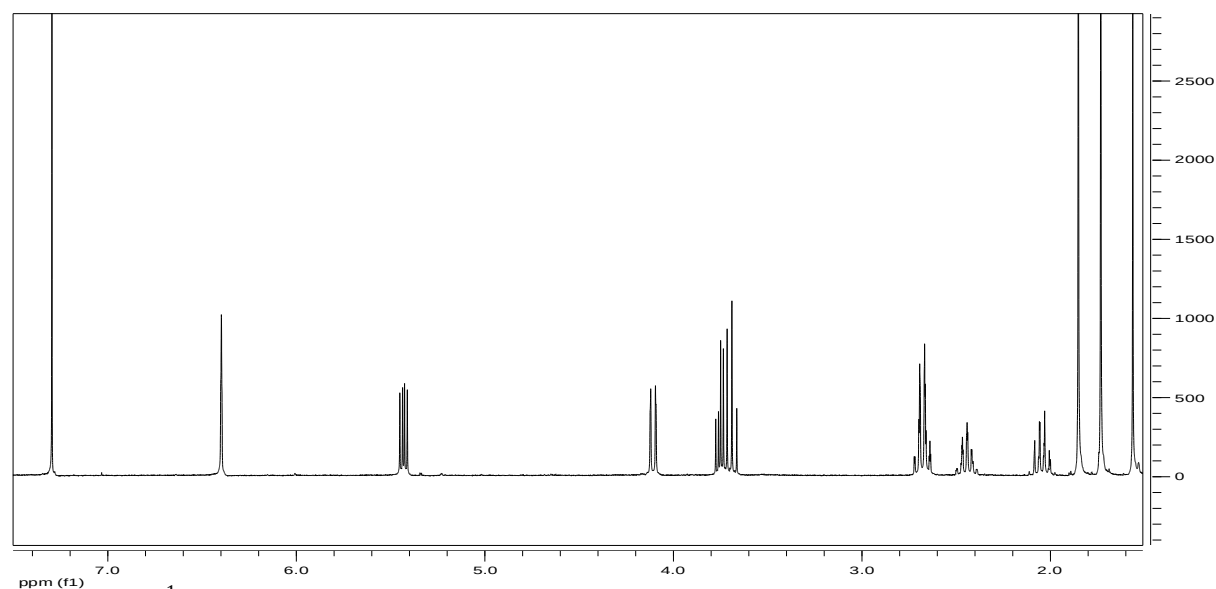


**Figure 3.4** Fraction 2 - CH<sub>2</sub>Cl<sub>2</sub> extract of NDK081212

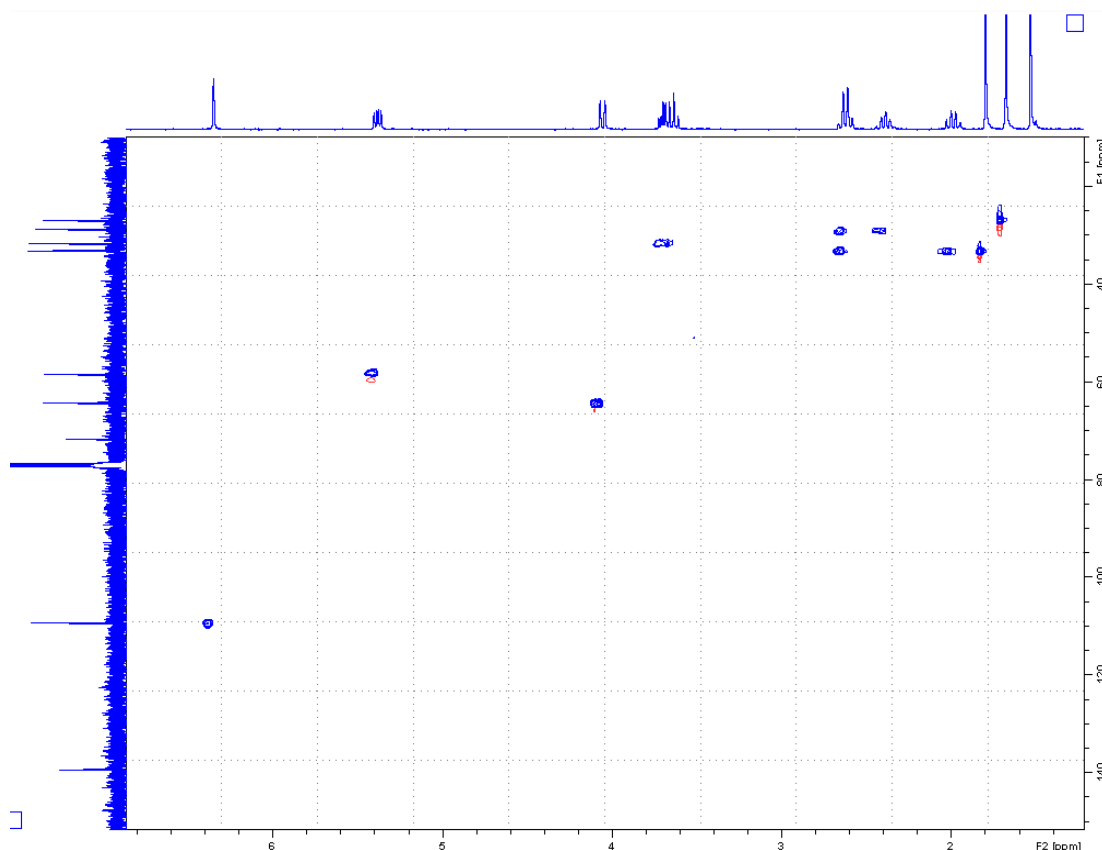
### 3.4.2 Characterisation of halogenated monoterpenes from *P. hornemannii*

The isolation and characterisation of three new linear halogenated monoterpene derivatives (**3.1-3.3**) as well as the identification of one known compound (**3.4**) from organic extracts of *P. hornemannii* (collected at Port Edward in Natal) are described below. In addition to this, the isolation and characterisation of one known compound (**3.5**), as well as one new compound, namely, portieric acid A (**3.6**) from organic extracts of *P. hornemannii* (collected at Noordhoek in the Eastern Cape) are also presented.

#### Structural elucidation of compound **3.1**



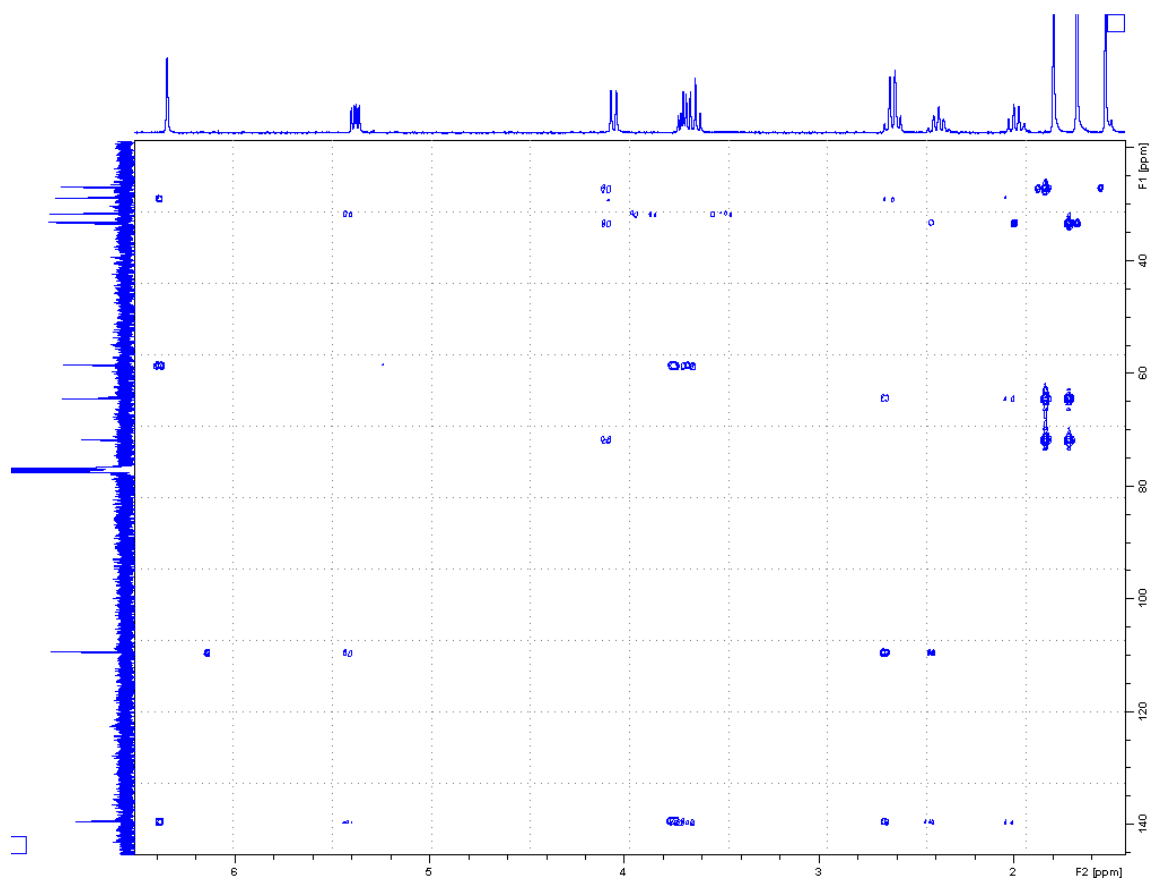
**Figure 3.5**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **3.1**



**Figure 3.6** HSQC spectrum of compound **3.1**

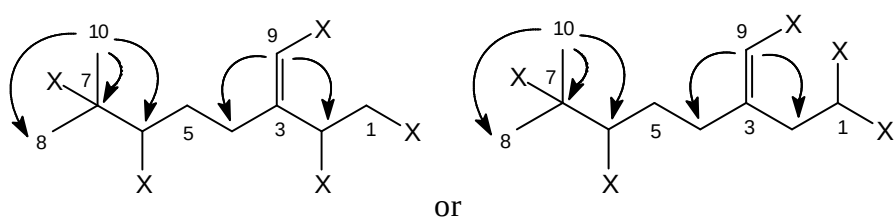
A molecular formula of  $C_{10}H_{15}Br_3Cl_2$  was deduced from HR-GCTOFMS and  $^{13}C$  NMR data for compound **3.1**. The  $^1H$  NMR spectrum of compound **3.1** (Figure 3.5), in combination with the HSQC (Figure 3.6) and DEPT-135 spectra, showed an olefinic methine singlet at  $\delta$  6.39, a deshielded methine double doublet at  $\delta$  5.43 ( $J = 5.9, 10.2$  Hz) and a deshielded methine doublet at  $\delta$  4.10 ( $J = 9.9$  Hz). A two proton methylene double doublet at  $\delta$  3.74 ( $J = 10.6$  and  $5.9$  Hz) and at 3.67 ( $J = 10.6$  and  $10.2$ ), two diastereotopic methylene protons at  $\delta$  2.67 and 2.44 ( $J = 10.8$  Hz) as well as and two diastereotopic methylene protons at  $\delta$  2.04 and 2.68 ( $J = 10.8$  Hz) was also noted. Finally, the  $^1H$  NMR spectrum showed the presence of two methyl singlets at  $\delta$  1.84 and 1.72, which were assigned as geminal dimethyl groups. Due to chemical shifts and the splitting patterns of the remainder of the protons; the doublet at  $\delta$  4.10 ( $J = 9.9$  Hz), the double-double doublet  $\delta$  3.74 ( $J = 10.6$  and  $5.9$  Hz) and at 3.67 ( $J = 10.6$  and  $10.2$ ), the double doublet at  $\delta$  5.43 ( $J = 5.9, 10.2$  Hz) and the singlet at  $\delta$  6.39 were all assigned to carbon bearing halogens. The  $^{13}C$  NMR data of compound **3.1** confirmed the presence of ten carbons. These carbons consisted of two quaternary carbons at  $\delta$  139.3 and 72.0, four methine carbons at  $\delta$  31.9, 58.3, 109.7 and 64.8, two methylene carbons  $\delta$  29.2 and

33.5, and two methyl groups at  $\delta$  33.6 and 27.3, respectively. These findings suggested that at least one double bond was present.



**Figure 3.7** HMBC spectrum for compound **3.1**

HSQC (Figure 3.6), HMBC (Figure 3.7) and COSY spectra were used to determine the arrangement of the ten identified carbons. The methyl group at  $\delta$  33.6 (CH<sub>3</sub>-10) showed HMBC correlations to carbons at  $\delta$  72.0 (C-7), 27.3 (CH<sub>3</sub>-8) and 64.8 (CH-6) as seen below (Figure 3.8; Table 3.1).



**Figure 3.8** Selected HMBC correlations for compound **3.1**



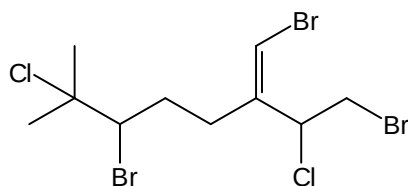
Correspondingly, the methyl group at  $\delta$  27.3 (CH<sub>3</sub>-8) also showed HMBC correlations to carbons at  $\delta$  72.0 (C-7), 33.6 (CH<sub>3</sub>-10) and 64.8 (CH-6) (Table 3.1). Since the proton shifts at C-8 and C-10 showed no other coupling to any other protons, it was established that C-8 and C-10 were both bonded to a quaternary carbon at  $\delta$  72.0 (C-7). HMBC data also confirmed that the halomethine at  $\delta$  4.10 (CH-6) correlated to the carbon at  $\delta$  72.0 (C-7).

HMBC data was also used to establish that the methylene protons at  $\delta$  2.04 and 2.68 are adjacent to the methine group at  $\delta$  4.10 (CH-6). The methylene moiety at  $\delta$  2.04 and 2.68 (CH<sub>2</sub>-5) showed clear COSY correlations with protons at  $\delta$  2.67 and 2.44 (CH<sub>2</sub>-4) and 4.10 (CH<sub>2</sub>-6). The two proton signal at  $\delta$  2.67 and 2.44 (CH<sub>2</sub>-4) only correlated with the proton signals at  $\delta$  2.04 and 2.68 (CH<sub>2</sub>-5), which suggested that the carbon adjacent to CH<sub>2</sub>-4 was the quaternary carbon at  $\delta$  139.3 (C-3). HMBC data of the proton attached to the carbon at  $\delta$  109.7 (C-9) clearly indicated that the carbon signals at  $\delta$  139.3 (C-3), 29.2 (CH<sub>2</sub>-4) and 58.3 (CH-2) are in close proximity, this suggests that C-2 must be a methine and not a methylene (Figure 3.8; Table 3.1).

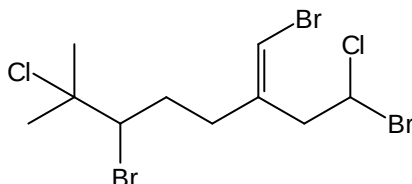
The chemical shift of  $\delta$  72.0 (C-7) implied that the fourth substituent was a Cl moiety (Fuller *et al.*, 1994). A Br atom was added at C-6 due to the carbon shift of  $\delta$  64.8 at this location, which is typical of a Br moiety (Fuller *et al.*, 1992). LREIMS showed a molecular ion peak at  $m/z$  441/443/445/447/449 with relative intensities, and this indicated the presence of three bromine and two chlorine atoms. So far, chlorine and bromine atoms were attached to C-7 and C-6, respectively. The chemical shift of the C-9 carbon ( $\delta$  109.7) was also consistent with a bromine moiety at this position. Therefore, the remaining chlorine and bromine atoms required by the molecular formula must be attached to either C-1 or C-2. The right hand side of the molecule is most likely –CHClCH<sub>2</sub>Br based on almost identical proton  $\delta$  3.62 (dd,  $J$  = 10.8, 5.4 Hz);  $\delta$  3.49 (dd,  $J$  = 10.8, 10.3 Hz) (H-1) and  $\delta$  5.41 (dd,  $J$  = 10.3, 5.4 Hz) (H-2) and carbon  $\delta$  31.5 (C-1) and  $\delta$  61.5 (C-2) shifts demonstrated by Fuller *et al.* (1994).

The *Z* geometry of the  $\Delta^{3,9}$  double bond was confirmed using a NOESY experiment. The proton CH-9 only showed correlations to the proton signal  $\delta$  2.67 (CH<sub>2</sub>-4) and 2.04/2.68 (CH<sub>2</sub>-5).

All the evidence derived from this experiment suggests that the compound is 3*Z*-1,6-dibromo-3-(bromomethylidene)-2,7-dichloro-7-methyloctane.

**3.1**

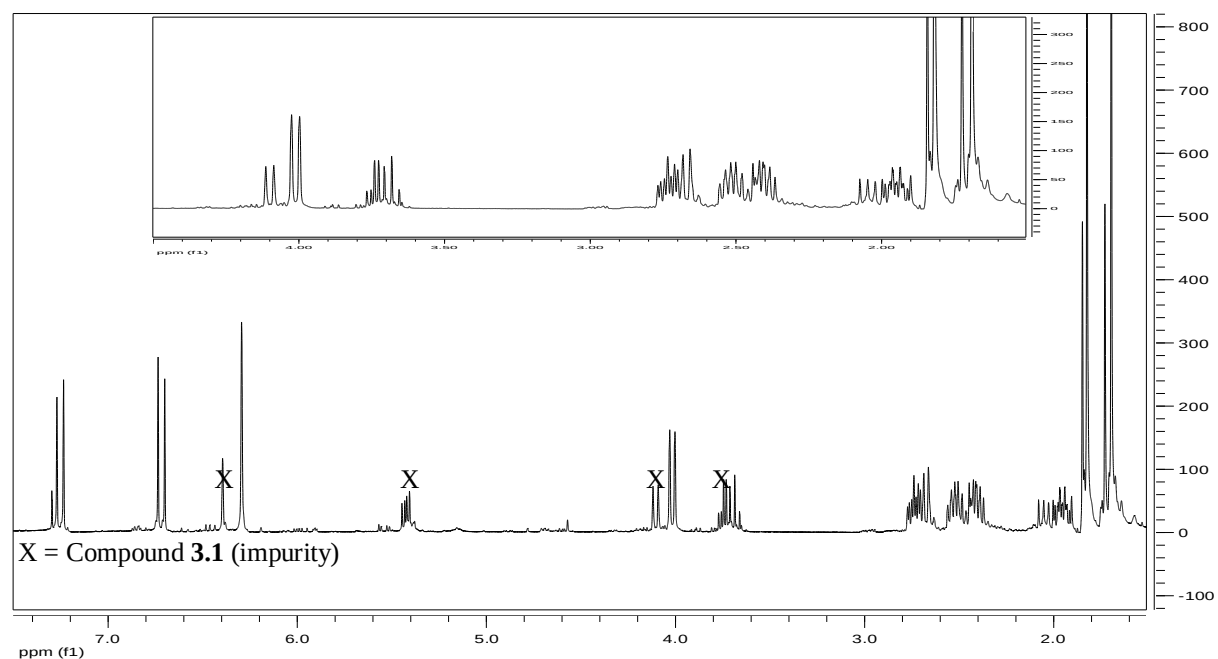
It was originally thought that compound **3.1** might be **3.1a** below. However, based on the intensity of the HMBC correlation between C-2 and H-9 (Figure 3.7) the author has decided to opt for **3.1**. Ignoring this HMBC correlation, the data presented for compound **3.1** supports both structures equally.

**3.1a**

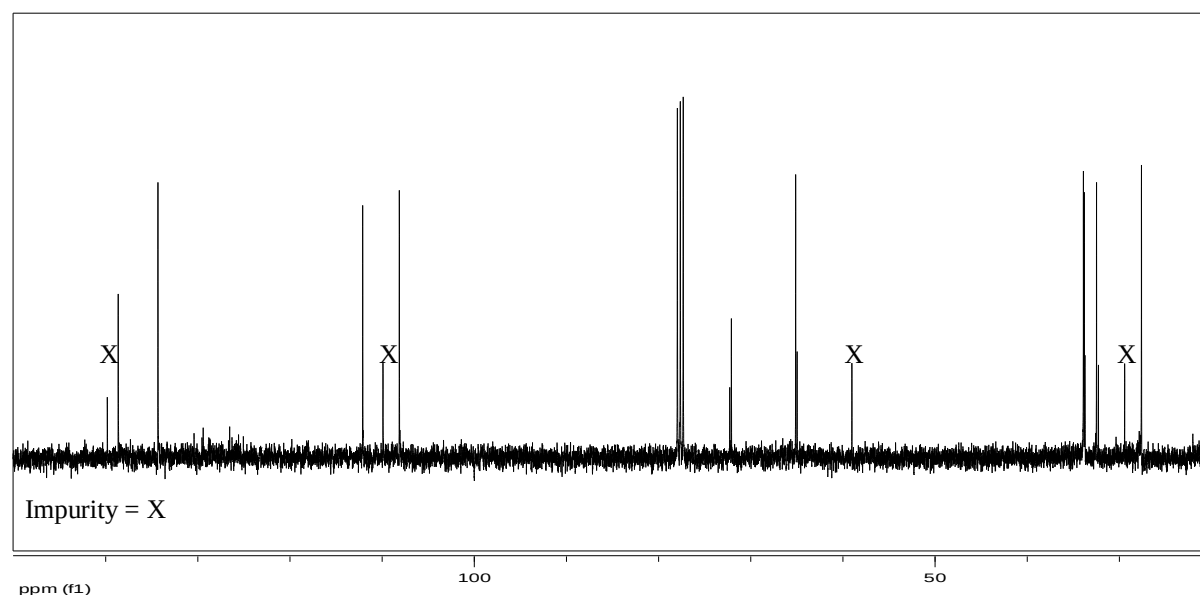
**Table 3.1**  $^1\text{H}$  NMR (400 MHz),  $^{13}\text{C}$  NMR (100 MHz), COSY, HMBC and NOESY data for compound **3.1** in  $\text{CDCl}_3$

| Carbon no. | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$ (Hz)       | $^1\text{H}$ - $^1\text{H}$ COSY | HMBC                       | NOESY    |
|------------|---------------------|---------------------------------------------|----------------------------------|----------------------------|----------|
| 1          | 31.9, $\text{CH}_2$ | 3.74, dd, 5.9, 10.6<br>3.67, dd, 10.2, 10.6 | H-2                              | C-2, C-3                   | H-2      |
| 2          | 58.3, CH            | 5.43, dd, 5.9, 10.2                         | H-1                              | C-1, C-3, C-9              | H-1, H-4 |
| 3          | 139.3, C            | -                                           |                                  |                            |          |
| 4          | 29.2, $\text{CH}_2$ | 2.44, q, 10.8<br>2.67, m                    | H-5                              | C-3, C-5, C-6,<br>C-3, C-9 | H-5      |
| 5          | 33.5, $\text{CH}_2$ | 2.04, q, 10.8<br>2.68, m                    | H-4, H-6                         | C-5, C-6                   | H-4, H-6 |
| 6          | 64.8, CH            | 4.10, d, 9.9                                | H-5                              | C-7, C-8, C-10             | H-5      |
| 7          | 72.0, C             | -                                           |                                  |                            |          |
| 8          | 27.3, $\text{CH}_3$ | 1.72, s                                     |                                  | C-6, C-7, C-8              | H-6      |
| 9          | 109.7, CH           | 6.39, s                                     |                                  | C-2, C-3, C-4              | H-4, H-5 |
| 10         | 33.6, $\text{CH}_3$ | 1.84, s                                     |                                  | C-6, C-7, C-10             | H-6      |

### Structural elucidation of compound 3.2



**Figure 3.9**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound 3.2



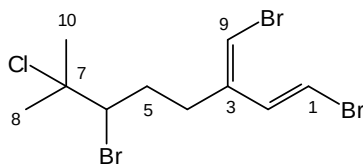
**Figure 3.10**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of compound 3.2

HREIMS for compound **3.2** did not give a molecular ion peak, instead a cluster of peaks at  $m/z$  327/329/331/333 corresponding to a fragment of  $[M-Br]^+$  was obtained. This suggested that the molecular formula of compound **3.2** was  $C_{10}H_{14}Br_3Cl$ . A comparison of the  $^1H$  and  $^{13}C$  NMR spectra of compound **3.2** and **3.1** (Figure 3.9; Figure 3.10; Table 3.2) indicated that the left-hand hemisphere from C-5 to C-8 are identical in compounds **3.1** and **3.2**.

The main difference between the two compounds was the presence of a *trans* double bond ( $\delta$  7.25, d,  $J = 14.6$  Hz and 6.72, d,  $J = 14.6$  Hz) in compound **3.2** which must be accommodated between C-1 and C-2. Since compounds **3.1** and **3.2** were so similar, the halogen atoms could be assigned as follows; bromine at C-6, chlorine at C-7 and bromine at C-9. A bromine substituent was assigned to C-1 on the basis of the carbon chemical shift.

A NOESY experiment was used to determine the *cis* or *trans* nature of the proton at  $\delta$  6.29 (CH-9). Similar to compound **3.1**, the proton at  $\delta$  6.29 (CH-9), correlated to protons at  $\delta$  2.37 (CH<sub>2</sub>-5) and  $\delta$  2.47/2.66 (CH<sub>2</sub>-4). This confirmed the *cis* nature of the  $\Delta^{3,9}$  double bond.

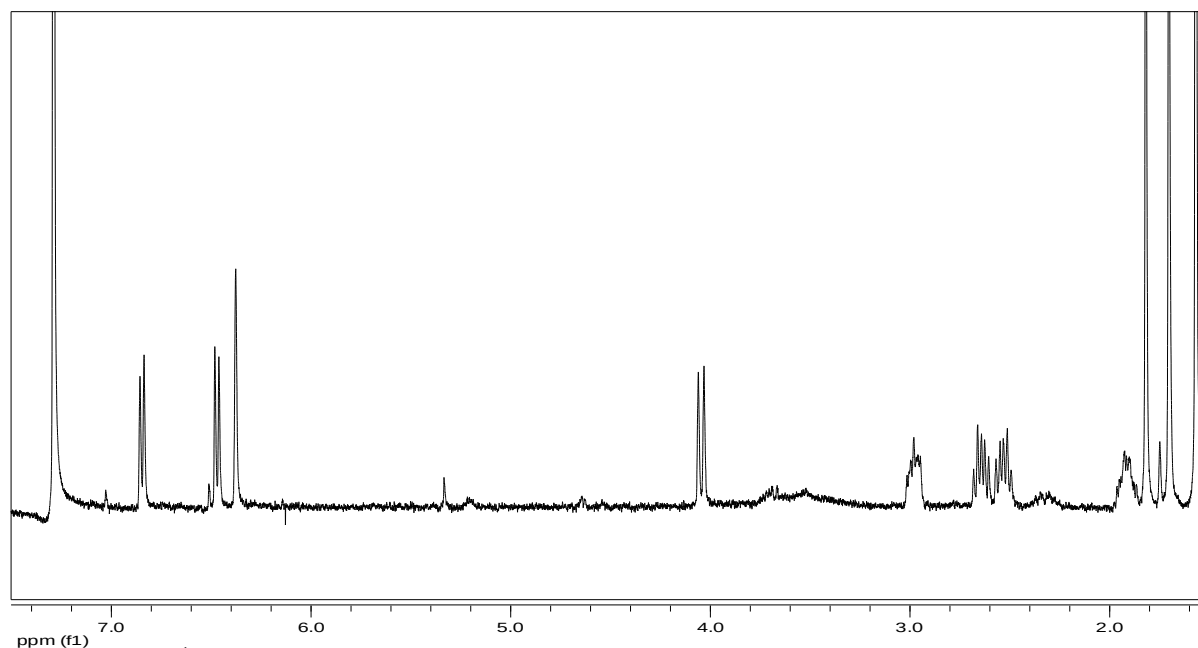
All the evidence suggested that compound **3.2** is 1*E*,3*Z*-1,6-dibromo-3-(bromomethylidene)-7-chloro-7-methyloct-1-ene.



**3.2**

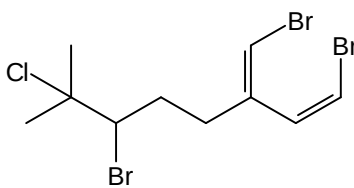
**Table 3.2**  $^1\text{H}$  NMR (400 MHz) and  $^{13}\text{C}$  NMR (100 MHz) data for compound **3.1** and **3.2** in  $\text{CDCl}_3$ 

| Compound 3.1 |                     |                                             | Compound 3.2        |                                       |
|--------------|---------------------|---------------------------------------------|---------------------|---------------------------------------|
| Carbon no.   | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$ (Hz)       | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$ (Hz) |
| 1            | 31.9, $\text{CH}_2$ | 3.74, dd, 5.9, 10.6<br>3.67, dd, 10.2, 10.6 | 111.9, CH           | 6.72, d, 14.6                         |
| 2            | 58.3, CH            | 5.43, dd, 5.9, 10.2                         | 134.2, CH           | 7.25, d, 14.6                         |
| 3            | 139.3, C            | -                                           | 138.7, C            | -                                     |
| 4            | 29.2, $\text{CH}_2$ | 2.44, q, 10.8;<br>2.67, m                   | 32.3, $\text{CH}_2$ | 2.47, m;<br>2.66, m                   |
| 5            | 33.5, $\text{CH}_2$ | 2.04, q, 10.8;<br>2.68, m                   | 33.5, $\text{CH}_2$ | 1.95, m;<br>2.37, m                   |
| 6            | 64.8, CH            | 4.10, d, 9.9                                | 64.9, CH            | 4.07, d, 11.1                         |
| 7            | 72.0, C             | -                                           | 72.1, C             | -                                     |
| 8            | 27.3, $\text{CH}_3$ | 1.72, s                                     | 27.4, $\text{CH}_3$ | 1.68, s                               |
| 9            | 109.7, CH           | 6.39, s                                     | 108.0, CH           | 6.29, s                               |
| 10           | 33.6, $\text{CH}_3$ | 1.84, s                                     | 33.7, $\text{CH}_3$ | 1.83, s                               |

Structural elucidation of compound **3.3**

**Figure 3.11**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **3.3**

Compound **3.3** was isolated as an unstable oil which rapidly degraded during the course of this study. As a result, only the NMR data for this compound could be obtained. The mutually coupled deshielded methine doublets at  $\delta$  6.45 ( $J = 8.3$  Hz, H-1) and  $\delta$  6.85 ( $J = 8.3$  Hz, H-2) (Figure 3.11) as observed in the  $^1\text{H}$  NMR spectrum of compound **3.3**, immediately suggested that it was the *cis*-isomer of compound **3.2** (Table 3.3). As a result, compound **3.3** was identified as 1*Z*,3*Z*-1,6-dibromo-3-(bromomethylidene)-7-chloro-7-methyloct-1-ene.

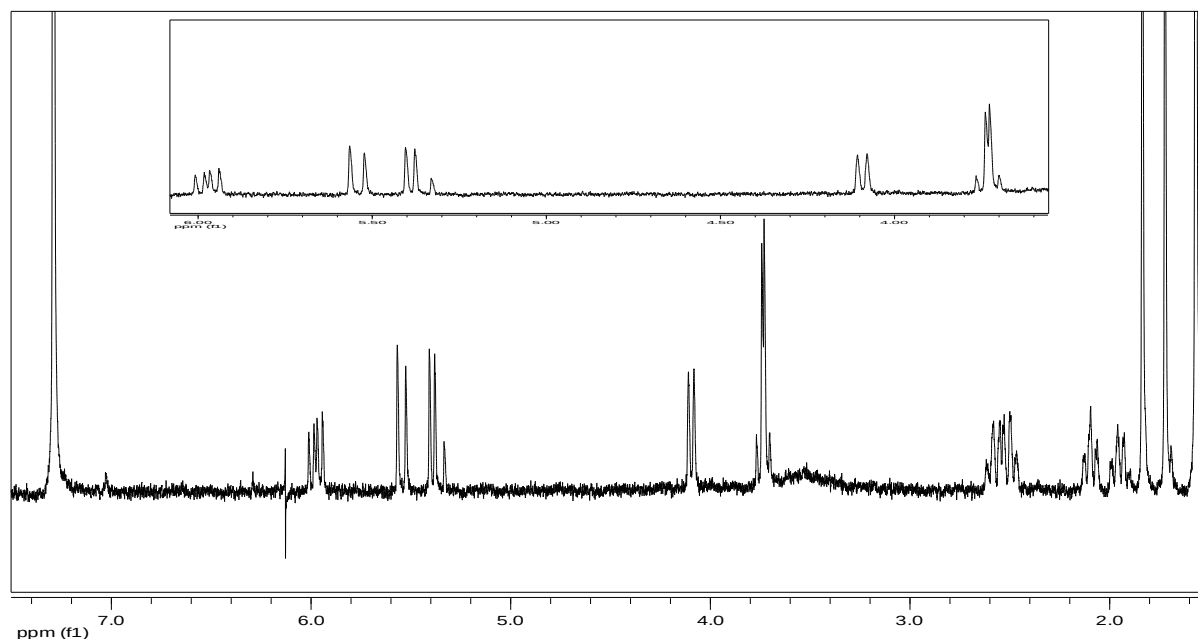


**3.3**

**Table 3.3**  $^1\text{H}$  NMR (400 MHz) and  $^{13}\text{C}$  NMR (100 MHz) data for compound **3.2** and **3.3** in  $\text{CDCl}_3$ 

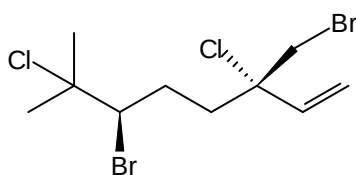
| Carbon no. | Compound 3.2        |                                       | Compound 3.3        |                                       |
|------------|---------------------|---------------------------------------|---------------------|---------------------------------------|
|            | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$ (Hz) | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$ (Hz) |
| 1          | 111.9, CH           | 6.72, d, 14.6                         | 110.5, CH           | 6.45, d, 8.3                          |
| 2          | 134.2, CH           | 7.25, d, 14.6                         | 130.7, CH           | 6.85, d, 8.3                          |
| 3          | 138.7, C            | -                                     | 139.5, C            | -                                     |
| 4          | 32.3, $\text{CH}_2$ | 2.47, m ;<br>2.66, m                  | 33.4, $\text{CH}_2$ | 2.60, m;<br>2.90, m                   |
| 5          | 33.5, $\text{CH}_2$ | 1.95, m;<br>2.37, m                   | 34.2, $\text{CH}_2$ | 1.95, m;<br>2.50, m                   |
| 6          | 64.9, CH            | 4.07, d, 11.1                         | 64.9, CH            | 4.10, d, 11.2                         |
| 7          | 72.1, C             | -                                     | 72.0, C             | -                                     |
| 8          | 27.4, $\text{CH}_3$ | 1.68, s                               | 27.7, $\text{CH}_3$ | 1.70, s                               |
| 9          | 108.0, CH           | 6.29, s                               | 108.7, CH           | 6.35, s                               |
| 10         | 33.7, $\text{CH}_3$ | 1.83, s                               | 33.0, $\text{CH}_3$ | 1.83, s                               |

### Structural elucidation of compound 3.4



**Figure 3.12**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound 3.4

Compound 3.4 showed a number of similarities to that of compound 3.1-3.3. Further analysis of the  $^1\text{H}$  NMR data and comparison with literature reports, confirmed that compound 3.4 was a previously isolated compound (Figure 3.12; Table 3.4). It is known as 3*S*,6*R*-6-bromo-3-(bromomethyl)-3,7-dichloro-7-methyloct-1-ene or 2-dechlorohalomon (Fuller *et al.*, 1994).



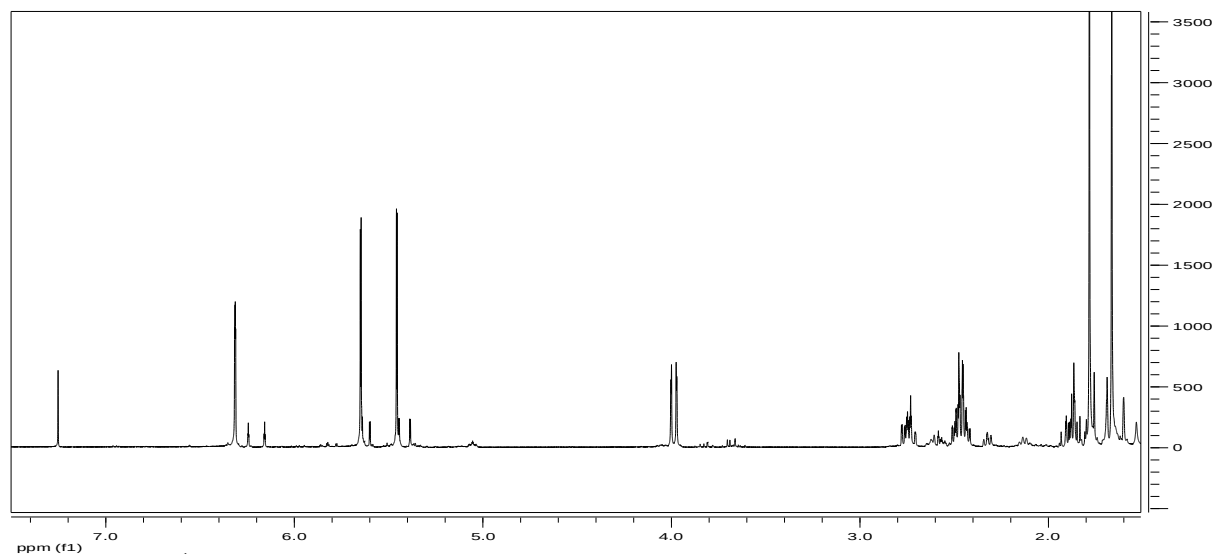
**3.4**



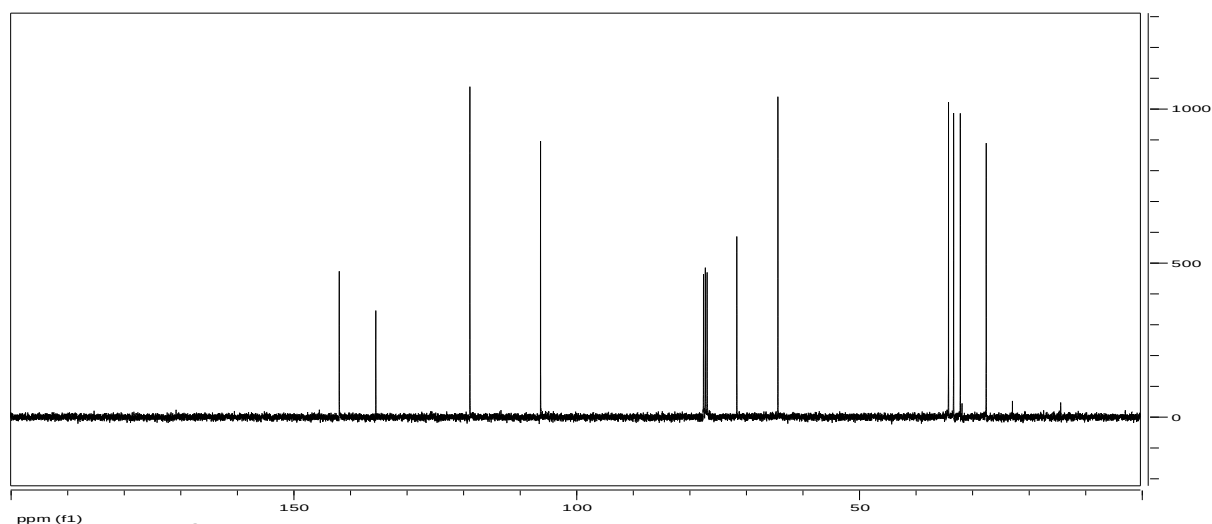
**Table 3.4**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) data for compound **3.4** and 2-dechlorohalomon (Fuller *et al.*, 1994)

| Proton no. | Compound 3.4<br>$\delta_{\text{H}}$ , multi, $J$ (Hz) | 2-dechlorohalomon<br>$\delta_{\text{H}}$ , multi, $J$ (Hz) |
|------------|-------------------------------------------------------|------------------------------------------------------------|
| 1          | 5.51, d, 16.9                                         | 5.51, dd, 0.5, 17.0                                        |
|            | 5.38, d, 10.6                                         | 5.36, dd, 0.5, 10.8                                        |
| 2          | 5.95, dd, 10.6, 16.5                                  | 5.94, dd, 10.8, 17.0                                       |
| 3          | -                                                     | -                                                          |
| 4          | 2.47, ddd, 2.2, 11.5, 13.3                            | 2.46, ddd, 3.7, 11.5, 13.7                                 |
|            | 2.08, ddd, 3.7, 11.5, 13.3                            | 2.06, ddd, 4.0, 11.6, 13.7                                 |
| 5          | 2.54, ddd, 4.4, 11.5, 13.3                            | 2.54, dddd, 1.7, 4.0, 11.5, 13.8                           |
|            | 1.93, ddd, 4.4, 11.5, 13.3                            | 1.91, dddd, 1.7, 4.0, 11.5, 13.8                           |
| 6          | 4.09, d, 10.9                                         | 4.06, dd, 1.7, 11.1                                        |
| 7          | -                                                     | -                                                          |
| 8          | 1.70, s                                               | 1.68, s                                                    |
| 9          | 3.71, d, 11.1                                         | 3.71, d, 10.5                                              |
|            | 3.69, d, 11.1                                         | 3.69, d, 10.5                                              |
| 10         | 1.81, s                                               | 1.80, s                                                    |

### Structural elucidation of compound 3.5

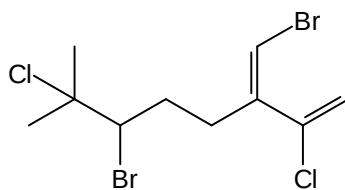


**Figure 3.13**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound 3.5



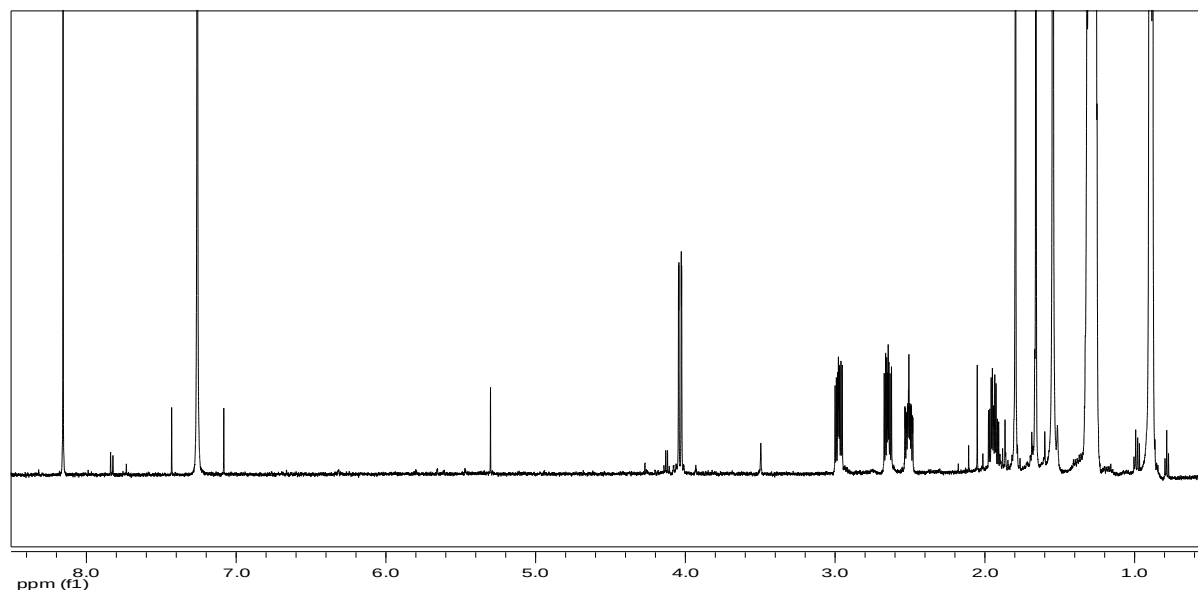
**Figure 3.14**  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) spectra of compound 3.5

Compound 3.5 was the major secondary metabolite of the NDK081212 extraction of *P. hornemannii*. However, it had already been reported by Andrianasolo (2006) who had previously isolated it from *P. hornemannii* collected off the Madagascar coastline (Table 3.5) (Andrianasolo *et al.*, 2006). Compound 3.5 is known as 3Z-6-bromo-3-(bromomethylidene)-2,7-dichloro-7-methyloct-1-ene.

**3.5****Table 3.5**  $^1\text{H}$  NMR (400 MHz) and  $^{13}\text{C}$  NMR (100 MHz) data for compound **3.5**, compared with known literature values (Andrianasolo *et al.*, 2006)

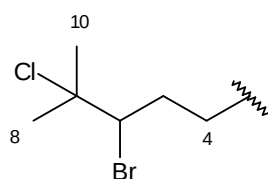
| Carbon no. | $\delta_{\text{C}}$   | $\delta_{\text{C}}$<br>Andrianasolo<br><i>et al.</i> , 2006 | $\delta_{\text{H}}$          | $\delta_{\text{H}}$<br>Andrianasolo<br><i>et al.</i> , 2006 |
|------------|-----------------------|-------------------------------------------------------------|------------------------------|-------------------------------------------------------------|
| 1          | 118.7, CH             | 118.9, CH                                                   | 5.45, d, 1.3<br>5.64, d, 1.1 | 5.49, d, 1.6<br>5.69, d, 1.6                                |
| 2          | 135.4, C              | 135.7, C                                                    |                              |                                                             |
| 3          | 141.8, C              | 142.3, C                                                    |                              |                                                             |
| 4          | 34.0, CH <sub>2</sub> | 34.4, CH <sub>2</sub>                                       | 2.75, m<br>2.50, m           | 2.80, m<br>2.50, m                                          |
| 5          | 31.9, CH <sub>2</sub> | 32.4, CH <sub>2</sub>                                       | 2.49, m                      | 2.49, m                                                     |
| 6          | 64.2, CH              | 64.5, CH                                                    | 4.00, d, 11.2                | 4.03, dd, 1.0. 11.3                                         |
| 7          | 71.5, C               | 72.4, C                                                     |                              |                                                             |
| 8          | 27.3, CH <sub>3</sub> | 27.7, CH <sub>3</sub>                                       | 1.67, s                      | 1.69, s                                                     |
| 9          | 106.2, CH             | 106.8, CH                                                   | 6.31, s                      | 6.35, s                                                     |
| 10         | 33.9, CH <sub>3</sub> | 33.6, CH <sub>3</sub>                                       | 1.77, s                      | 1.81, s                                                     |

### Structural elucidation of compound **3.6**



**Figure 3.15**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **3.6**

LR-TOFMS analysis of compound **3.6** indicated the presence of a molecular ion at  $m/z$  345/347/349. This suggested a molecular formula of  $\text{C}_9\text{H}_{13}\text{O}_2\text{Br}_2\text{Cl}$ . The number and nature of the halogens present was also confirmed by the halogen cluster of the MS data. The presence of only nine carbons indicated that this compound was a halogenated nor-monoterpene. Nor-monoterpenes have been previously reported by others (Bates *et al.*, 1979).



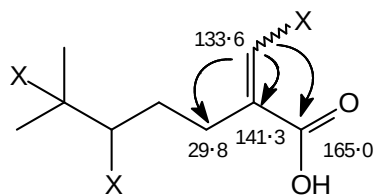
**Figure 3.16** Common fragment found in halomon related compounds

All the compounds discussed until now, including compound **3.6**, contained the same C-4 to C-10 fragment (Figure 3.16). The three remaining carbon signals ( $\delta_C$  165.0, 133.6, 141.3) still needed to be ‘put in order’.

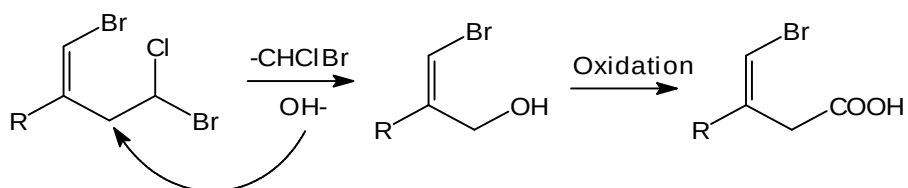
The  $^1\text{H}$  NMR spectrum of the remainder of the ‘unknown’ part of the structure for compound **3.6** indicated a one proton singlet at  $\delta$  8.15 and a two proton multiplet at  $\delta$  2.94 ( $J = 2.8$  Hz) and  $\delta$  2.63 ( $J = 2.8$  Hz) (Figure 3.15).

The most important piece of evidence for the assignment of compound **3.6** came from HMBC data. The olefinic methine at  $\delta$  8.15 (HSQC to  $\delta$  133.6) indicated several correlations to carbon signals at  $\delta$  165.0, 141.3 and 29.8 respectively (Figure 3.17). Unfortunately, the compound degraded it was not possible to run a NOESY experiment on the  $\Delta^{3,9}$  double bond. In addition to this, the carbon shift at  $\delta_C$  165.0 indicated the presence of a carboxylic acid derivate, something very unusual for *Portieria* sp. The presence of a COOH functional group was also confirmed using IR. IR data showed a broad band between 2800-3000 $\text{cm}^{-1}$ , as well as a band at near 1700 $\text{cm}^{-1}$  and 1400 $\text{cm}^{-1}$ . Together, these three bands indicated the presence of a COOH functional group (Figure 3.21).

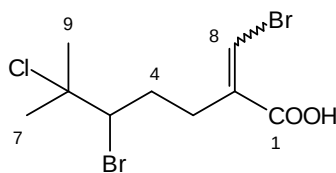
The data suggested that compound **3.6** be identified as 5-bromo-2-(bromomethylidene)-6-chloro-6-methylheptanoic acid. Compound **3.6** was given the name portieric acid. According to the literature (see 3.8 Appendix 1), this is the first time that a secondary metabolite containing carboxylic acid has been isolated and characterised from *P. hornemannii*.



**Figure 3.17** Selected HMBC correlations data for compound **3.6**



**Figure 3.18** Possible mechanism of synthesis of portieric acid A

**3.6****Table 3.6**  $^1\text{H}$  NMR (400 MHz),  $^{13}\text{C}$  NMR (100 MHz), COSY and HMBC data for compound **3.6** in  $\text{CDCl}_3$ 

| Carbon no. | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$ (Hz) | $^1\text{H}$ - $^1\text{H}$ COSY | HMBC          |
|------------|---------------------|---------------------------------------|----------------------------------|---------------|
| 1          | 165.0, C            | -                                     |                                  |               |
| 2          | 141.3, C            | -                                     |                                  |               |
| 3          | 29.8, $\text{CH}_2$ | 2.94, m<br>2.63, m                    | H-4                              | C-1, C-4      |
| 4          | 32.4, $\text{CH}_2$ | 2.49, m<br>1.92, m                    | H-3, H-5                         | C-3, C-5, C-6 |
| 5          | 64.2, CH            | 4.02, dd, 1.9, 11.1                   | H-4                              |               |
| 6          | 71.5, C             | -                                     |                                  |               |
| 7          | 27.0, $\text{CH}_3$ | 1.64, s                               |                                  |               |
| 8          | 133.6, CH           | 8.15, s                               |                                  | C-1, C-2, C-3 |
| 9          | 33.6, $\text{CH}_3$ | 1.78, s                               |                                  | C-5, C-6      |

*Possible explanation for isolating different secondary metabolites from two different Portieria hornemannii samples*

Globally, *Portieria hornemannii* has demonstrated extreme site-to-site and temporal variations with regards to the concentrations and types of halogenated monoterpenes that have been isolated during relevant extractions. From the evidence presented in this chapter, it seems that the South African *Portieria hornemannii* samples collected from Port Edward and Noordhoek were no exception; as each sample contained different halogenated monoterpenes as major secondary metabolites.

It should be noted that the crude extract of *Portieria hornemannii* collected from Noordhoek (in the Eastern Cape) contained the same compound that was isolated from *Portieria hornemannii* in Madagascar (Andrianasolo *et al.*, 2006), while this compound was not found in the sample of *Portieria hornemannii* collected from Port Edward (in Natal). This raises some interesting questions about the nature of the *Portieria hornemannii* samples that were used. Bolton (2011) was confident that all the extractions were performed on genuine *Portieria hornemannii* species. However, this research demonstrated that *Portieria hornemannii* in South Africa has at least two distinctly different chemical profiles. This begs the question, can  $^1\text{H}$  NMR spectra of crude *Portieria hornemannii* extracts be used as a tool to assist in the taxonomy of different South African *Portieria hornemannii* species?

### Bioactivity

Compounds (3.1-3.2) showed that they were non-toxic towards MDA-MB-231 breast cancer cell lines (metastatic) at 100  $\mu\text{M}$ . Interestingly, Fuller (1994) suggested that a halogen at C-6 is essential for biological activity, but in addition to this, a halogen at C-2 is also required for 'halomon-like' activity. Compounds (3.1-3.2) lack a halogen at C-2. This may be the reason that these halomon related compounds showed little activity towards the MDA-MB-231 breast cancer cell lines. Unfortunately, compound 3.3, 3.4 and 3.5 could not be tested because the samples degraded. It should be noted that compound 3.2 was not a pure sample and this would have had an effect on the bioactivity results.

Compound 3.6 showed slight cytotoxic activity towards MDA-MB-231 breast cancer cell lines (metastatic). The effect of compound 3.6 on the survival and proliferation of MDA-MB-231 cells were performed in triplicate and cell viability was assessed using the MTT assay. The half-maximal inhibitory concentrations ( $\text{IC}_{50}$ ), as calculated from the linear dose-response region was 77.7  $\mu\text{M}$  on average (with a standard deviation of 5.7  $\mu\text{M}$ ). In addition, only 20% of the MDA-MB-231 cells survived after being exposed to 80.0  $\mu\text{M}$  of portieric acid A.



### 3.5 Experimental section

#### 3.5.1 General experimental procedures

Normal phase TLC was performed on DC Alufolie Kieselgel 60F<sub>254</sub> and plates were viewed under UV light (254 nm). Column chromatography was performed on silica gel 60 (0.04 – 0.063 mm) from Merck, Darmstadt, Germany. Semi-preparative HPLC was carried out with a Spectra-Physics IsoChrom pump, a Whatman Partisil 10 semi-preparative column (1x50cm) and a Waters R451 Refractive Index detector (ATTx64), or a SpectraSERIES UV100 detector and a Rikadenki chart recorder. All solvents were of HPLC grade (HiPerSolv<sup>TM</sup> and Merck).

The <sup>1</sup>H (400 MHz), <sup>13</sup>C (100 MHz) and DEPT-135 NMR spectra were all recorded on a Bruker 400 NMR spectrometer using standard pulse sequences. HMQC, HMBC, COSY-90 and NOESY NMR spectra were recorded on a Bruker 600 NMR spectrometer using standard pulse sequences. Spectra were referenced to residual protonated solvent resonances (CHCl<sub>3</sub>  $\delta_H$  7.25,  $\delta_C$  77.0). Chemical shifts were reported in ppm, while coupling constants were reported in Hz.

Optical rotations were measured on a Perkin-Elmer 141 polarimeter. UV data was obtained using the Perkin Elmer Precisely Lambda 25 UV/VIS spectrometer. IR spectra were obtained using a Perkin Elmer Spectrum 100 FT-IR spectrometer with a universal ATR sampling accessory.

LREIMS and HREIMS (70 eV) was performed by Marelize Ferreira at Wits University in Johannesburg, South Africa. The experiments were performed using a VG SE70 magnetic sector spectrometer. Samples were run on the EI mode by direct probe insertion using perfluorokersen as reference material. HR-GCTOFMS was performed at Stellenbosch University in Stellenbosch, South Africa. The experiments were performed by Fletcher Hiten using a Waters GCT Premier mass spectrometer. HR-GCTOFMS (70 eV) experiments were performed using a HP-5 column (30 m, 0.25 mm ID, 0.25  $\mu$ m film thickness); injector temperature 260 °C; split injection (1:10); the carrier gas was helium 1ml/min; scanning range from 35 to 600 *m/z* (perfluorotri-N-butylamine as mass reference); and temperature ramp 40-300 °C at 20 °C/min over a 5 minute period. LR-TOFMS was also performed by Fletcher Hiten at Stellenbosch University. These experiments were conducted on a Waters

API Q-TOF Ultima mass spectrometer. The conditions were as follows; capillary voltage: 3.5 kV; cone voltage: 35; RFI:40; Source: 100 °C; desolvation temperature: 350 °C; desolvation gas: 350 L/h and cone gas 50 L/h and scan 200-2000.

### 3.5.2 Plant material

*P. hornemannii* (PE07-4) was collected from the intertidal zone at Port Edward in KwaZulu-Natal, South Africa on the 19<sup>th</sup> January 2007 and immediately frozen. A voucher specimen (PE07-4) is retained at the Faculty of Pharmacy, Rhodes University, South Africa.

*P. hornemannii* (NDK-081212) was collected from the intertidal zone at Noordhoek (in the Eastern Cape) during December 2008 and immediately frozen. A voucher specimen is retained at the Faculty of Pharmacy (NDK-081212), Rhodes University, South Africa.

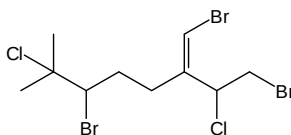
### 3.5.3 Extraction and isolation

The frozen alga (PE07-4) (25.5 g dry weight after extraction) was submerged in MeOH at 4 °C for 1 hour. The MeOH was decanted and the alga was re-extracted three times with 500 ml CH<sub>2</sub>Cl<sub>2</sub>-MeOH (2:1) and heated to 37 °C for 30 minutes. The MeOH and CH<sub>2</sub>Cl<sub>2</sub>-MeOH extracts were combined and concentrated to give 2.9 g of crude organic extract. The latter was partitioned between MeOH-H<sub>2</sub>O (9:1, 50 ml) and hexane (3 x 50 ml). The hexane partition layers were combined, dried over anhydrous sodium sulfate, filtered and concentrated to give 1.54 g of hexane partition. The water content of the aqueous methanol layer was increased to 20% and partitioned with CH<sub>2</sub>Cl<sub>2</sub> (3 x 50 ml). The latter was dried over anhydrous sodium sulfate, filtered and concentrated to give 1.40 g of CH<sub>2</sub>Cl<sub>2</sub> extract. The aqueous methanol layer was concentrated and extracted with EtOAc (3 x 50 ml). The EtOAc partition was combined with the aqueous phase and stored in the refrigerator at 4 °C. The hexane and CH<sub>2</sub>Cl<sub>2</sub> fractions were concentrated to give two crude organic extracts; fraction 1 and fraction 2 (or A and B). A portion of fraction 1 (776.6 mg) was fractionated on a silica gel column (10 g) using 50 ml portions of a hexane-EtOAc mixture in a ratio of 1:0, 9:1, 8:2, 7:3, 6:4, 4:6, 2:8, 1:9 and finally with a 1:1 mixture of MeOH-EtOAc to give nine fractions (A1-A9). Fraction 2 or extract B (866.9 mg) was also fractionated on a silica gel column (10.0 g) using 50 ml portions as mentioned above. From this, 9 fractions (B1-B9) were also obtained. The first two fractions from extract A; Fn 3 (from 100% hexane) and Fn 4 (from hexane:EtOAc 9:1), were subjected to normal phase HPLC using 100% hexane as the

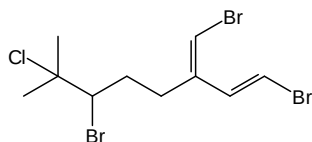
mobile phase. From Fn 3 (A1) (118.7 mg) was injected and a further seven sub-fractions were obtained from the HPLC. From Fn 4 (A2) (119.4 mg) a further six sub-fractions were obtained from the HPLC. Of these six sub-fractions obtained from A2 (or Fn 4), four compounds were pure; sub-fraction 9 weighed 15.4 mg (0.060% dry weight) (**3.1**), sub-fraction 11 weighed 25.4 mg (0.099% dry weight) (**3.2**), sub-fraction 10 weighed 6.4 mg (0.025% dry weight) (**3.4**) and sub-fraction 7 weighed 6.4 mg (0.025% dry weight) (**3.3**) respectively.

From Noordhoek (NDK081212), the frozen sample (110.5 g dry weight) was initially steeped in a mixture of  $\text{CH}_2\text{Cl}_2$  - MeOH (2:1) for 30 minutes per extraction at 37 °C (this was repeated three times). The combined  $\text{CH}_2\text{Cl}_2$ - MeOH (2:1) mixture was then partitioned three times with hexane (50 ml). This was followed by three  $\text{CH}_2\text{Cl}_2$  (50 ml) partitions with the same crude  $\text{CH}_2\text{Cl}_2$ - MeOH (2:1) mixture. The hexane extract weighed 3.37 g and the  $\text{CH}_2\text{Cl}_2$  extract weighed 2.72 g. A portion (1.06 g) of the above hexane extract was dissolved in a small amount of hexane and added to 10 g silica solution which had been equilibrated in hexane. 40 ml amounts of hexane and increasing proportions of EtOAc were used as the eluent. The 100% hexane fraction (741.9 mg) was placed on a 10 g silica column (as mentioned above), and 12 fractions were separated and grouped on the basis of TLC. Fraction 5 was obtained from hexane - EtOAc (6:4) and was a pure but known compound **3.5** (100.5 mg, 0.282% dry weight). Fraction 4 (hexane-EtOAc mixture in a ratio of 8:2; 7:3) was grouped together (307.8 mg) for possible further HPLC separation. Fraction 4 (100 mg) was then injected onto the HPLC using 100% hexane. Five sub-fractions were obtained. Unfortunately, the UV detector was not suitable for these compounds since they often lack conjugation bonds. As a result of this, only a minor metabolite was isolated from sub-fraction 6, compound **3.6** (3.5 mg, 0.010% dry weight).

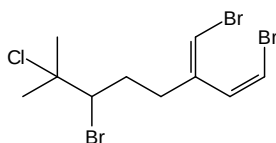
## 3.5.4 Compounds



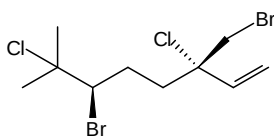
**3Z-1,6-dibromo-3-(bromomethylidene)-2,7-dichloro-7-methyloctane (3.1):** Colourless oil;  $[\alpha]_D^{28} 13^\circ$  (c 0.008, Hexane); UV (Hexane)  $\lambda_{\max} 357$  ( $\epsilon$  109); IR (dry film)  $\nu_{\max}$  2982, 2931, 1454, 1439, 1388, 1368, 1223, 1203, 1104, 814  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) see Table 3.1; HR-GCTOFMS  $m/z$  445.8086 (calculated for the formula  $\text{C}_{10}\text{H}_{15}\text{Br}_3\text{Cl}_2$  445.8075)



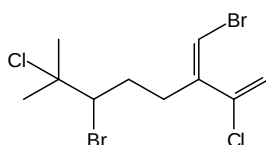
**1E,3Z-1,6-dibromo-3-(bromomethylidene)-7-chloro-7-methyloct-1-ene (3.2):** Colourless oil;  $[\alpha]_D^{28} 8^\circ$  (c 0.006, Hexane); UV (Hexane)  $\lambda_{\max} 307$  ( $\epsilon$  430); IR (dry film)  $\nu_{\max}$  3068, 2928, 2850, 1456, 1371, 1216, 1104, 943, 797, 720  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) see Table 3.2; HR-GCTOFMS  $m/z$   $[\text{M}-\text{Br}]^+$  328.9236 (calculated for the formula  $\text{C}_{10}\text{H}_{14}\text{Br}_3\text{Cl}$  409.3801)



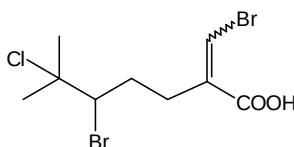
**1Z,3Z-1,6-dibromo-3-(bromomethylidene)-7-chloro-7-methyloct-1-ene (3.3):** Colourless oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) see Table 3.3



**3S,6R-6-bromo-3-(bromomethyl)-3,7-dichloro-7-methyloct-1-ene (3.4):** As previously reported (Fuller *et al.*, 1994) see Table 3.4



**3Z-6-bromo-3-(bromomethylidene)-2,7-dichloro-7-methyloct-1-ene (3.5):** As previously reported (Andrianasolo *et al.*, 2006) see Table 3.5



**Portieric acid A or 5-bromo-2-(bromomethylidene)-6-chloro-6-methylheptanoic acid (3.6):**

Colourless oil;  $[\alpha]_D^{28} -33^\circ$  (c 0.0003, CH<sub>2</sub>Cl<sub>2</sub>); UV (CH<sub>2</sub>Cl<sub>2</sub>)  $\lambda_{\max}$  252 ( $\epsilon$  345); IR (dry film)  $\nu_{\max}$  2931, 1745, 1595, 1446, 1388, 1370, 1120, 905, 756, 668 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) see Table 3.7; LR-TOFMS  $m/z$  344.9/346.9/348.9 (calculated for the formula C<sub>9</sub>H<sub>13</sub>O<sub>2</sub>Br<sub>2</sub>Cl 344.9/346.9/348.9)

### 3.5.5 Cytotoxicity assays

As discussed in chapter two.

### 3.6 References

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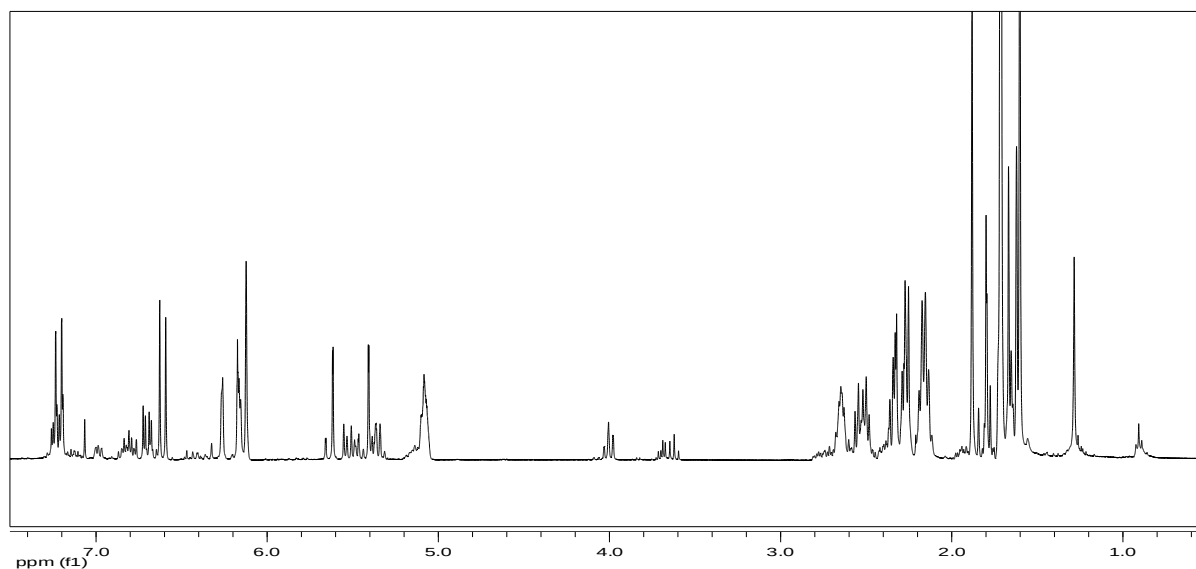
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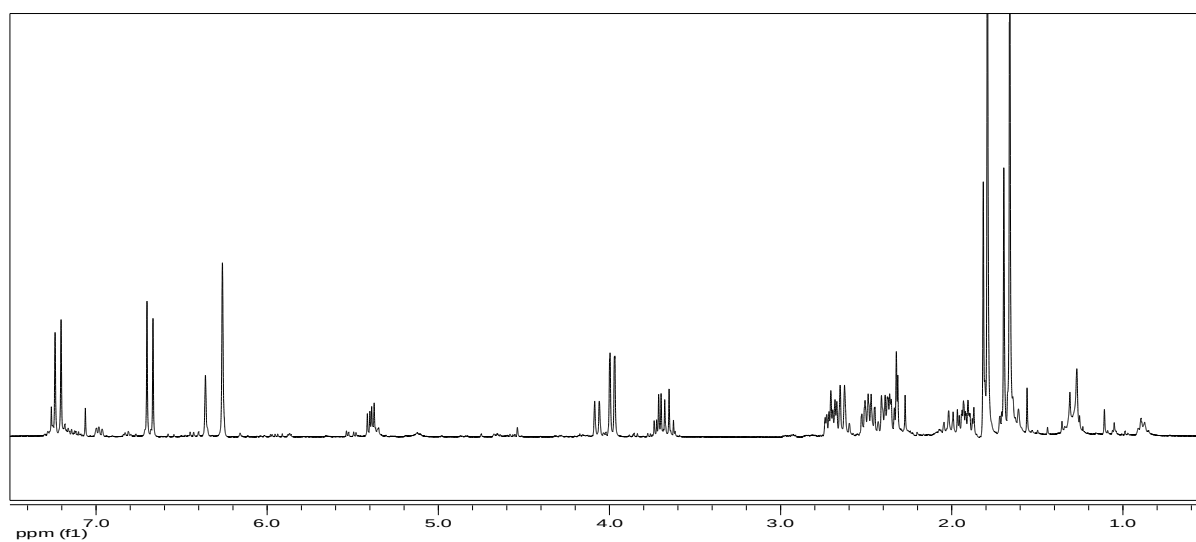
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### 3.7 Supplementary data



**Figure 3.19** PE07-4A1 (Fraction 3 - 100% hexane fraction obtained from the silica column)



**Figure 3.20** PE07-4A2 (Fraction 4 - 90% hexane fraction obtained from the silica column)



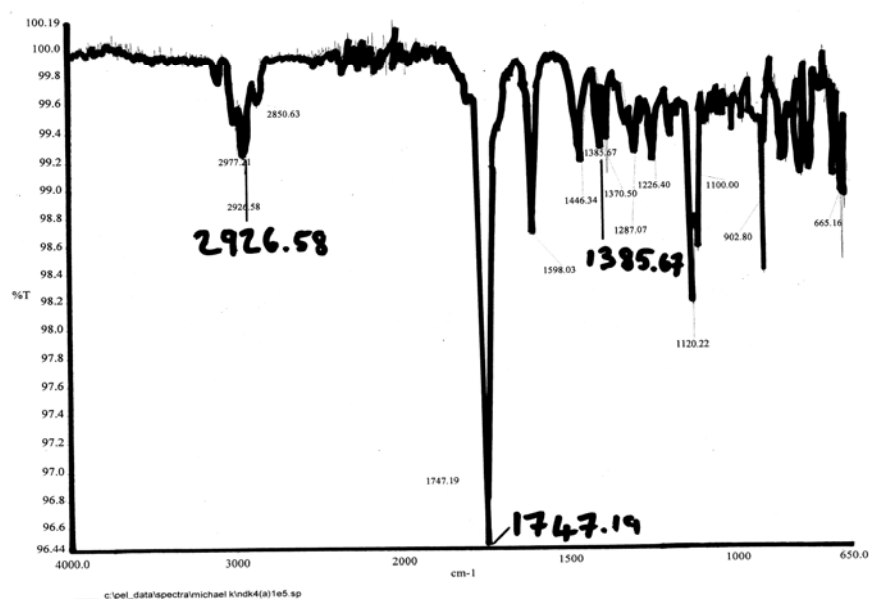


Figure 3.21 IR spectra for compound 3.6

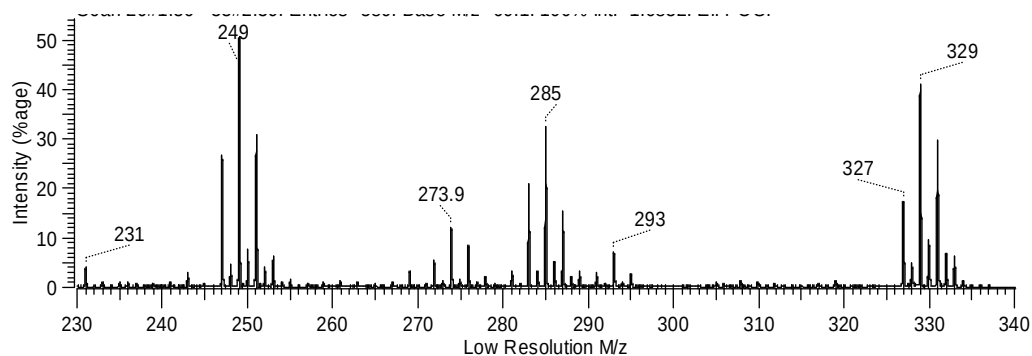
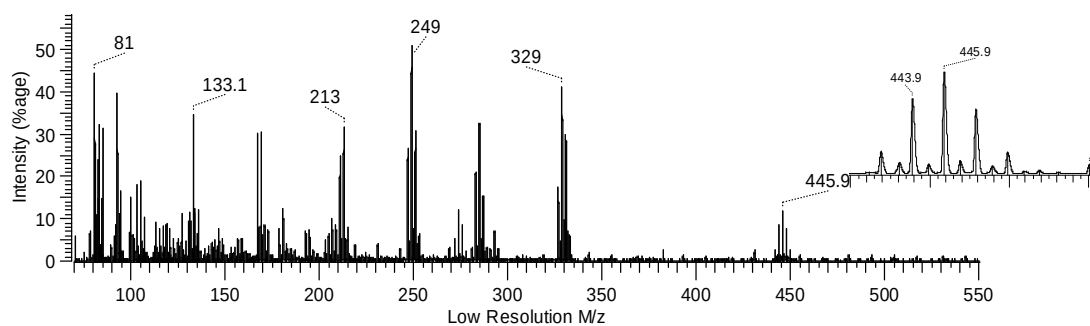
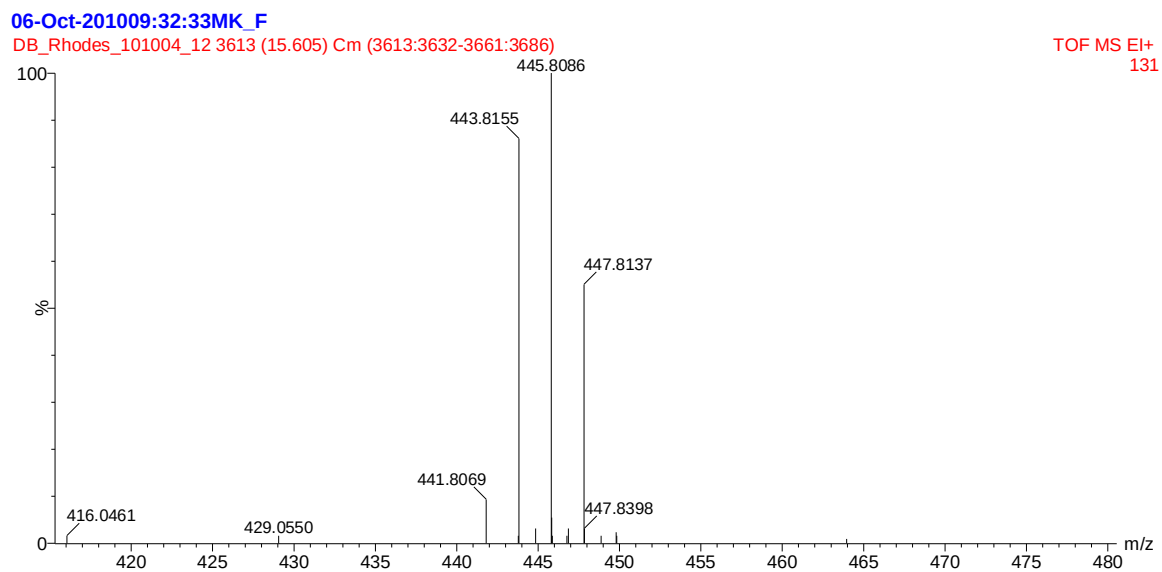
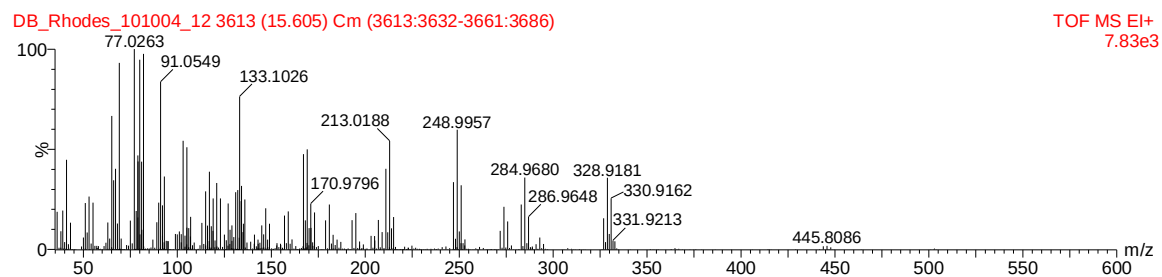
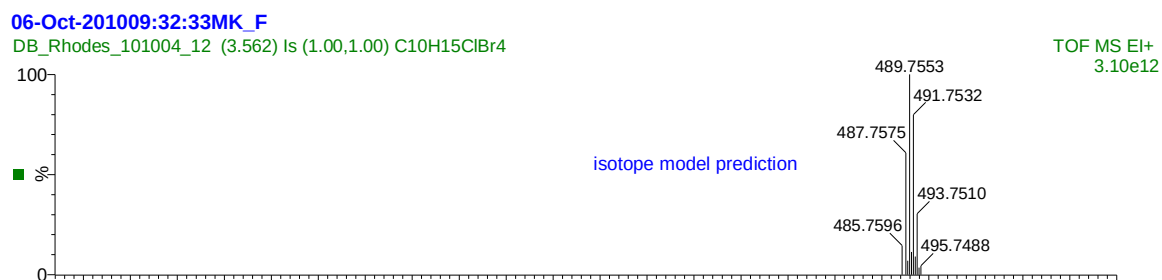
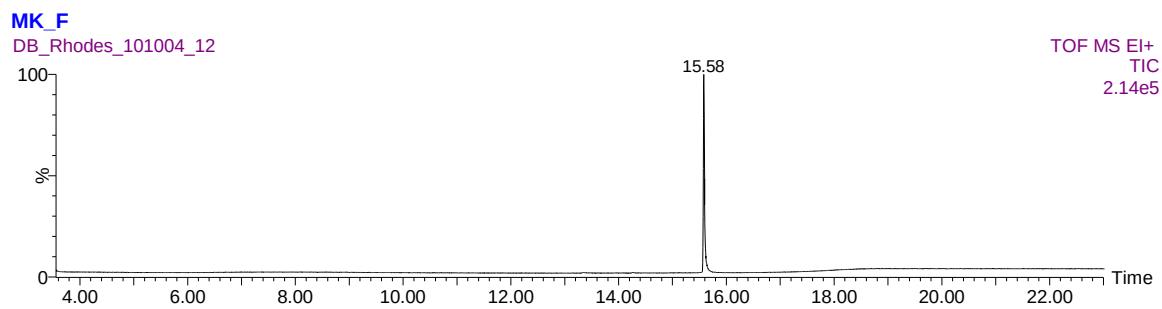
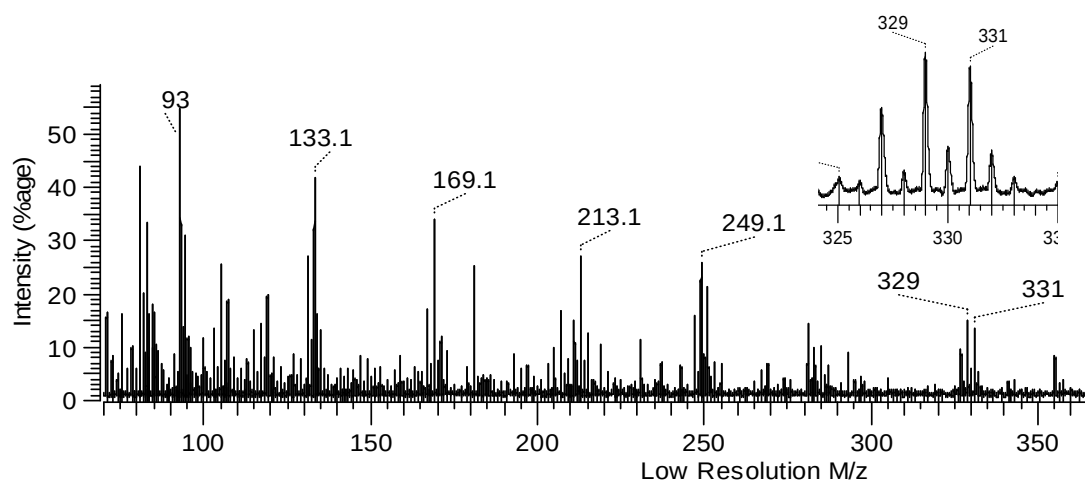


Figure 3.22 LREIMS data for compound 3.1

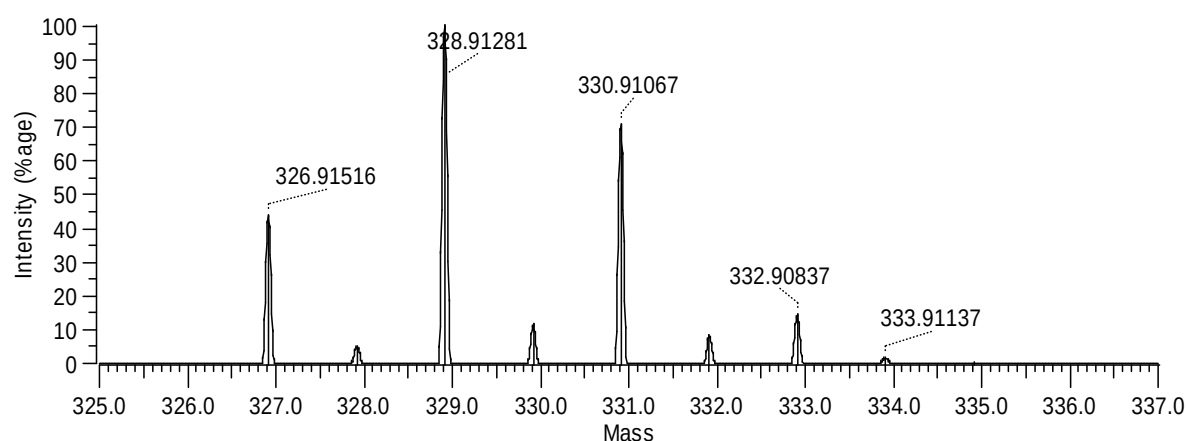
### Total Ion Chromatogram:



**Figure 3.24** HR-GCTOFMS data for compound **3.1**

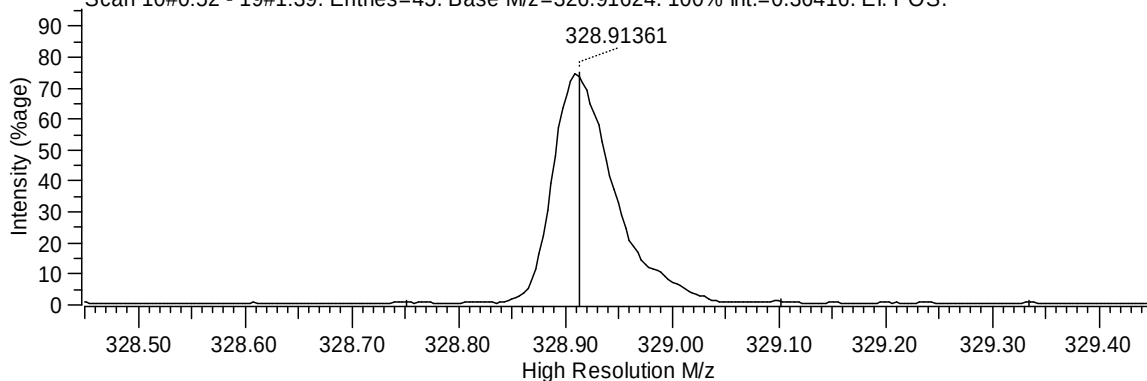


Predicted distribution:

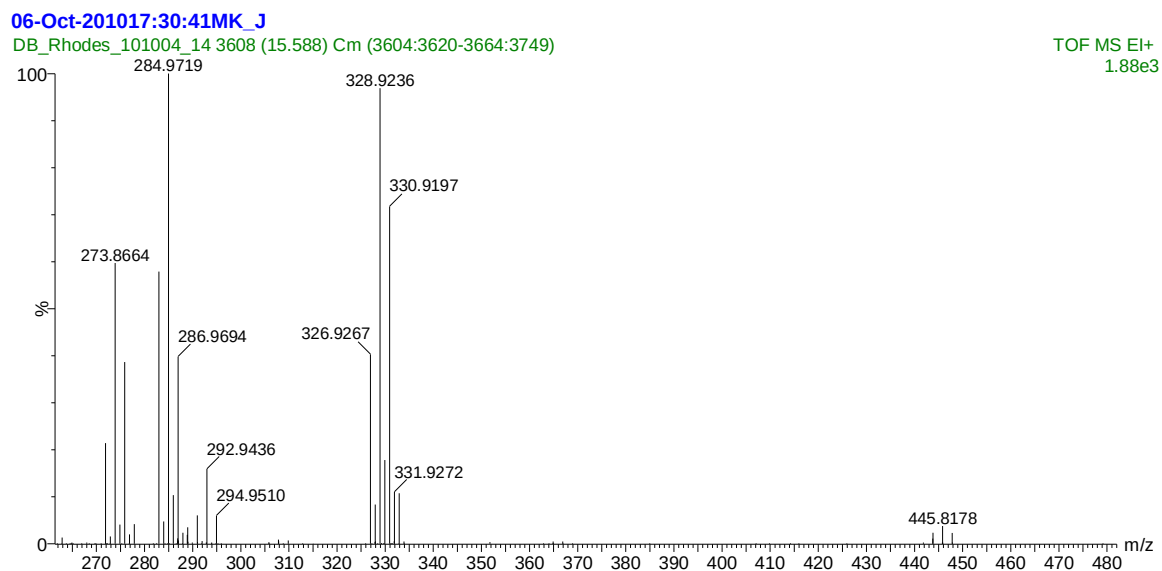
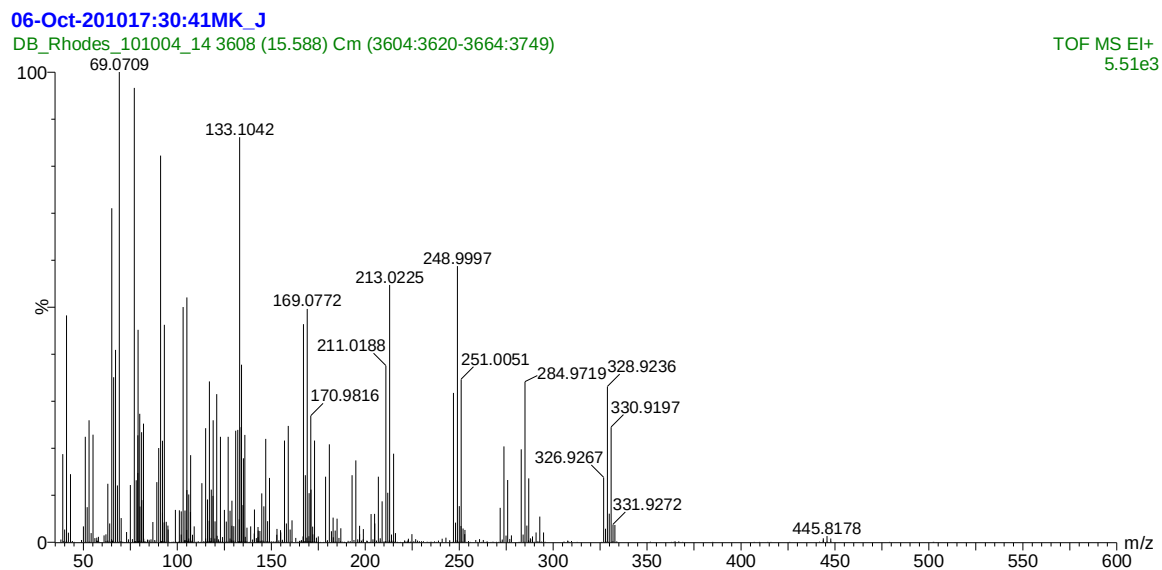
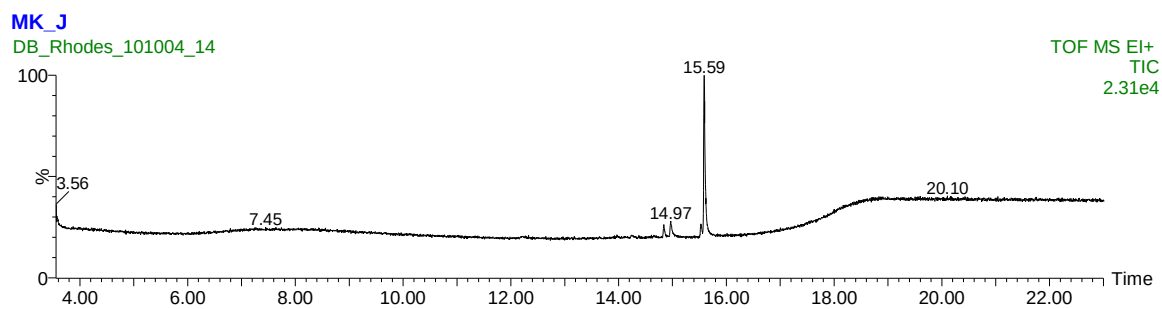


Mass measurement

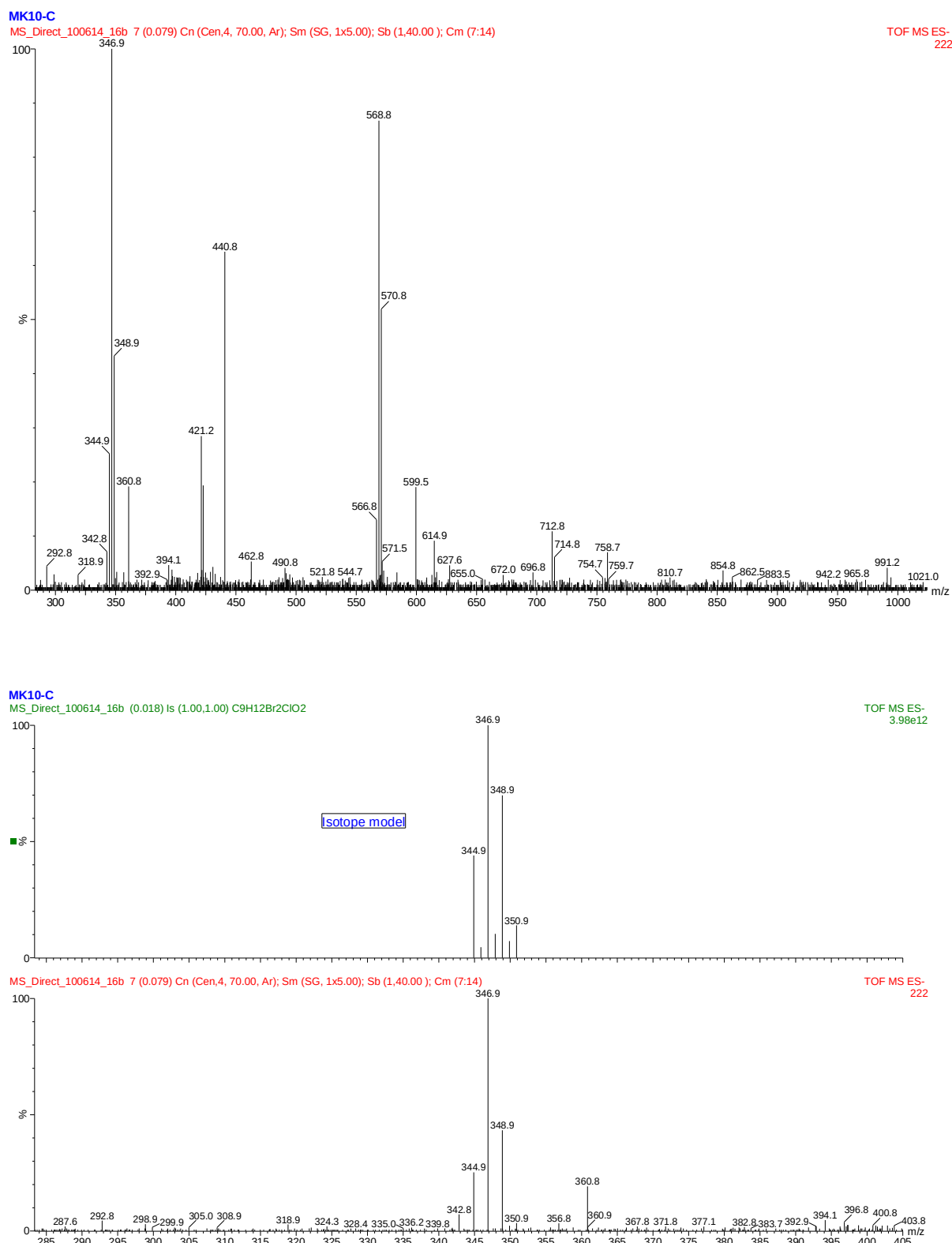
SCAN GRAPH. Flagging=High Resolution M/z. Ctd=[Thr:1000 $\mu$ V, Min.Hgt:1000 $\mu$ V, Min.Wid(Mlt):10(7), Inc:50%, Res:1%]. H Scan 10#0:52 - 19#1:39. Entries=45. Base M/z=326.91624. 100% Int.=0.36416. El. POS.



**Figure 3.23** LREIMS and HREIMS data for compound **3.2**

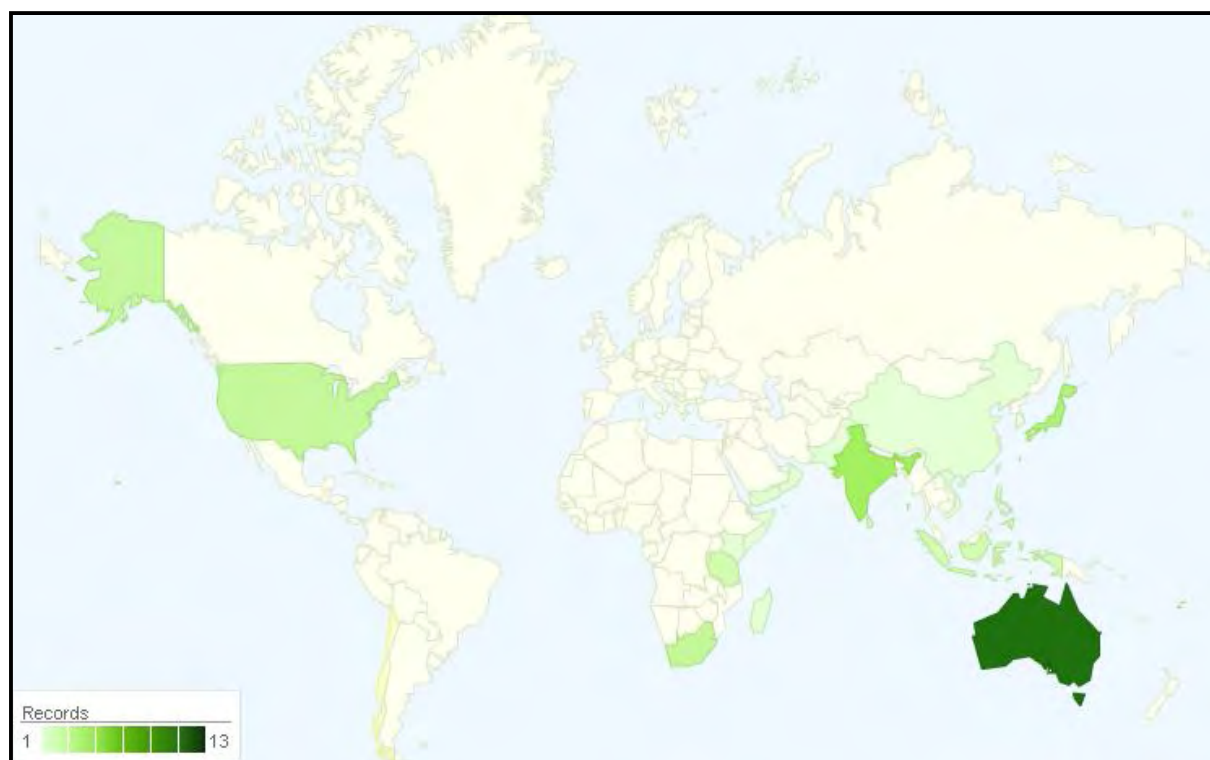


**Figure 3.25** HR-GCTOFMS data for compound 3.2



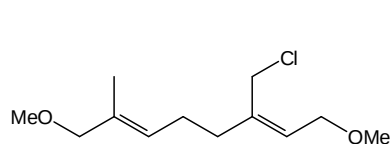
**Figure 3.26** LR-TOFMS data of portieric acid A (3.6)

### 3.8 Appendix 1

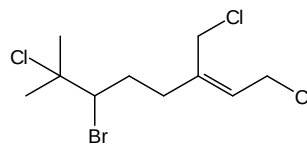


**Figure 3.27** Worldwide distribution of the number of different kinds of *P. hornemannii* found in each country (Guiry and Guiry, 2011).

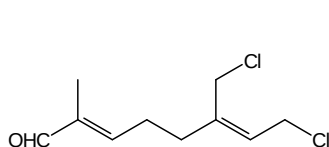
The genus *Portieria* as well as the genus *Chondrococcus* both belong to the family Rhizophyllidaceae. From the data below, it can be seen that *P. hornemannii* exhibits notable site-to-site variation in secondary metabolite production and that *P. hornemannii* contains large amounts of both acyclic and cyclic polyhalogenated monoterpenes.



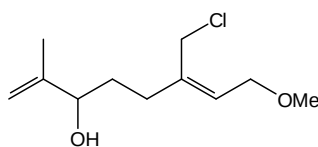
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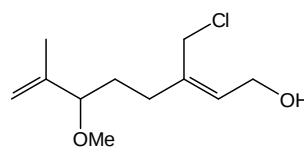
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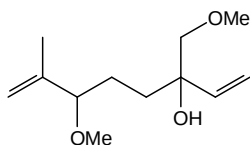
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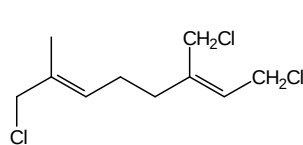
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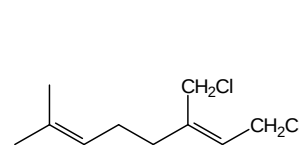
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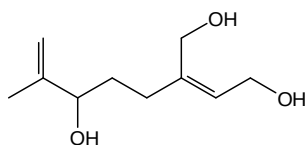
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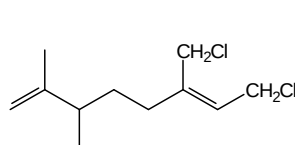
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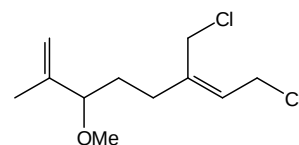
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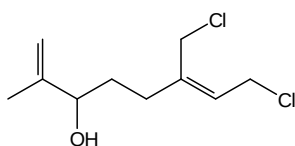
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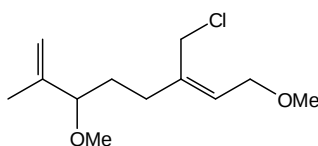
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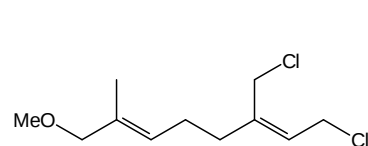
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**3.18**

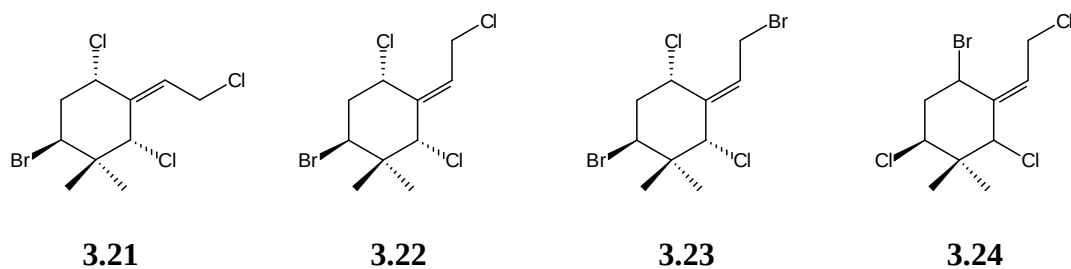


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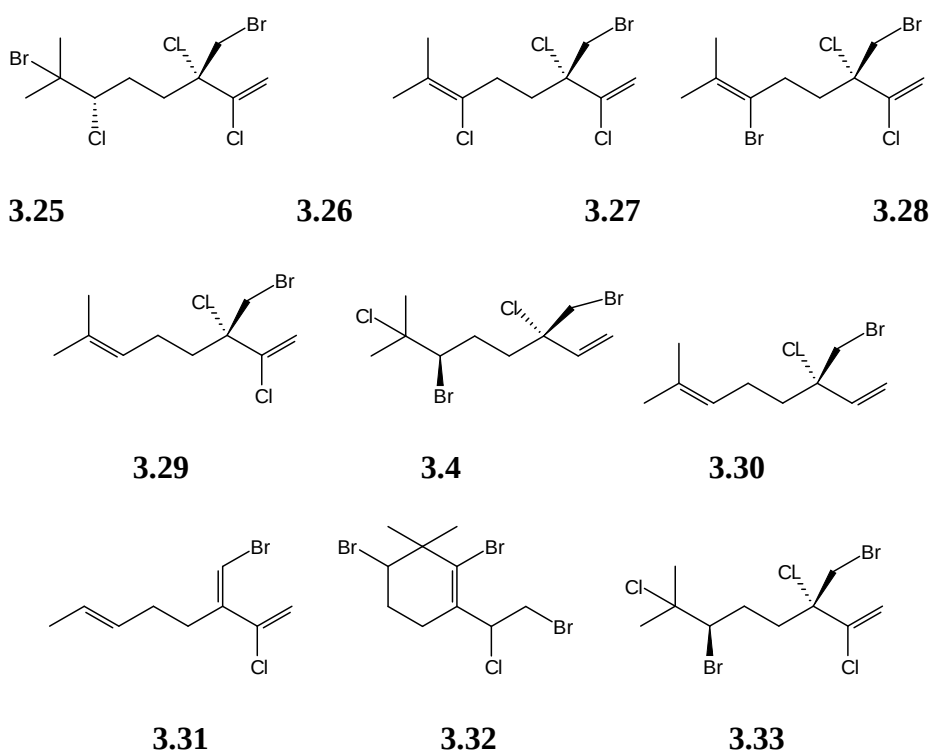


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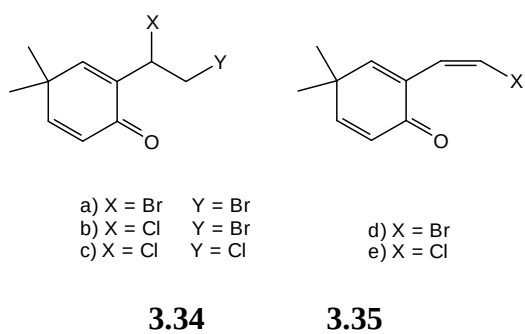
Australia (Wright *et al.*, 1991)



Guam (Gunatilaka *et al.*, 1999)

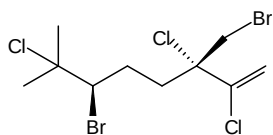


Hawaii (Fuller *et al.*, 1994)

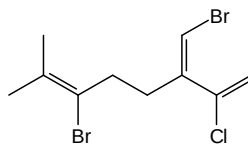


Japan (Kuniyoshi *et al.*, 2003)

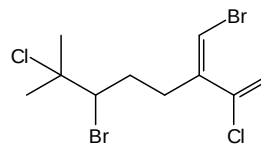




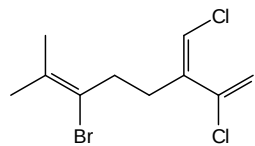
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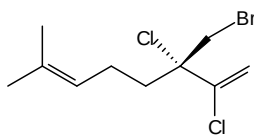
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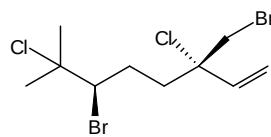
**3.5**



**3.38**

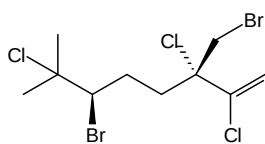


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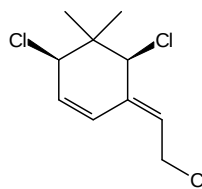


**3.40**

Madagascar (Andrianasolo *et al.*, 2006)



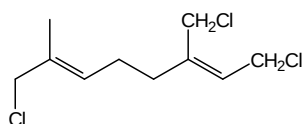
**3.41**



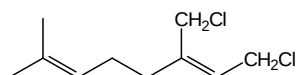
**3.42**

Philippines (Fuller *et al.*, 1992)

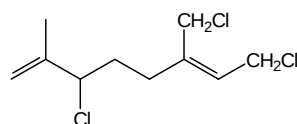
The genera *Chondrococcus* has also shown considerable structural and geographical variation with regards to its monoterpene content (Coll and Wright, 1987). *Chondrococcus hornemannii* from different locations around the world have also yielded a considerable number of halogenated monoterpenes.



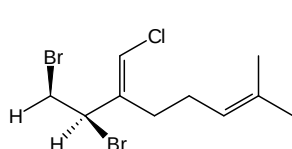
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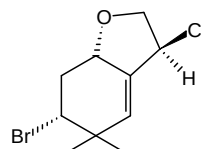
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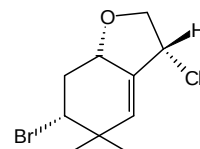
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3.46

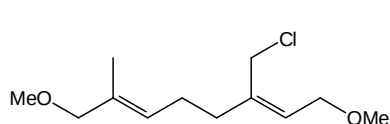


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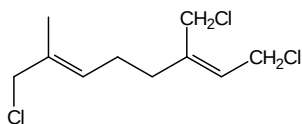


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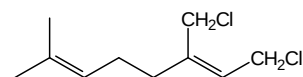
Australia (Coll and Wright, 1987)



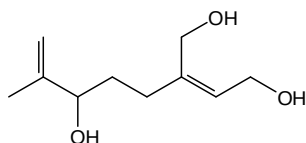
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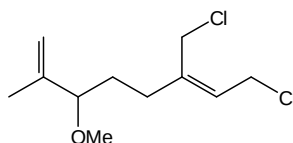
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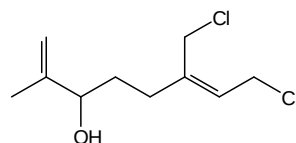
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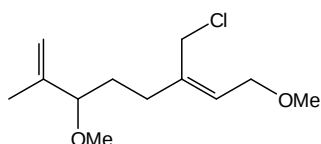
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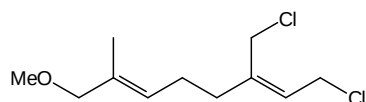
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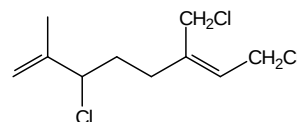
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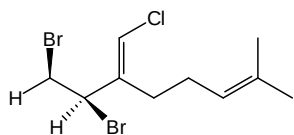
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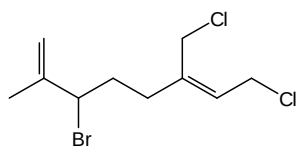
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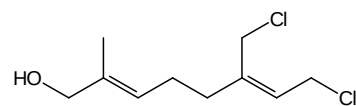
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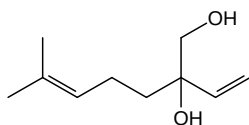
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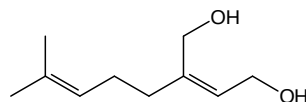
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**3.60**

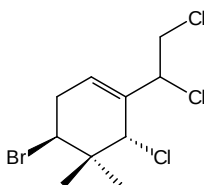


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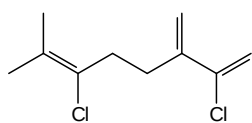
**3.62**

Australia (Coll and Wright, 1989)

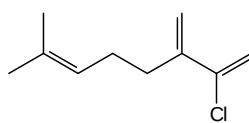


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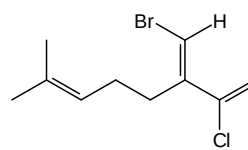
Bora Bora (Crews *et al.*, 1984)



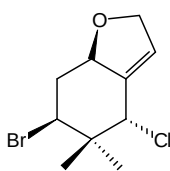
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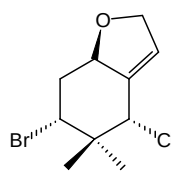
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**3.66**

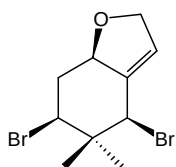


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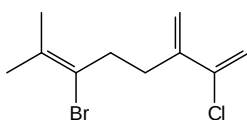


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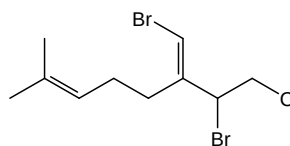
Hawaii (Burrenson *et al.*, 1975)



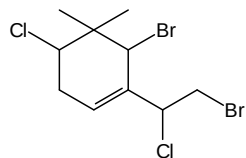
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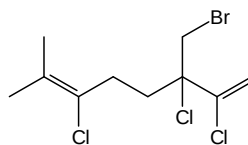
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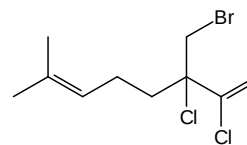
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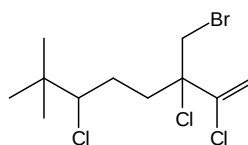
**3.72**



**3.73**

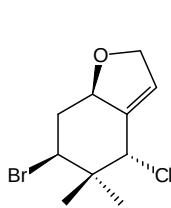


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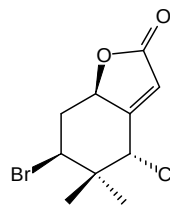


**3.75**

Hawaii (Burrenson *et al.*, 1975)

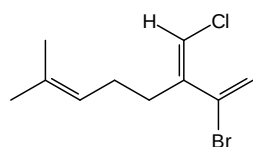


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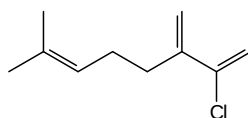


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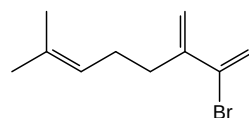
Hawaii (Woolard and Moore, 1978)



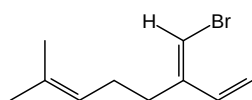
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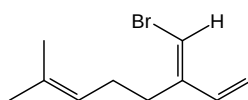
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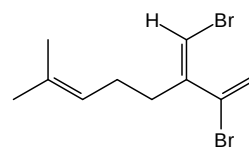
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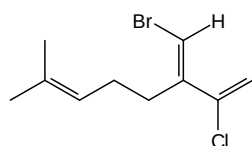
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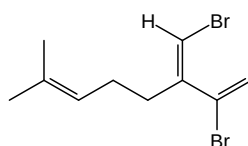
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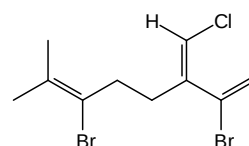
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**3.84**

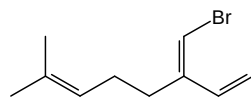


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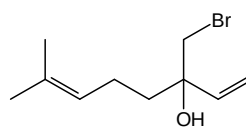


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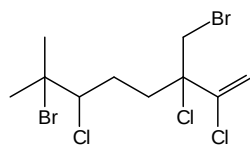
Japan (Ichikawa *et al.*, 1974)



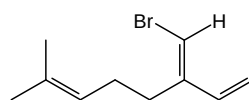
**3.87**



**3.88**



**3.89**



**3.90**

Sri Lanka (Woolard *et al.*, 1976)

## References for Appendix 1

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## Chapter Four

# The natural product chemistry of selected South African *Laurencia* species

### 4.1 Abstract

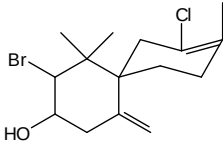
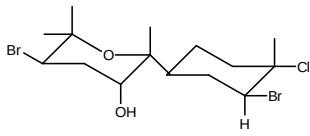
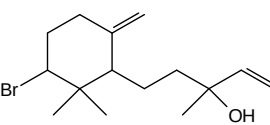
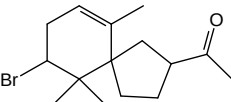
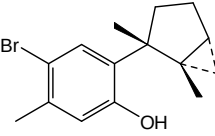
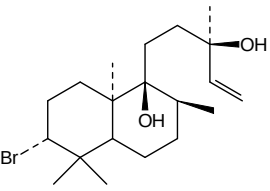
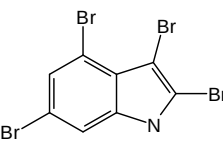
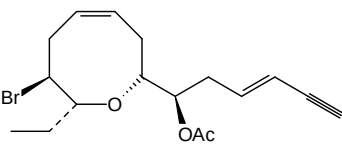
From the red algae *Laurencia glomerata* (Kützinger) two known compounds, 7-hydroxylaurene (4.9) and *cis*-neolaurencenyne (4.12), as well as a chamigrane (4.11) were isolated; while, from *Laurencia flexuosa* (Kützinger), a major known secondary metabolite called 3Z-bromofucin (4.13) was noted. Although both these *Laurencia* species had already been investigated by Elsworth and Thomson (1989) and Mann (2008) respectively, these species were re-investigated to assess the possibility of finding other secondary metabolites that previous researches might have omitted.

**Keywords:** 3Z-bromofucin; 7-hydroxylaurene; *cis*-neolaurencenyne; *Laurencia* species

### 4.2 Introduction

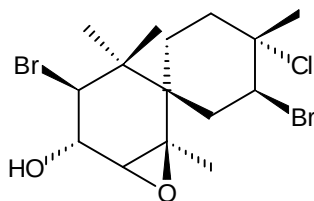
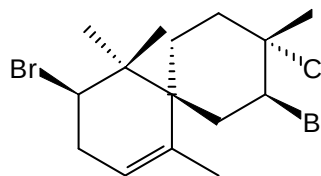
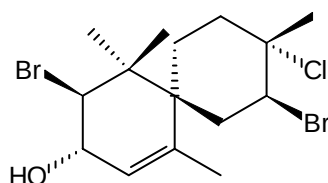
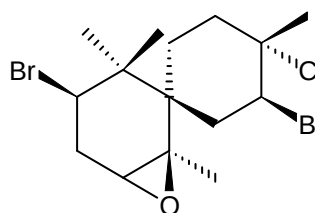
Red algae from the genus *Laurencia* (Ceramiales, Rhodomelaceae) are a well known source of biologically active secondary metabolites (Blunt *et al.*, 2011). It is believed that these compounds are produced as feeding deterrents against marine herbivores (Kladi *et al.*, 2004). These compounds vary and are predominantly chamigrenes, sesquiterpenes, diterpenes, triterpenes and C<sub>15</sub>-acetogenins, which are usually characterised by the presence of halogen atoms. Many novel structural groups of compounds are only found in this genus. A full review of all compounds isolated from *Laurencia* species would go beyond the scope of this thesis. For this reason, only the major classes of secondary metabolites isolated from *Laurencia* species have been presented below (Table 4.1).

**Table 4.1** Major classes of carbon skeletons produced by *Laurencia* species (adapted from Mann, 2008)

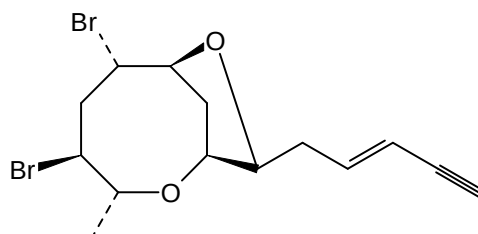
| Structural Class                   | Example                                                                             | Name           |
|------------------------------------|-------------------------------------------------------------------------------------|----------------|
| Chamigrenes                        |    | Elatol         |
| Bisabolene addition products       |    | Caespitol      |
| Bromomonocyclofarnesyl derivatives |    | Snyderol       |
|                                    |   | Spirolaurenone |
| Cuparanes                          |  | Laurinterol    |
| Diterpenes                         |  | Concinndiol    |
| Polybromoindoles                   |  | -              |
| Non-terpenoids                     |  | Laurencin      |

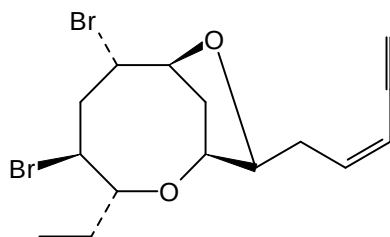


Previous investigations into *L. glomerata* from South Africa, revealed the isolation of four halogenated chamigrenes; one new epoxide (**4.1**) as well as the following metabolites (**4.2-4.4**) (Elsworth and Thomson, 1989).

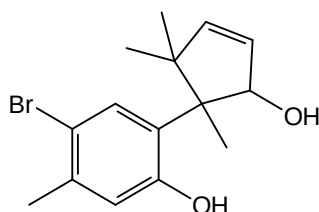
**4.1****4.2****4.3****4.4**

Four compounds (**4.5-4.8**) were previously isolated from a South African species of *L. flexuosa* (Mann, 2008). 3*E*-bromofucin (**4.5**) had previously been isolated from *L. implicata* (Coll and Wright, 1989), while its geometric isomer 3*Z*-bromofucin (**4.6**) had previously been isolated from the digestive gland of the South African sea hare, *Aplysia parvula* (McPhail and Davies-Coleman, 2005). Compound **4.8** is believed to be a degradation product of compound **4.7**. Interestingly, during this work, Mann (2008) also isolated a halogenated monoterpene typical of those found in *Plocamium* species. This compound has not been included below, as it is doubtful whether it is an authentic secondary metabolite of *Laurencia* species.

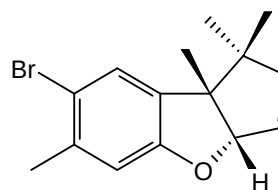
3*E*-bromofucin (**4.5**)



3Z-bromofucin (4.6)



4.7



4.8

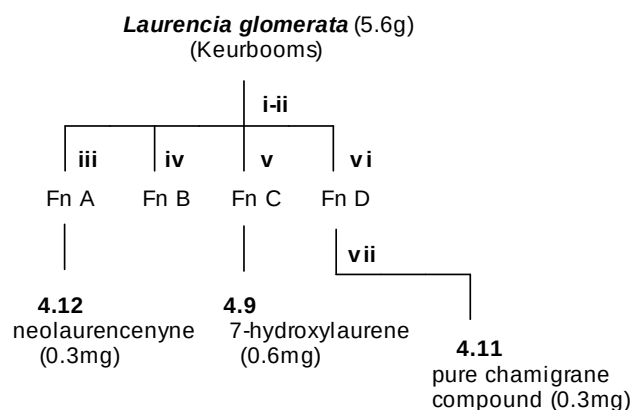
### 4.3 Chapter aims

The aim of this study was to extract new or known compounds from South African *Laurencia* species that might have been omitted by other researchers and then test these compounds for potential cytotoxic activity. Even if known compounds were re-isolated, this would still provide the opportunity to test known compounds on cell lines which have not yet been screened.

### 4.4 Results and discussion

#### 4.4.1 Collection, extraction and isolation

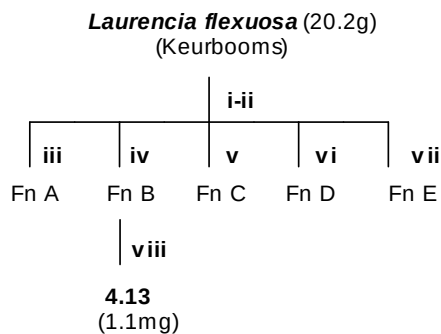
See the experimental section; paragraphs 4.5.2 and 4.5.3 for a full explanation of the collection, extraction and isolation procedure illustrated in Scheme 4.1 below.



**Scheme 4.1** Extraction and isolation of metabolites from *Laurencia glomerata* (Keurbooms, September 2010)

Conditions: i)  $\text{CH}_2\text{Cl}_2$ -MeOH (2:1) 100 ml overnight extraction ii) 7.4 mg of this extract was dissolved in hexane and chromatographed on a silica column which had been equilibrated with hexane. The silica column was eluted with hexane and increasing proportions of  $\text{CH}_2\text{Cl}_2$  iii) Fraction A was the 100% hexane extract, and re-chromatographed on another silica column using 100% hexane as the eluent. From this, 0.3 mg of *cis*-neolaurencenyne was isolated iv) Fraction B was derived from hexane: $\text{CH}_2\text{Cl}_2$  (9:1) v) Fraction C was the hexane: $\text{CH}_2\text{Cl}_2$  (8:2) fraction and was a pure compound identified as 7-hydroxylaurene vi) Fraction D came from the hexane: $\text{CH}_2\text{Cl}_2$  (1:1) eluent vii) Fraction D was re-chromatographed on another silica column and the hexane: $\text{CH}_2\text{Cl}_2$  (6:4) eluent yielded a pure chamigrane compound (0.3 mg).

See the experimental section; paragraphs 4.5.2 and 4.5.3 for a full explanation of the collection, extraction and isolation procedure illustrated in Scheme 4.2 below.



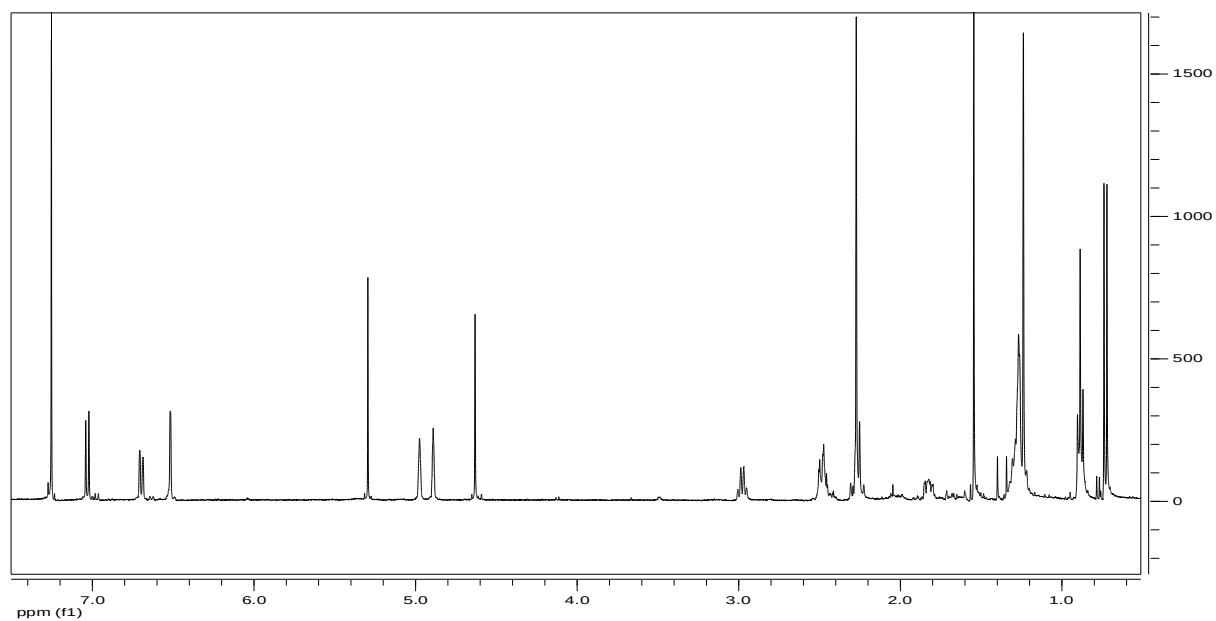
**Scheme 4.2** Extraction and isolation of metabolites from *Laurencia flexuosa* (Keurbooms, September 2010)

Conditions: i) Sample steeped in MeOH (150 ml) for 1 hour ii) Sample re-extracted with CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) and left to stand for 12 hours. Both extracts (i-ii) were then combined, dried over MgSO<sub>4</sub> then filtered and dried. Of the dried crude extract (206.5 mg), 40.2 mg was placed onto a silica column. From this, Fractions A-E were obtained iii) Fraction A was obtained from 100% hexane iv) Fraction B was obtained from hexane:EtOAc (95:5) v) Fraction C was obtained from hexane:EtOAc (9:1) vi) Fraction D was obtained from hexane:EtOAc (8:2) vii) Fraction E was obtained from hexane:EtOAc (1:1) viii) Fraction B was further purified *via* another silica column. The hexane:EtOAc (98:2) fraction was 3Z-bromofucin (1.1 mg)

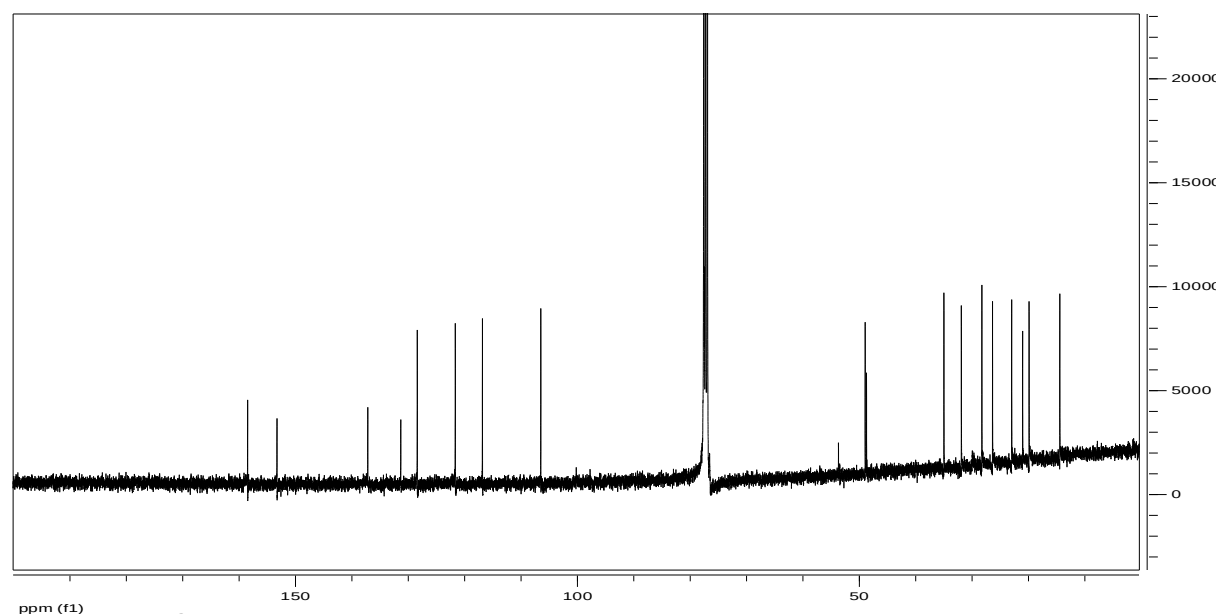
#### 4.4.2 Characterisation of metabolites from selected *Laurencia* species

The isolation and characterisation of four compounds from two different *Laurencia* species is presented below.

##### Structural elucidation of compound **4.9**



**Figure 4.1**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **4.9**

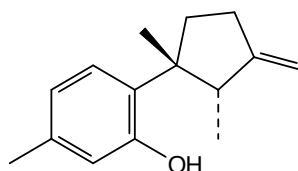
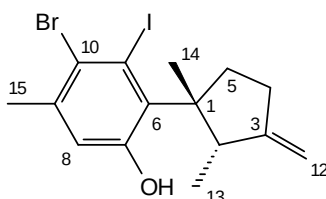


**Figure 4.2**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of compound **4.9**

Compound **4.9** was the major compound obtained from *L. glomerata*. The structure of this compound was initially determined by comparing the  $^1\text{H}$  NMR chemical shifts of 7-hydroxylaurene or 2-(1*R*,2*S*)-1,2-dimethyl-3-methylenecyclopentyl-5-methylphenol (Wratten and Faulkner, 1977). As this compound was already known, a full set of 2D data was not obtained, instead, identification was based on literature values of a similar halogenated compound, 10-bromo-7-hydroxy-11-iodolaurene (**4.10**) (Welch and Prakasa Rao, 1979) (Table 4.2). The identity of this known compound was further confirmed by examining at the  $^{13}\text{C}$  and COSY data.

An important distinguishing factor between the two compounds was that at positions 10 and 11. Coupling constants for both the one proton doublets at  $\delta$  7.27 ( $J = 8.0$  Hz) and  $\delta$  6.69 ( $J = 8.0$  Hz), as well as COSY data, confirmed that these protons were indeed vicinal, and this suggested that unlike compound **4.10**, compound **4.9** did not have bromine or iodine substituents in the six-membered ring system.

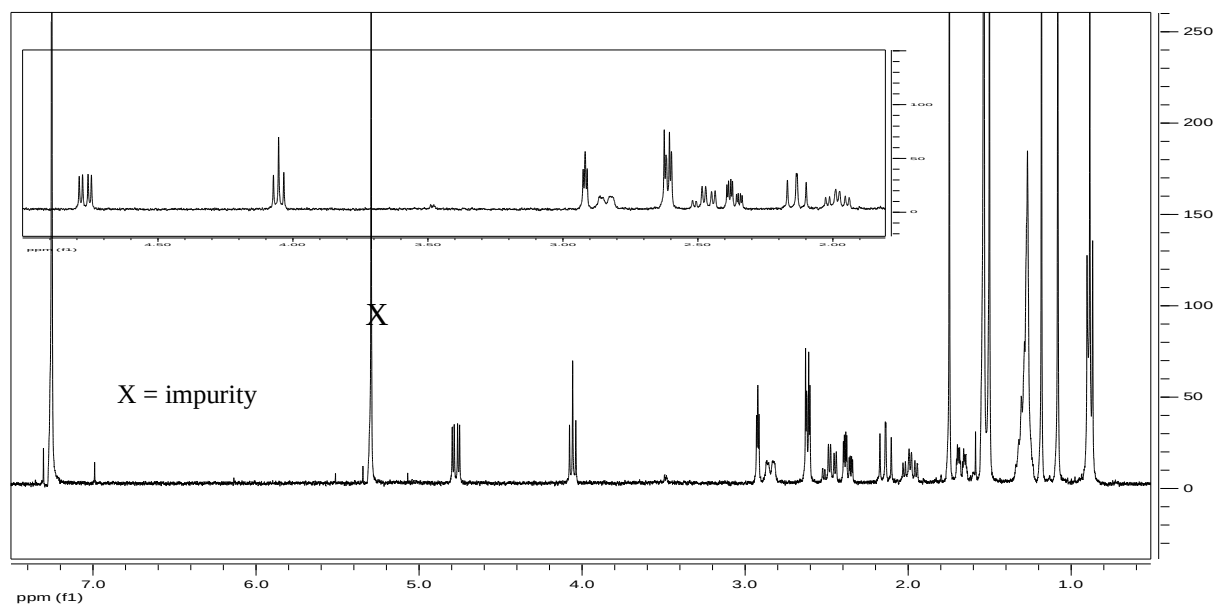
Although this compound is known, this is the first time that 7-hydroxylaurene has been isolated from *L. glomerata*. Interestingly, 7-hydroxylaurene has shown anti-microbial properties and has demonstrated the ability to inhibit the growth of *Staphylococcus aureus* at 100  $\mu\text{g}$  (Wratten and Faulkner, 1977).

**4.9****4.10**

**Table 4.2**  $^1\text{H}$  NMR (400 MHz), COSY and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) data of 7-hydroxylaurene, as well as the  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) data for compound **4.10**.

| Carbon no. | $\delta_{\text{C}}$  | $\delta_{\text{H}}$ , multi, $J$ (Hz) | $^1\text{H}$ - $^1\text{H}$ COSY | $\delta_{\text{C}}$  |
|------------|----------------------|---------------------------------------|----------------------------------|----------------------|
|            | <b>4.9</b>           | <b>4.9</b>                            | <b>4.9</b>                       | <b>4.10</b>          |
| 1          | 48.7, C              |                                       |                                  | 49.1, C              |
| 2          | 48.4, CH             | 2.98, q, 6.9                          | H-13                             | 48.2, CH             |
| 3          | 158.4, C             |                                       |                                  | 157.7, C             |
| 4          | 28.0, $\text{CH}_2$  | 2.48, m                               | H-5; H-12, lr                    | 28.0, $\text{CH}_2$  |
| 5          | 34.7, $\text{CH}_2$  | 1.90, m                               | H-4                              | 34.6, $\text{CH}_2$  |
| 6          | 137.0, C             |                                       |                                  | 135.4, C             |
| 7          | 153.1, C             |                                       |                                  | 150.7, C             |
| 8          | 116.7, CH            | 6.51, s                               |                                  | 133.1, CH            |
| 9          | 131.2, C             |                                       |                                  | 134.9, C             |
| 10         | 121.5, CH            | 6.68, d, 8.0                          | H-11; H-15, lr                   | 121.8, C             |
| 11         | 128.2, CH            | 7.02, d, 8.0                          | H-10                             | 116.5, C             |
| 12         | 106.3, $\text{CH}_2$ | 4.97, s<br>4.89, s                    | H-4                              | 106.7, $\text{CH}_2$ |
| 13         | 19.6, $\text{CH}_3$  | 0.72, d, 6.9                          | H-2                              | 19.8, $\text{CH}_3$  |
| 14         | 26.1, $\text{CH}_3$  | 1.23, s                               |                                  | 25.4, $\text{CH}_3$  |
| 15         | 20.7, $\text{CH}_3$  | 2.26, s                               | H-10, lr                         | 20.0, $\text{CH}_3$  |

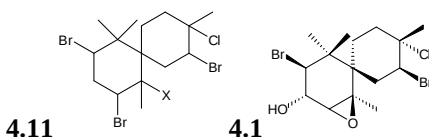
Note - Positions 10 and 11 may be interchangeable for 7-hydroxylaurene

Structural elucidation of compound **4.11**

**Figure 4.3**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **4.11**

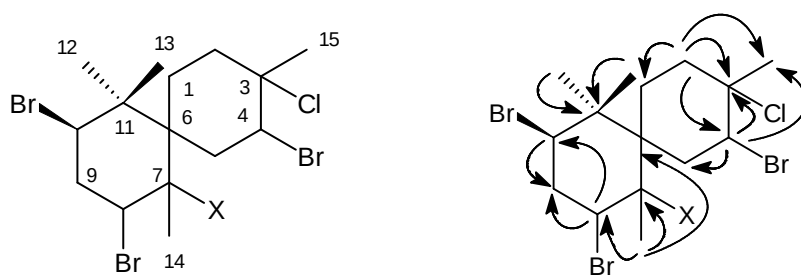
A chamigrane derivative (**4.11**) was isolated as a minor compound from *L. glomerata*. The nature of compound **4.11** was determined by examining the  $^1\text{H}$  NMR shifts of related compounds isolated from Elsworth and Thomson (1989) as well as Howard and Fenical (1975) (Table 4.3; Table 4.4), and well as taking note of the corresponding 2D NMR data of compound **4.11**.



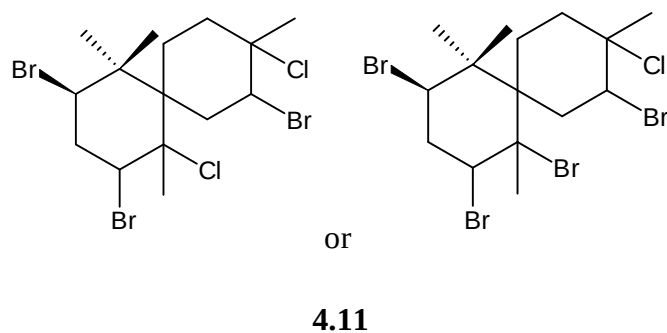
**Table 4.3**  $^{13}\text{C}$  NMR (100 MHz) data for compound **4.11** as well as compound **4.1**


| C. no. | $\delta_{\text{C}}$   | $\delta_{\text{C}}$   |
|--------|-----------------------|-----------------------|
| 1      | 25.9, CH <sub>2</sub> | 25.3, CH <sub>2</sub> |
| 2      | 40.5, CH <sub>2</sub> | 38.8, CH <sub>2</sub> |
| 3      | 70.7, C               | 70.7, C               |
| 4      | 62.2, CH              | 61.83, CH             |
| 5      | 39.2, CH <sub>2</sub> | 39.9, CH <sub>2</sub> |
| 6      | 44.5, C               | 44.5, C               |
| 7      | 71.0, C               | 64.4, C               |
| 8      | 57.2, CH              | 65.1, CH              |
| 9      | 32.7, CH <sub>2</sub> | 70.6, CH              |
| 10     | 61.8, CH              | 69.6, CH              |
| 11     | 42.8, C               | 42.8, C               |
| 12     | 22.2, CH <sub>3</sub> | 20.1, CH <sub>3</sub> |
| 13     | 31.8, CH <sub>3</sub> | 28.3, CH <sub>3</sub> |
| 14     | 25.5, CH <sub>3</sub> | 25.4, CH <sub>3</sub> |
| 15     | 23.7, CH <sub>3</sub> | 23.9, CH <sub>3</sub> |

Based on literature values and 2D data (Table 4.3, Table 4.4) at positions 7, 8 and 9, key differences were noted between compound **4.1** and compound **4.11**. The triplet at  $\delta$  4.06 (H-8) of compound **4.11** had an HSQC carbon correlation of  $\delta$  57.2 (C-8), based on the carbon shift, a Br moiety at C-8 was suggested, as a Cl moiety would have had a corresponding carbon shift of approximately  $\delta$  65.0 (Mann, 2008). The triplet at  $\delta$  4.06 (H-8) also had a strong COSY correlation to the two proton double doublet at  $\delta$  2.59 (H-9). Both COSY and HMBC correlations confirmed that the proton triplet at  $\delta$  4.06 was placed at position H-8.

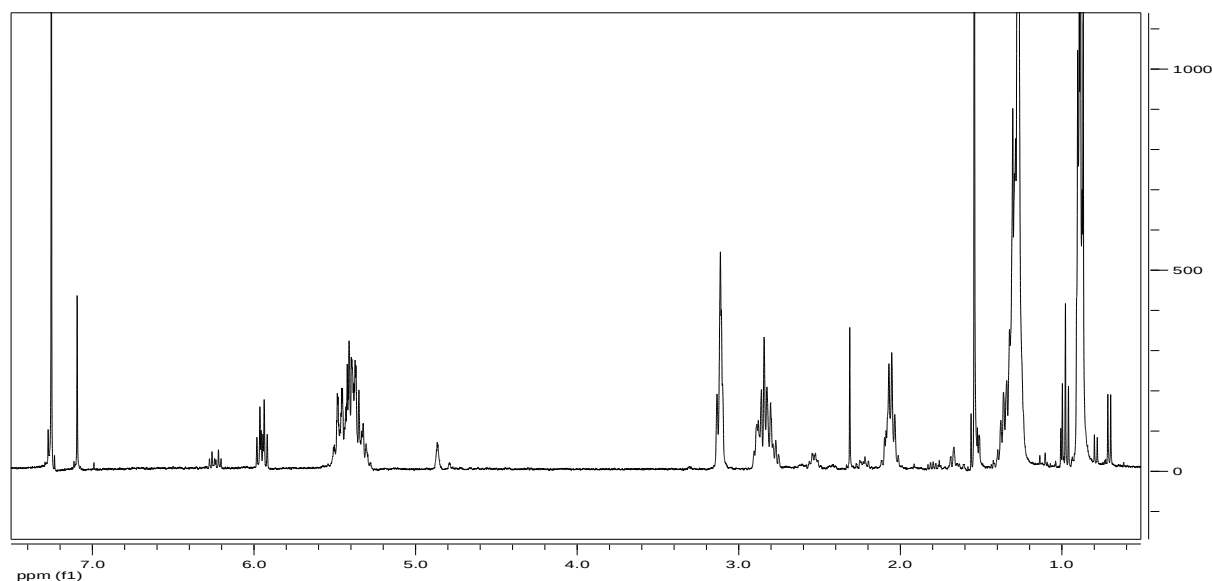
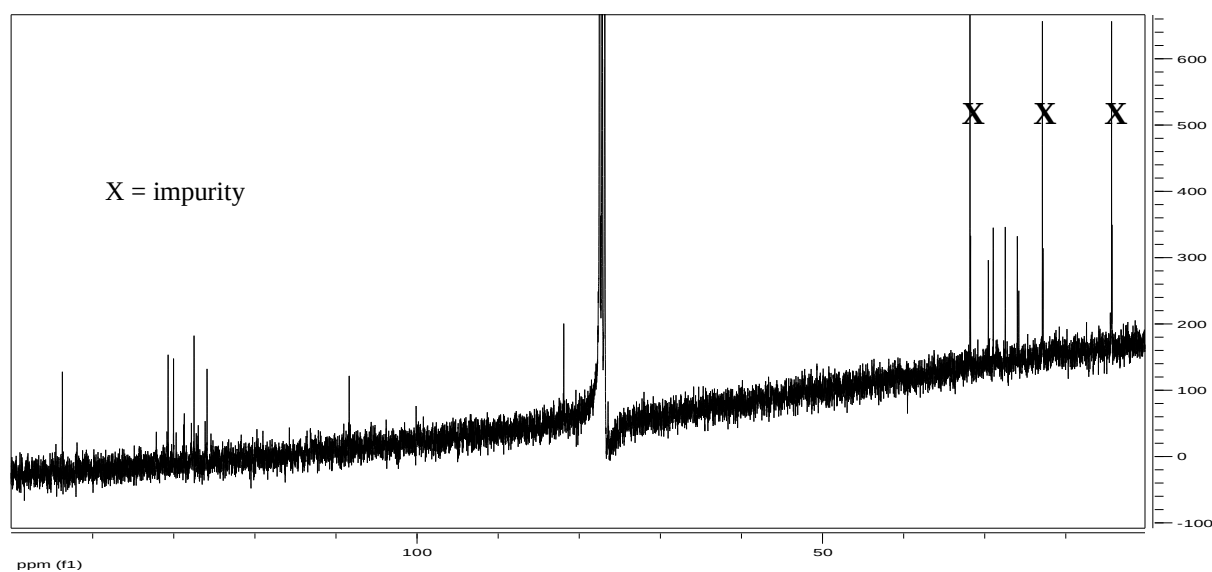
**Figure 4.4** Numbering and selected HMBC for compound **4.11**

The quaternary carbon at position 7 had one unknown substituent which was most likely a Cl or Br or an OH group. IR data lacked evidence to suggest that an OH group was present. Due to the MS experiments being unsuccessful, further structural elucidation was not possible. An alternative method of acquiring the MS spectrum using LC-MS could perhaps be used in the future, as this compound might not elute from a GC column. Compound **4.11** is most likely one of the following previously unreported compounds below.

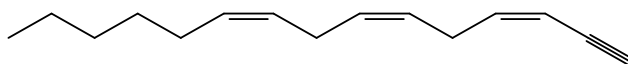


**Table 4.4**  $^1\text{H}$  NMR (400 MHz),  $^{13}\text{C}$  NMR (100 MHz), COSY and HMBC data for compound **4.11**, as well as data to corresponding proton shifts for compound **4.4**

| Carbon no. | $\delta_{\text{C}}$ | $\delta_{\text{H}}$ , multi, $J$ (Hz)                   | $^1\text{H}$ - $^1\text{H}$ COSY | HMBC                     | $\delta_{\text{H}}$ , multi, $J$ (Hz) |
|------------|---------------------|---------------------------------------------------------|----------------------------------|--------------------------|---------------------------------------|
|            | <b>4.11</b>         | <b>4.11</b>                                             | <b>4.11</b>                      | <b>4.11</b>              | <b>4.4</b>                            |
| 1          | 25.9, $\text{CH}_2$ | H-1eq 1.95, ddd, 5.68, 5.81, 5.94<br>H-1ax 1.65, m      | H-2                              |                          | 1.70-2.75, $m$ (8H)                   |
| 2          | 40.5, $\text{CH}_2$ | H-2eq 2.44, dd, 5.9, 14.4<br>H-2ax 2.34, dd, 2.53, 5.94 | H-1                              | C-1, C-3, C-4, C-6, C-15 | 1.70-2.75, $m$ (8H)                   |
| 3          | 70.7, C             |                                                         |                                  |                          |                                       |
| 4          | 62.2, CH            | 4.76, dd, 5.3, 13.2                                     | H-5                              | C-3, C-5, C-15           | 4.77, dd, 12.5                        |
| 5          | 39.2, $\text{CH}_2$ | H-5eq 2.83, m<br>H-5ax 2.09, t, 13.9                    | H-4                              | C-4, C-15                | 1.70-2.75, $m$ (8H)                   |
| 6          | 44.5, C             |                                                         |                                  |                          |                                       |
| 7          | 71.0, C             |                                                         |                                  |                          |                                       |
| 8          | 57.2, CH            | 4.06, t, 7.96                                           | H-9                              | C-9, C-10                | 4.05, dd, 7.7                         |
| 9          | 32.7, $\text{CH}_2$ | 2.59, dd, 3.03, 7.60                                    | H-8, H-10                        |                          | 1.70-2.75, $m$ (8H)                   |
| 10         | 61.8, CH            | 2.91, t, 3.03                                           | H-9                              | C-9                      | 2.93, dd, 3.3                         |
| 11         | 42.8, C             |                                                         |                                  |                          |                                       |
| 12         | 22.2, $\text{CH}_3$ | 1.24                                                    |                                  | C-11                     | 1.16, s                               |
| 13         | 31.8, $\text{CH}_3$ | 1.23                                                    |                                  | C-11                     | 1.04, s                               |
| 14         | 25.5, $\text{CH}_3$ | 1.50, s                                                 | H-8, lr                          | C-6, C-7, C-8            | 1.48, s                               |
| 15         | 23.7, $\text{CH}_3$ | 1.74, s                                                 | H-2, lr                          | C-2, C-3, C-4            | 1.73, s                               |

Structural elucidation of compound **4.12****Figure 4.5**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **4.12****Figure 4.6**  $^{13}\text{C}$  ( $\text{CDCl}_3$ , 100 MHz) NMR spectra of compound **4.12**

Neolaurencenyne was originally isolated from *Laurencia okamurai* (Kigoshi *et al.*, 1981), and was re-isolated on this occasion from *L. glomerata*. It was identified by direct comparison of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data (Figure 4.5; Figure 4.6) with that of published literature values (Kigoshi *et al.*, 1981) (Table 4.5).



## 4.12

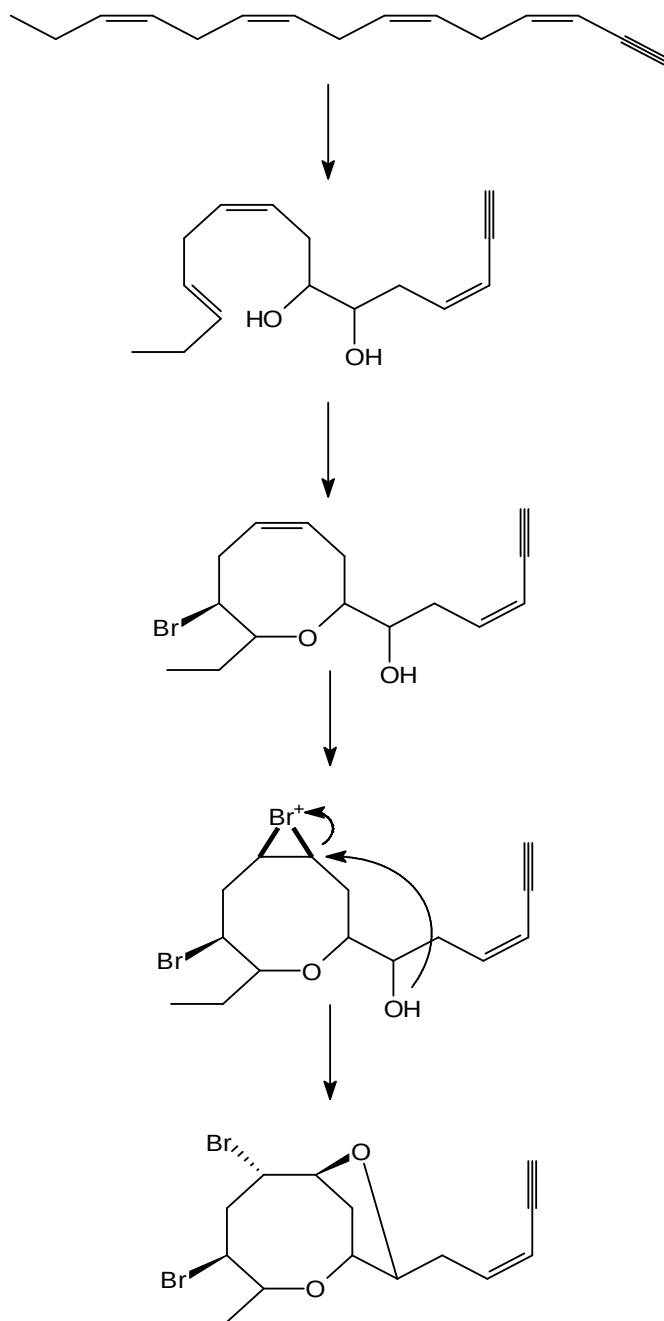
**Table 4.5**  $^1\text{H}$  NMR (400 MHz) and  $^{13}\text{C}$  NMR (100 MHz) data (in  $\text{CDCl}_3$ ) comparing *cis*-neolaurencenyne from *L. glomerata*, with the results obtained by Kigoshi (1981) from *L. okamurai*

| Carbon no. | $\delta_{\text{H}}$ , multi, <i>J</i> (Hz)<br>Kigoshi (1981) | $\delta_{\text{C}}$<br>Kigoshi (1981) | $\delta_{\text{H}}$ , multi, <i>J</i> (Hz) | $\delta_{\text{C}}$ |
|------------|--------------------------------------------------------------|---------------------------------------|--------------------------------------------|---------------------|
| 1          | 3.11, d, 2.0                                                 | 81.7, CH                              | 3.12, m                                    | 81.7, CH            |
| 2          |                                                              | 80.2, C                               |                                            | 80.4, C             |
| 3          | 5.2-5.6, m                                                   | 108.1, CH                             | 5.2-5.6, m                                 | 108.2, CH           |
| 4          | 5.97, dt, 11.7                                               | 143.5, CH                             | 5.95, dt, 11.8                             | 143.6, CH           |
| 5          | 3.13, br. dd, 7.0                                            | 25.7, $\text{CH}_2$                   | 3.15, br. dd, 7.9                          | 25.7, $\text{CH}_2$ |
| 6          | 5.2-5.6, m                                                   | 125.7, CH                             | 5.2-5.6, m                                 | 125.8, CH           |
| 7          | 5.2-5.6, m                                                   | 127.3, CH                             | 5.2-5.6, m                                 | 127.4, CH           |
| 8          | 2.83, m                                                      | 27.2, $\text{CH}_2$                   | 2.82, m                                    | 27.2, $\text{CH}_2$ |
| 9          | 5.2-5.6, m                                                   | 129.8, CH                             | 5.2-5.6, m                                 | 129.9, CH           |
| 10         | 5.2-5.6, m                                                   | 130.5, CH                             | 5.2-5.6, m                                 | 130.6, CH           |
| 11         | 2.07, m                                                      | 28.7, $\text{CH}_2$                   | 2.06, m                                    | 28.7, $\text{CH}_2$ |
| 12         | 1.2-1.5, m                                                   | 29.3, $\text{CH}_2$                   | 1.2-1.5, m                                 | 29.3, $\text{CH}_2$ |
| 13         | 1.2-1.5, m                                                   | 31.5, $\text{CH}_2$                   | 1.2-1.5, m                                 | 31.6, $\text{CH}_2$ |
| 14         | 1.2-1.5, m                                                   | 22.5, $\text{CH}_2$                   | 1.2-1.5, m                                 | 22.7, $\text{CH}_2$ |
| 15         | 0.9, br. t, 6.0                                              | 14.0, $\text{CH}_3$                   | 0.9, br. t, 6.2                            | 14.1, $\text{CH}_3$ |

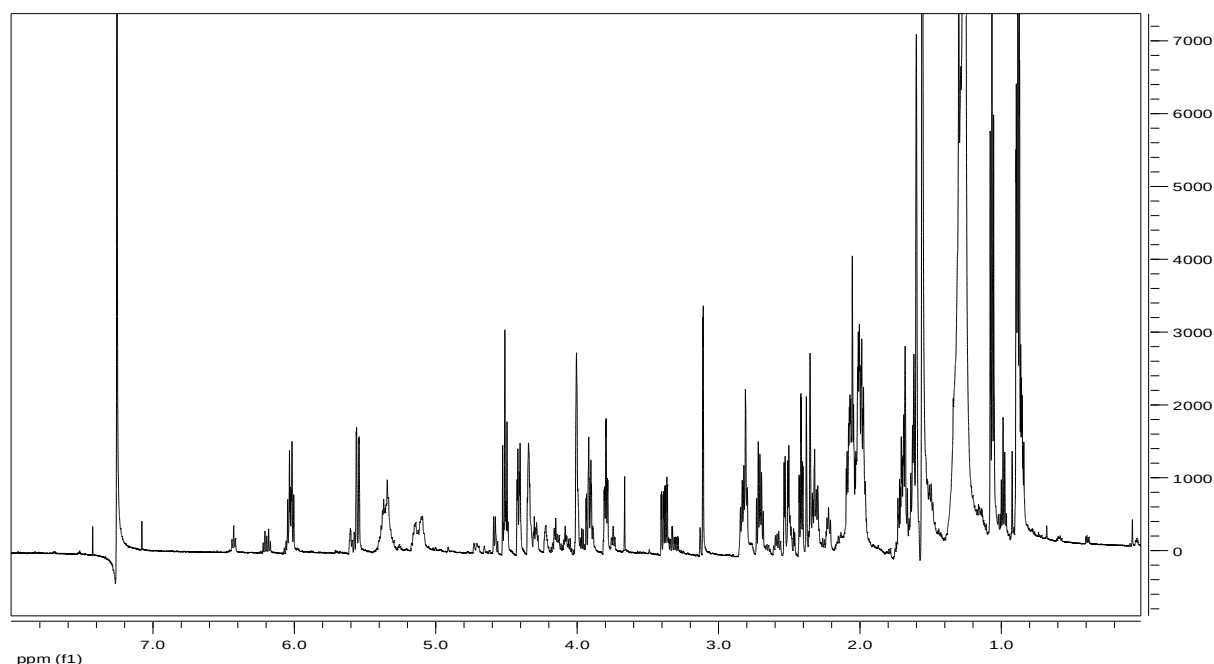
Note – Carbons shifts at position 6, 7, 9 and 10 for the CH groups maybe interchangeable; as well as at positions 5, 8, 11 and at 12, 13, 14 for the  $\text{CH}_2$  groups respectively.

Typically, for the *cis* geometry, C-2 has a shift of approximately  $\delta$  80.2 (Kigoshi *et al.*, 1981). For *trans* geometry, C-2 has a shift of approximately  $\delta$  76.0 (Kigoshi *et al.*, 1982). It also should be noted that the geometry of the  $\Delta^{3,4}$  double bond could also be determined by examining the coupling constants of the proton at the  $\Delta^{3,4}$  double bond. Compound **4.12** was identified as *cis*-neolaurencenyne or 3*Z*,6*Z*,9*Z*-pentadeca-3,6,9-trien-1-yne.

Neolaurencenyne is related to laurencenyne, the only difference is that laurencenyne has an extra double bond at positions C-12 and C-13. It is believed that laurencenyne is the biosynthetic precursor to C<sub>15</sub> halogenated cyclic ethers such as bromofucin below (Kigoshi *et al.*, 1981, Kigoshi *et al.*, 1982 ) (Scheme 4.3). Interestingly, laurencenyne was recently isolated from a South African sample of *Laurencia natalensis* (Fakee, 2011).

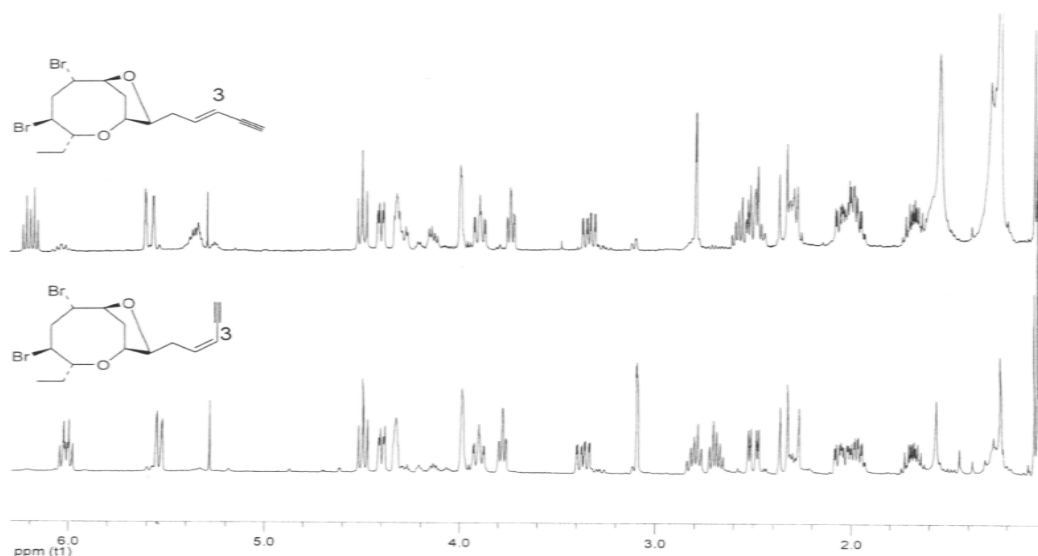


**Scheme 4.3** Proposed mechanism for the synthesis of 3Z-bromofucin from laurencenyne

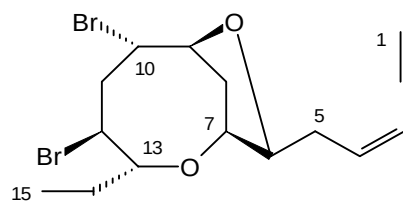
Structural elucidation of compound **4.13**

**Figure 4.7**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of compound **4.13**

This complex  $^1\text{H}$  NMR spectrum (Figure 4.7) of the *L. flexuosa* crude extract indicated the presence of 3Z-bromofucin (Table 4.6). Although the NMR spectrum was not pure, it was identified by direct comparison of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra which had previously been published (Figure 4.8) (Mann, 2008).



**Figure 4.8**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of bromofucin (adapted from Mann, 2008)



## 4.13

**Table 4.6**  $^1\text{H}$  NMR (400 MHz), and  $^{13}\text{C}$  NMR data comparing the NMR data of 3Z-bromofucin that was recently isolated, with the results obtained by Mann (2008)

| Carbon no. | $\delta_{\text{C}}$<br>4.13 | $\delta_{\text{C}}$<br>Mann, 2008 | $\delta_{\text{H}}$<br>4.13                      | $\delta_{\text{H}}$<br>Mann, 2008                |
|------------|-----------------------------|-----------------------------------|--------------------------------------------------|--------------------------------------------------|
| 1          | 82.2, CH                    | 82.0, CH                          | 3.10, d, 2.1                                     | 3.10, d, 2.1                                     |
| 2          | 80.1, C                     | 80.0, C                           |                                                  |                                                  |
| 3          | 110.4, CH                   | 110.4, CH                         | 5.54, d, 10.8                                    | 5.54, d, 10.9                                    |
| 4          | 141.2, CH                   | 141.1, CH                         | 6.02, dd, 7.4, 10.9                              | 6.02, dd, 7.5, 10.8                              |
| 5          | 30.1, $\text{CH}_2$         | 30.1, $\text{CH}_2$               | 2.70, m<br>2.80, m                               | 2.70, m<br>2.81, m                               |
| 6          | 84.2, CH                    | 84.3, CH                          | 3.79, dt, 1.7, 14.3                              | 3.79, dt, 1.7, 15.9                              |
| 7          | 70.1, CH                    | 70.0, CH                          | 3.98, br s                                       | 3.99, br s                                       |
| 8          | 33.5, $\text{CH}_2$         | 33.6, $\text{CH}_2$               | 2.06, m<br>2.35, d, 15.6                         | 2.06, m<br>2.35, d, 15.6                         |
| 9          | 79.7, CH                    | 79.6, CH                          | 4.41, dd, 3.5, 9.9                               | 4.40, dd, 3.4, 9.6                               |
| 10         | 55.3, CH                    | 55.3, CH                          | 4.33, m                                          | 4.33, m                                          |
| 11         | 38.6, $\text{CH}_2$         | 38.5, $\text{CH}_2$               | 2.55, dd, 4.5, 16.8<br>3.38, ddd, 2.4, 8.8, 15.8 | 2.51, dd, 4.7, 15.8<br>3.37, ddd, 2.2, 8.9, 15.8 |
| 12         | 53.1, CH                    | 53.0, CH                          | 4.51, t, 9.2                                     | 4.50, t, 9.2                                     |
| 13         | 82.8, CH                    | 82.7, CH                          | 3.90, dt, 2.8, 11.6                              | 3.90, dt, 2.6, 11.8                              |
| 14         | 23.0, $\text{CH}_2$         | 23.0, $\text{CH}_2$               | 1.60, m<br>1.95, m                               | 1.65, m<br>1.99, m                               |
| 15         | 11.8, $\text{CH}_3$         | 11.8, $\text{CH}_3$               | 1.06, t, 7.3                                     | 1.06, t, 7.4                                     |



### Bioactivity

Unfortunately, the mass of the *Laurencia* compounds that were extracted was not sufficient to carry out cytotoxic bioassays. These compounds need to be re-extracted and isolated in larger quantities before they can be accurately tested for cytotoxic activity.

To re-isolate these compounds and test them for cytotoxic activity, another collection had to be undertaken to find exactly the same species and type of *Laurencia* which had been originally worked on. This raised a number of further questions as the taxonomy of *Laurencia* species is very complex and has undergone several changes based on the use of new vegetative morpho-anatomical and reproductive features, cladistic analyses of morphological characters and molecular approaches based on the plastidial *rbcL* gene (Fujii *et al.*, 2011). Many morphologically similar, but chemically distinct populations have been found in the red alga *Laurencia*, for example *Laurencia nipponica* (Masuda *et al.*, 1997). A species of *Laurencia* which produces diverse secondary metabolites may include several different species or varieties (Fenical and Norris, 1975) and their taxonomic status requires re-examination at the morphological level (Masuda *et al.*, 1997). When a species of *Laurencia* produces diverse secondary metabolites, three explanations are possible; a) Sibling species that are morphologically indistinguishable may be present. b) The wrong species might have been identified. c) Or the species includes a different entity that has not yet been described or is poorly known. Something which is morphologically similar to, but distinct from the identified species (Masuda *et al.*, 1997).

From the discussion, it became clear that there was a need to be able to accurately and rapidly identify *Laurencia* species.

Interestingly, the compounds isolated from *Laurencia glomerata* were not the same as those previously isolated by Elsworth and Thomson (1989), while 3Z-bromofucin was once again re-isolated from *Laurencia flexuosa* (Mann, 2008). This variation may be related to a number of different factors such as; the misidentification of *Laurencia glomerata* or perhaps *Laurencia* species are susceptible to seasonal and geographical variation of their secondary metabolites.

## 4.5 Experimental section

### 4.5.1 General experimental procedures

Normal phase TLC was performed on DC Alufolie Kieselgel 60F<sub>254</sub> and plates were viewed under UV light (254 nm). Column chromatography was performed on silica gel 60 (0.04 – 0.063 mm) from Merck, Darmstadt (Germany). All solvents were of HPLC grade (HiPerSolv<sup>TM</sup> and Merck).

The <sup>1</sup>H (400 MHz), <sup>13</sup>C (100 MHz) and DEPT-135 NMR spectra were all recorded on a Bruker 400 NMR spectrometer using standard pulse sequences. HMQC, HMBC, COSY-90 and NOESY NMR spectra were recorded on a Bruker 600 NMR spectrometer using standard pulse sequences. Spectra were referenced to residual protonated solvent resonances (CHCl<sub>3</sub>  $\delta_H$  7.25,  $\delta_C$  77.0). Chemical shifts were reported in ppm, while coupling constants were reported in Hz.

Optical rotations were measured on a Perkin-Elmer 141 polarimeter. UV data was obtained using the Perkin Elmer Precisely Lambda 25 UV/VIS spectrometer. IR spectra were obtained using a Perkin Elmer Spectrum 100 FT-IR spectrometer with a universal ATR sampling accessory.

### 4.5.2 Plant material

*L. glomerata* was collected on the 23<sup>rd</sup> September 2010 (D874), while *L. flexuosa* was collected on the 22<sup>nd</sup> September 2010. Both samples were collected at Keurbooms by Prof. Rob Anderson.

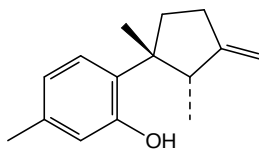
### 4.5.3 Extraction and isolation

5.6 g (dry weight) of *L. glomerata* was soaked overnight in CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) (100 ml). The CH<sub>2</sub>Cl<sub>2</sub>:MeOH mixture was then filtered and vacuumed down. This dried extract (7.4 mg) was then made up with a very small amount of hexane (0.1 ml) and put onto a silica column (0.59 g). From this, fractions A-D were obtained. Fraction A was obtained from the 100% hexane extract. Fraction B consisted of a yellow layer which was derived from a

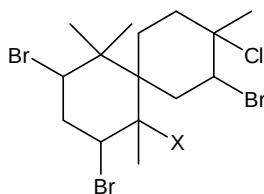
combination of hexane:CH<sub>2</sub>Cl<sub>2</sub> (9:1), fraction C was obtained from hexane:CH<sub>2</sub>Cl<sub>2</sub> (8:2) and was a pure compound known as 7-hydroxylaurene **4.9** (0.6 mg, 0.011% dry weight). Fraction D was obtained from the hexane:CH<sub>2</sub>Cl<sub>2</sub> (1:1) eluent and NMR data confirmed that at least two compounds were present. Fraction D was then dried and placed onto another silica column (0.59 g). The hexane:CH<sub>2</sub>Cl<sub>2</sub> (6:4) wash of fraction D yielded a white substance which turned out to be a pure minor chamigrane compound **4.11** (0.3 mg, 0.054% dry weight). In an effort to clean up fraction A, the sample was placed on a silica column (0.59 g) for a second time - 100% hexane was used as the eluent. This purified the sample and yielded compound **4.12** or known as *cis*-neolaurencenyne (0.3 mg, 0.054% dry weight).

20.2 g (dry weight) of *L. flexuosa* was soaked in MeOH (150 ml) for one hour at 25 °C. *L. flexuosa* was then re-extracted with CH<sub>2</sub>Cl<sub>2</sub>:MeOH (2:1) and left to stand for 12 hours. Both extracts were then combined, dried over MgSO<sub>4</sub>, filtered and vacuumed down. This dried crude extract (206.5 mg) was then dissolved in 1 ml of CH<sub>2</sub>Cl<sub>2</sub>. 40.2mg of this was placed onto a silica column. From this, fractions A-E were obtained. Fraction A was obtained by using 100% hexane, fraction B was obtained from hexane:EtOAc (95:5), and fraction C was obtained from the hexane:EtOAc (9:1) eluent. Fraction D was obtained from a hexane:EtOAc (8:2) eluent while fraction E came from using hexane:EtOAc (1:1) as the solvent system. NMR data showed that the hexane:EtOAc (95:5) fraction contained the most interesting chemistry. This substance came off as a yellow/orange oil. Although the compound was not yet fully purified, <sup>1</sup>H NMR data revealed that it was mostly that of 3Z-bromofucin. The yellow/orange oil was then dissolved in hexane and placed on a silica column which had been equilibrated with hexane. The 100% hexane fraction yielded no compounds, but the hexane:EtOAc (98:2) fraction released a yellow band which was collected. This turned out to be a slightly 'cleaner' version of 3Z-bromofucin **4.13** (1.1 mg, 0.005% dry weight).

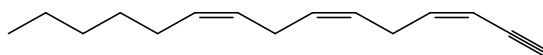
## 4.5.4 Compounds



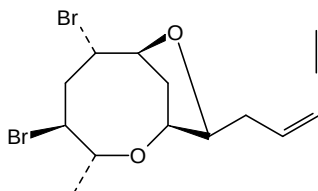
**7-hydroxylaurene OR 2-(1R,2S)-1,2-dimethyl-3-methylidenecyclopentyl-5-methylphenol (4.9):** As previously reported (Wratten and Faulkner, 1977)  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) see Table 4.2.



**4, 8, 10-tribromo-3-chloro- $\alpha$ -chamigrane related compound (4.11):** Whitish oil; UV (Hexane)  $\lambda_{\text{max}}$  273 ( $\epsilon$  860); IR (dry film)  $\nu_{\text{max}}$  2978, 2925, 1468, 1379, 1310, 1187, 1055, 983, 810, 696  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) see Table 4.4



**cis-neolaurencenyne OR 3Z,6Z,9Z-pentadeca-3,6,9-trien-1-yne (4.12):** As previously reported (Kigoshi *et al.*, 1981)  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) see Table 4.5



**3Z-bromofucin (4.13):** As previously reported (Mann, 2008)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) see Table 4.6

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## Chapter Five

# Molecular systematics and chemical taxonomy of selected South African *Laurencia* species (a preliminary study)

## 5.1 Abstract

By investigating the  $^1\text{H}$  NMR and gas chromatography (GC) chemical profiles of different *Laurencia* species with their unique genetic sequences, a correlation between the natural product chemistry and molecular systematics of selected *Laurencia* species became apparent. This suggested that  $^1\text{H}$  NMR and/or GC may be used as a new method of taxonomy, in assisting to identify different *Laurencia* species for South Africa.

**Keywords:** GC;  $^1\text{H}$  NMR; *Laurencia* species; molecular systematics

## 5.2 Introduction

Identifying different *Laurencia* species is not a trivial task, as a result, the idea of using natural product chemistry as a tool to assist in the taxonomy of South African *Laurencia* species was explored. For this to happen, the development of a method for the rapid chemical screening of *Laurencia* species using  $^1\text{H}$  NMR and/or GC, as well as a good understanding of the molecular systematics of *Laurencia* species first had to be established.

### *Molecular systematics in red algae*

A natural product or a secondary metabolite is a phenotype, this means it is an expression of the DNA of the organism. A natural product is produced by enzymes, which are in turn encoded in the DNA of the organism. Therefore, different metabolites should show up as different DNA sequences. This process may be complicated in that certain genes may be silent or require an environmental factor to be 'switched on'. This brings up further fundamental questions such as, 'What constitutes a species? A phenotype or the genes?' (Beukes, 2011)

‘Typically, marine algae can be classified according to morphological, ultra-structural, molecular and chemical analysis. Red algal systematics has a good morphological foundation, based on analysis of female reproductive structures and post-fertilisation development. Molecular approaches to systematics (molecular systematics) have further advanced our understanding of red algae and have led to the proposal of several new orders. Currently, known molecular/phylogenetic markers include; four plastid, three mitochondrial, and two nuclear genomes,’ (Maggs *et al.*, 2007).

‘The diversity of certain species of red algae has often been underestimated due to cryptic or pseudo-cryptic species which exist within a morphologically defined species. These cryptic species are sometimes referred to as sibling species, and are defined as species that are impossible to distinguish based on morphological characteristics alone. Pseudo-cryptic species are species that are readily distinguished morphologically once the appropriate characters are considered. Examination of molecular systematics used on a species level, it is evident that the biodiversity of red algae has been massively underestimated. For example, the recent discovery of pseudo-cryptic entities within morphologically identical species occurred in the genus *Plocamium*,’ (Maggs *et al.*, 2007). Using molecular markers, *Plocamium cartilagineum* was shown to contain at least eight different pseudo-cryptic species (Saunders and Lehmkuhl, 2005). This information was used to define morphological boundaries for four European species of *Plocamium*. Similarly, Yano (2004, 2006) showed that traditional morphological markers used to identify Japanese *Plocamium* species did not correspond with their molecular clusters. Although they could not find definite morphological features specific to their molecular entities, they showed that colour, bromine content and cell acidity levels could also be used as clues for identification purposes (Maggs *et al.*, 2007).

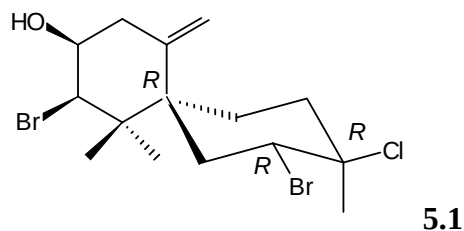


*Combining chemistry (  $^1\text{H}$  NMR and/or GC) and molecular systematics, for the purpose of understanding the relationship between classification and natural product chemistry for Laurencia species*

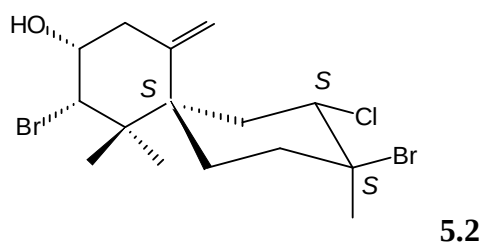
Using GC and GC-MS during routine natural product investigations on marine algae is not uncommon (Knott, 2003). However, few have used this technique in combination with  $^1\text{H}$  NMR to identify different species of marine algae, such as *Laurencia* species. Various chemical methods have been used to assist in the taxonomy of *Laurencia* species. For the purpose of this chapter, the author found Francesco Pietra's work on the evolution of secondary metabolites *versus* evolution of the species as being particularly interesting (Pietra, 2002).

'The construction of phylogenetic trees and evolutionary events concerning species is based on molecules which are found in all organisms, such as rRNA coding fractions and highly conserved proteins, or polymorphic enzymes for population structure and dynamics.' Pietra (2002) continues to say that, 'The correlation between gene and protein expression is low, and that the correlation between proteins and secondary metabolites is even lower (within the scope of the *Laurencia* genus for example). This casts doubt over these "lower" species that compete for space and resources, or defend themselves from predators with the use of secondary metabolites; as secondary metabolites are often used as taxonomic and phylogenetic markers.' As a result, Pietra (2002) argued that there is little *trait d' union* (connecting link or correlation) between the molecular biologist's work, and that of the natural product chemist. Pietra (2002) also added, 'That with a few exceptions, the natural product chemistry of red seaweeds in the genus *Laurencia* was characterised by fragmentary observations that parallel a confusion at the taxonomic level of the species.'

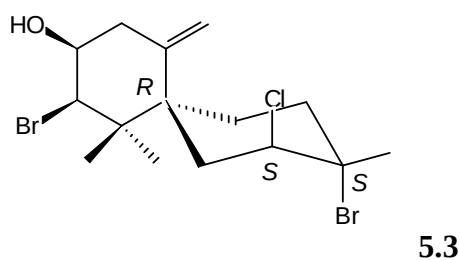
At positions C-2, C-3, C-4 and C-6, Pietra (2002) identified four stereochemical series of diastereomeric polyhalogenated chamigrane sesquiterpenes (5.1-5.4) unique to several different species of *Laurencia* found around the world, and put forth the idea of there being four lineages of red seaweeds within the genus *Laurencia*. It was further suggested that these stereochemical features of sesquiterpene metabolites reflected phylogeny, as they must be closely related to the organisation of the functional genes coding for the relevant enzymes of the particular *Laurencia* species involved.



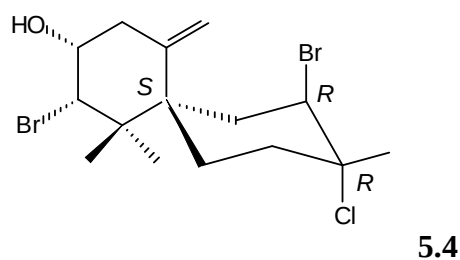
Rogiolol (5.1) found in *L. microcladia* from Rogiolo and *L. majuscula* from the Great Barrier Reef



Obtusol (5.2) found in *L. obtusa* from Tenerife



Isoobtusol (5.3) found in *L. obtusa* from Tenerife, *L. obtusa* from Jamaica, *L. majuscula* from Western Australia and *Aplysia dactylomela* from Puerto Rico



Cartilagineol (5.4) found in *L. cartilaginea* from Ohau as well as *Laurencia* sp. from the Philippines

Based on this alone, Pietra (2002) postulated that there are at least four distinct *Laurencia* lineages, and that these observations called for a taxonomic revision of this algal genus. Pietra (2002) concluded that his studies, from one side, have indicated a phylogenetic *trait d'union* in the taxonomy of this confused area of seaweeds in the genus *Laurencia*. Currently, the future of red algal systematics is based on using molecular tools to investigate for phylogenetic markers, in combination with traditional, morphological taxonomic work. It is believed that this is sufficient to learn more about evolutionary diversification from genetic, physiological and ecological perspectives of marine algae (Maggs *et al.*, 2007). To further one's understanding of marine algae, it was postulated that molecular systematics ought to be combined with GC and  $^1\text{H}$  NMR data (as these two chemical screening methods are easily and rapidly performed and provide a good indication of the chemical consistency of the crude extract being investigated), which can be used to assist in finding chemical markers. Secondary metabolites are produced as a result of protein gene expression, and are thus a reflection of the molecular make-up of a particular alga. By combining the phylogenetic molecular markers of a particular marine alga, with their associated crude secondary metabolite chemistry profile, a new and valuable dimension to one's understanding of marine algae might become apparent. For example, by comparing *L. flexuosa* and *L. glomerata* using molecular systematics,  $^1\text{H}$  NMR data and GC analysis, one may be able to learn more about the relationship that exists between the chemistry and genetics of different *Laurencia* species. This technique might assist in attempting to answer a number of important questions pertaining to different South African *Laurencia* species. For example;

- 1) How much of an impact does the environment have on secondary metabolite production of South African *Laurencia* species?
- 2) With regards to South African *Laurencia* species, do the same molecular markers mean similar major secondary metabolite chemistry and *vice versa*?

### 5.3 Chapter aims

Exploring the relationship between the major natural product secondary metabolite profile of *Laurencia* species and the molecular systematics of *Laurencia* species in an attempt to improve our understanding of the taxonomy of different South African *Laurencia* species.

### 5.4 Results and discussion

#### 5.4.1 Collection and extraction

##### *Rapid chemical screening of Laurencia species using <sup>1</sup>H NMR and/or GC*

On the 18<sup>th</sup> February 2011, Prof. John Bolton collected several species of *Laurencia* from De Hoop in the Western Cape. These samples were frozen and labelled as follows by Prof. Bolton;

- 1) D928 – *L. flexuosa*
- 2) D930 – *L. natalensis*
- 3) D943 – *L. glomerata*
- 4) D947 – *Laurencia* sp. (growing as a clump – perhaps *L. glomerata*)
- 5) D903 – *Laurencia* sp. (attenuated)
- 6) D922 – *Laurencia* sp.
- 7) D926 – *L. glomerata*
- 8) D927 – *Laurencia* sp. (+ parasite – not to be confused with *L. flexuosa*)

From the labels it was noted that simple field identification alone is often not sufficient for the purposes of identifying *Laurencia* species on-sight. As a result, identification of *Laurencia* species is not a trivial task. Using <sup>1</sup>H NMR (and/or GC) as a method to rapidly screen *Laurencia* crude extracts profiles for similarity or differences between *Laurencia* species was suggested as a method to assist in the classification of different South African *Laurencia* species.

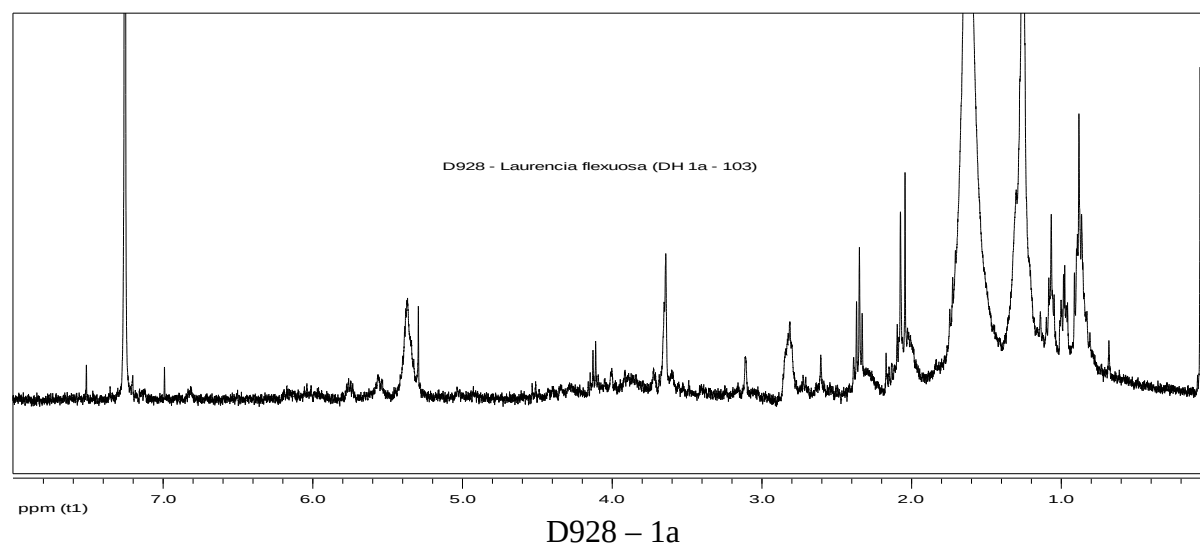
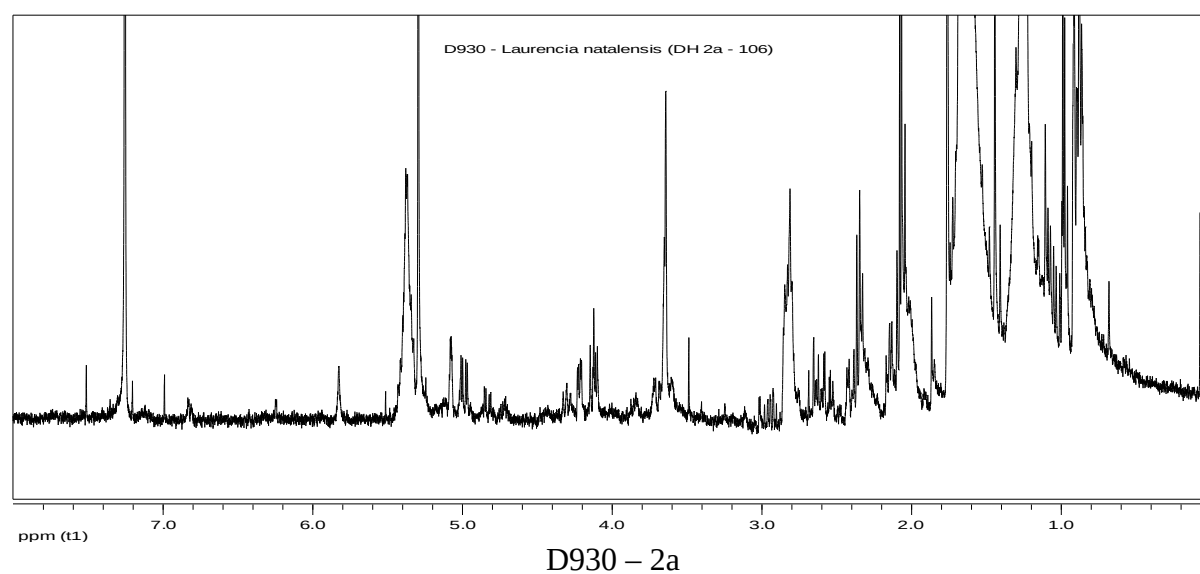
### 5.4.2 $^1\text{H}$ NMR and GC crude extracts from selected *Laurencia* species

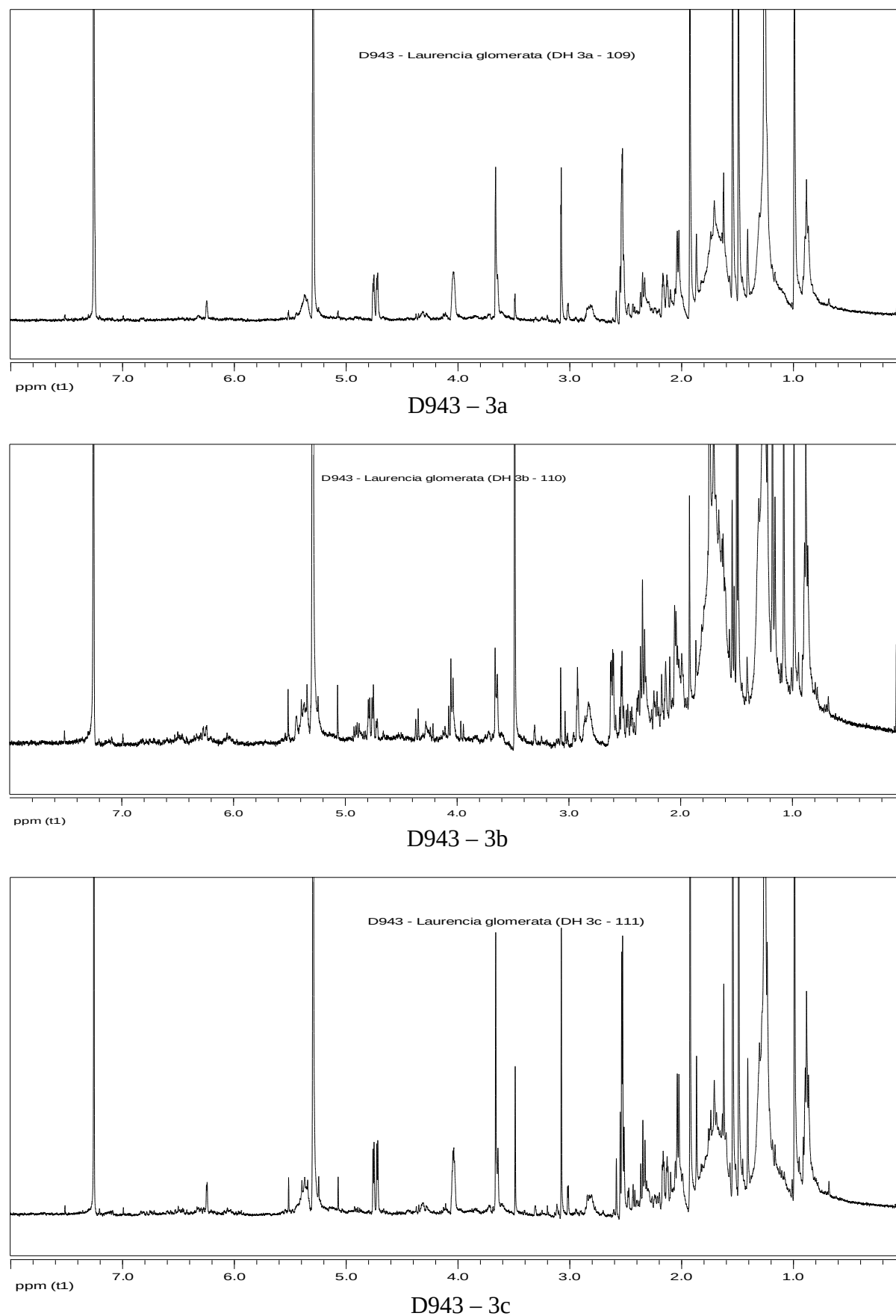
See section 5.5.3 ‘Method for the rapid chemical screening of *Laurencia* species using  $^1\text{H}$  NMR’ for the extraction method and NMR parameters which were adopted for the purpose of extracting polar and non-polar secondary metabolites from *Laurencia* species.

**Table 5.1** Wet weight, dry weight and total extract weight of different *Laurencia* species

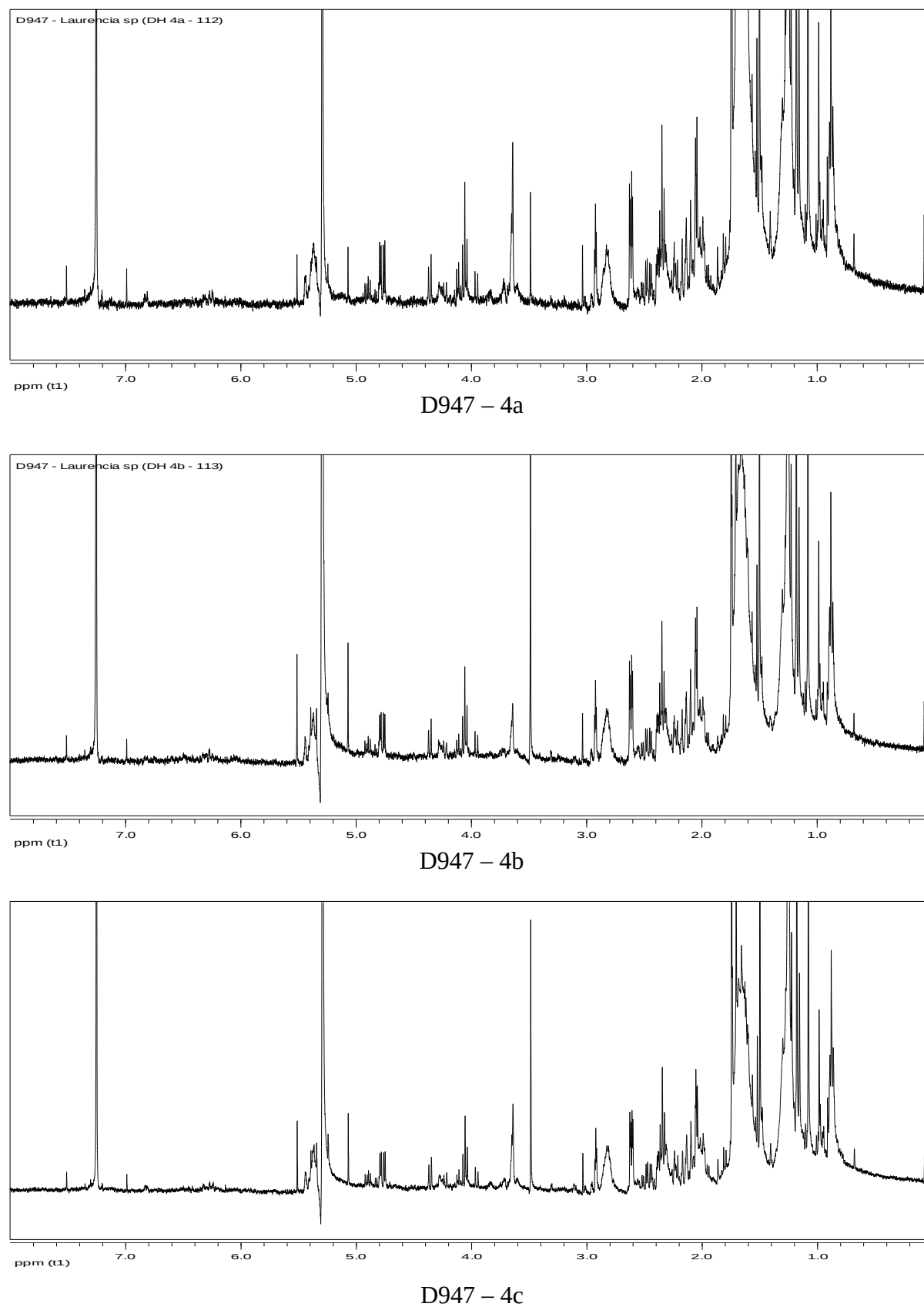
| Identification no. | Wet weight (g) | Dry weight (g) | Total extract weight (mg) | Total extract vs dry weight (%)* |
|--------------------|----------------|----------------|---------------------------|----------------------------------|
| D928 – 1a          | 1.90g          | 0.29g          | 3.3mg                     | 0.11%                            |
| D928 – 1b          | 2.01g          | 0.31g          | 8.9mg                     | 0.28%                            |
| D928 – 1c          | 2.02g          | 0.43g          | 14.1mg                    | 0.32%                            |
| D930 – 2a          | 2.20g          | 0.37g          | 6.4mg                     | 0.17%                            |
| D930 – 2b          | 1.91g          | 0.30g          | 9.1mg                     | 0.30%                            |
| D930 – 2c          | 1.97g          | 0.31g          | 9.3mg                     | 0.30%                            |
| D943 – 3a          | 1.75g          | 0.37g          | 15.9mg                    | 0.43%                            |
| D943 – 3b          | 2.09g          | 0.53g          | 48.2mg                    | 0.91%                            |
| D943 – 3c          | 1.84g          | 0.40g          | 22.3mg                    | 0.56%                            |
| D947 – 4a          | 2.07g          | 0.58g          | 15.2mg                    | 0.26%                            |
| D947 – 4b          | 2.10g          | 0.32g          | 24.1mg                    | 0.75%                            |
| D947 – 4c          | 1.94g          | 0.30g          | 21.9mg                    | 0.73%                            |
| D903 – 5a          | 2.01g          | 0.20g          | 20.2mg                    | 1.01%                            |
| D903 – 5b          | 1.96g          | 0.25g          | 21.0mg                    | 0.84%                            |
| D903 – 5c          | 2.25g          | 0.29g          | 27.9mg                    | 0.96%                            |
| D922 – 6a          | 2.21g          | 0.17g          | 20.0mg                    | 1.17%                            |
| D922 – 6b          | 1.91g          | 0.13g          | 21.5mg                    | 1.65%                            |
| D922 – 6c          | 2.09g          | 0.18g          | 26.1mg                    | 1.45%                            |
| D926 – 7a          | 2.11g          | 0.31g          | 30.3mg                    | 0.98%                            |
| D926 – 7b          | 1.91g          | 0.28g          | 31.6mg                    | 1.13%                            |
| D926 – 7c          | 2.07g          | 0.30g          | 36.3mg                    | 1.21%                            |
| D927 – 8a          | 1.88g          | 0.26g          | 37.5mg                    | 1.44%                            |
| D927 – 8b          | 2.15g          | 0.36g          | 39.8mg                    | 1.11%                            |
| D927 – 8c          | 2.21g          | 0.34g          | 45.6mg                    | 1.34%                            |

\*Due to the presence of water the total extract weight is higher than expected in some cases.

**$^1\text{H}$  NMR data of crude *Laurencia* extracts collected at De Hoop, during February 2011****Figure 5.1**  $^1\text{H}$  NMR spectra of the D928 (*L. flexuosa*) crude extract**Figure 5.2**  $^1\text{H}$  NMR spectra of the D930 (*L. natalensis*) crude extract

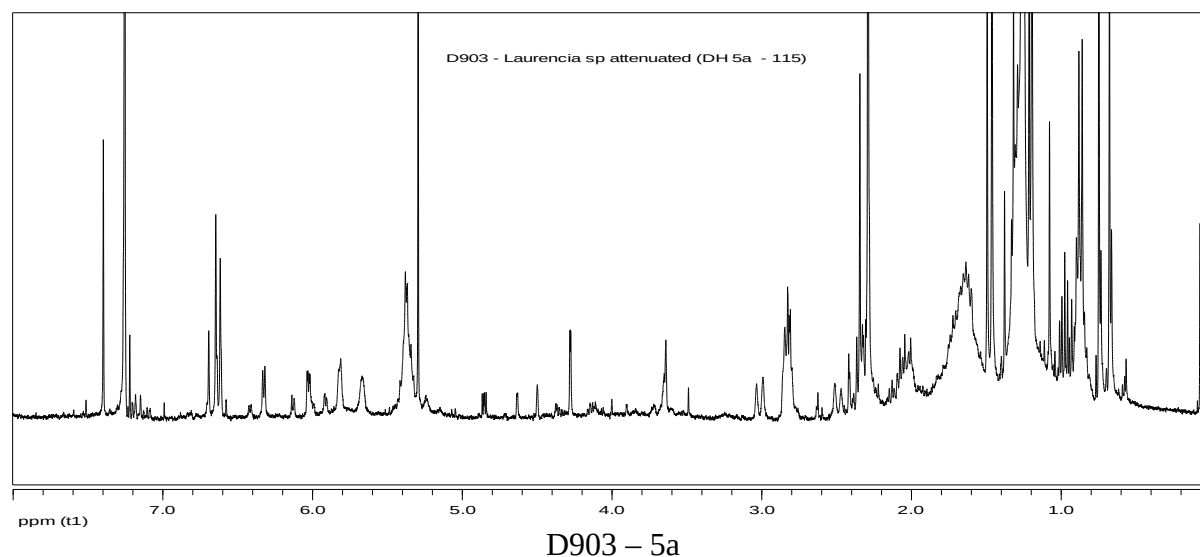


**Figure 5.3**  $^1\text{H}$  NMR spectra of the D943 (*L. glomerata*) crude extract

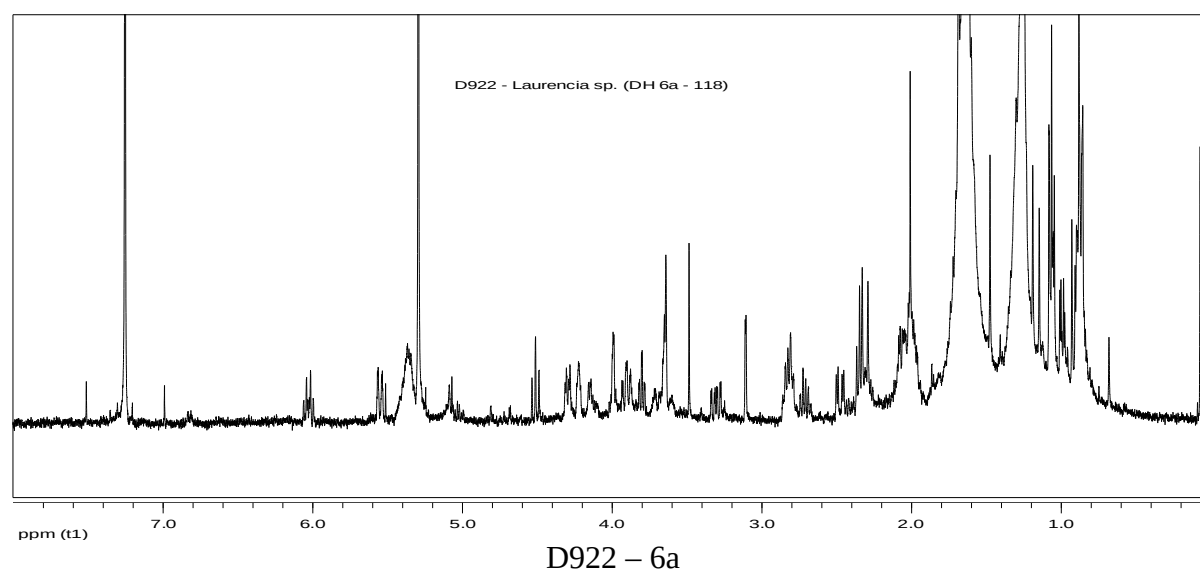


**Figure 5.4**  $^1\text{H}$  NMR spectra of the D947 (*Laurencia* sp. (growing as a clump) – perhaps *L. glomerata*) crude extract

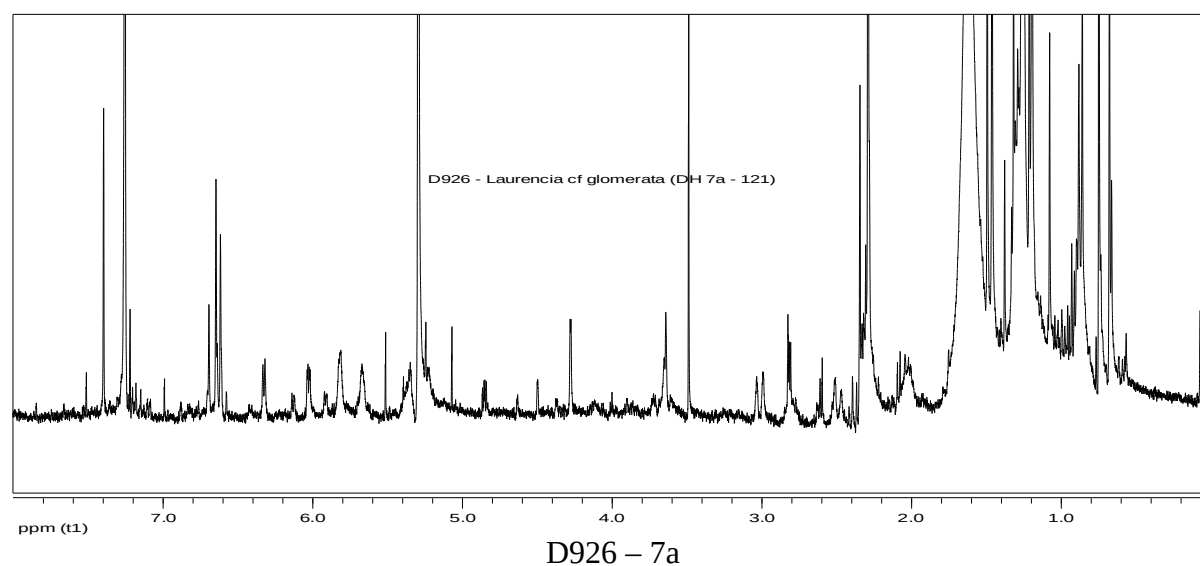




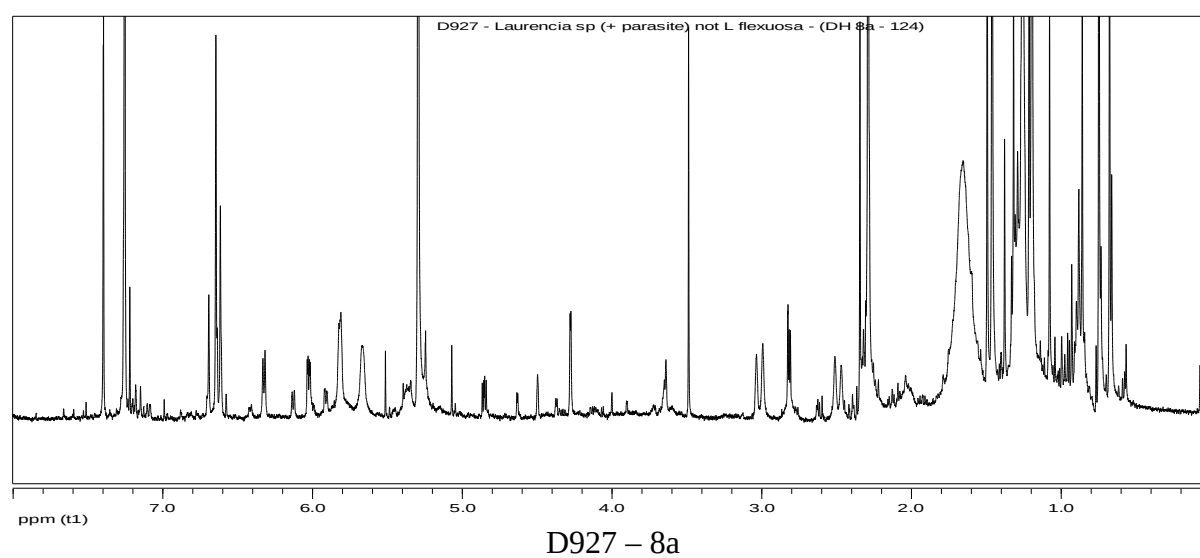
**Figure 5.5**  $^1\text{H}$  NMR spectra of the D903 (*Laurencia* sp. (attenuated)) crude extract



**Figure 5.6**  $^1\text{H}$  NMR spectra of the D922 (*Laurencia* sp.) crude extract



**Figure 5.7**  $^1\text{H}$  NMR spectra of the D926 (*L. glomerata*) crude extract



**Figure 5.8**  $^1\text{H}$  NMR spectra of the D927 crude extract

Each sample was tested in triplicate (See 5.7 Appendix 1b) and in most cases, comparable  $^1\text{H}$  NMR results were obtained with regards to the major compounds that were present in each species. However, in some cases (even though each sample was collected from the same *Laurencia* specimen), there were slight differences with regards to some of the minor compounds that were present. For example, D943-3b showed that it had minor metabolites that were not seen in either D943-3a or D943-3c. Sample D947 also showed very slight differences between the three samples that were tested. This raises further questions with regards to both the extraction procedure that was used, as well as the uniformity of the distribution of secondary metabolites throughout the sample that was tested. These slight spectral differences might also provide some insight as to why the compounds isolated from *Laurencia glomerata* in chapter four, were not the same as those isolated by Elsworth and Thomson (1989). Interestingly, with this extraction procedure, ~2.0g (wet weight) of *L. flexuosa* was not sufficient in terms of mass to gain meaningful spectral information relating to the secondary metabolite profile of *L. flexuosa*. However, ~2.0g (wet weight) of *L. glomerata* (as well as other *Laurencia* species) presented a reasonable  $^1\text{H}$  NMR spectrum. In addition to searching for new ways to improve the extraction procedure of *Laurencia* species, another way to improve these results was to use the 600 MHz NMR (instead of using the 400 MHz NMR - as seen above). This was done for some of the samples (see 5.7 Appendix 1a).

$^1\text{H}$  NMR data obtained from the crude extracts of *L. natalensis* (D930), *L. flexuosa* (D928) and *L. glomerata* (D943) (which were collected from the same geographical location) each demonstrated different profiles. D922 (*Laurencia* species) showed that it was not related to any of the other samples (i.e. it was neither *L. natalensis*, *L. flexuosa* nor *L. glomerata*).

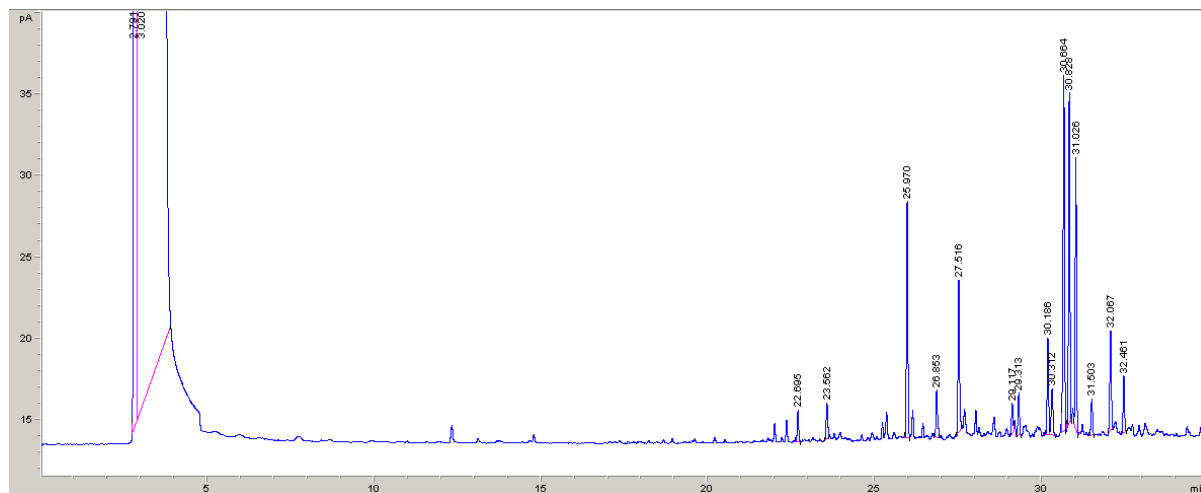
Interestingly, *L. glomerata* exhibited two distinctly different chemical profiles.

Samples D926 (*L. glomerata*), D927 (*Laurencia* species + parasite) and D903 (*Laurencia* species – attenuated) all showed a similar  $^1\text{H}$  NMR chemical profile; while samples D947 (*Laurencia* species) and D943 (*L. glomerata*) demonstrated a similar  $^1\text{H}$  NMR chemical profile.

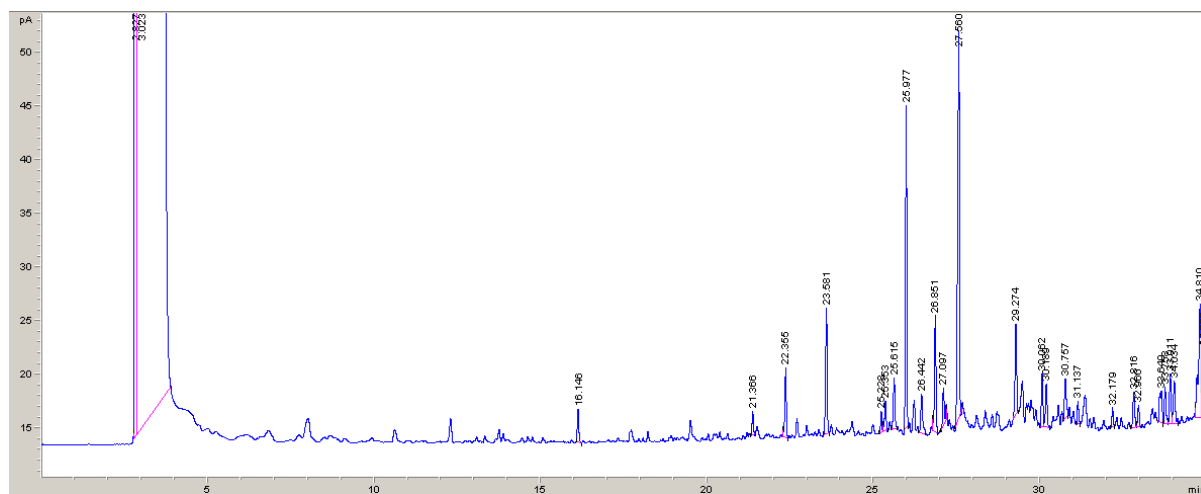
The chemistry of D943 (*L. glomerata*) and D926 (*L. glomerata*) is markedly different. This data suggested that either the chemistry of *L. glomerata* varies depending on external environmental factors, or that one of the samples is not a genuine *Laurencia glomerata* species.

**Crude GC traces of *Laurencia* extracts collected at De Hoop, during February 2011**

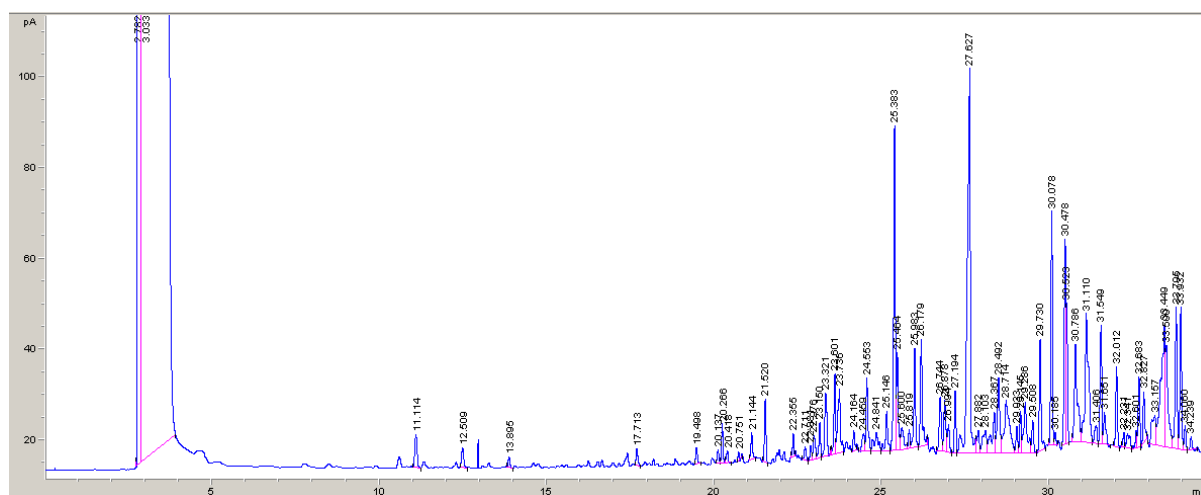
Note: The reproducibility of the GC chromatograms was consistent. For this reason only one GC trace is shown for each sample.



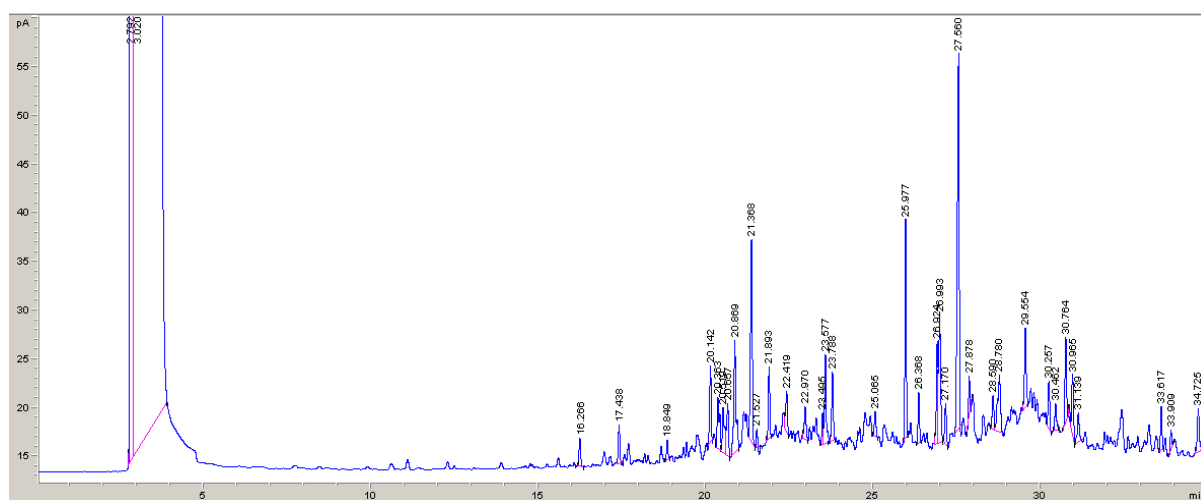
**Figure 5.9** GC trace of D928 (*L. flexuosa*) crude extract



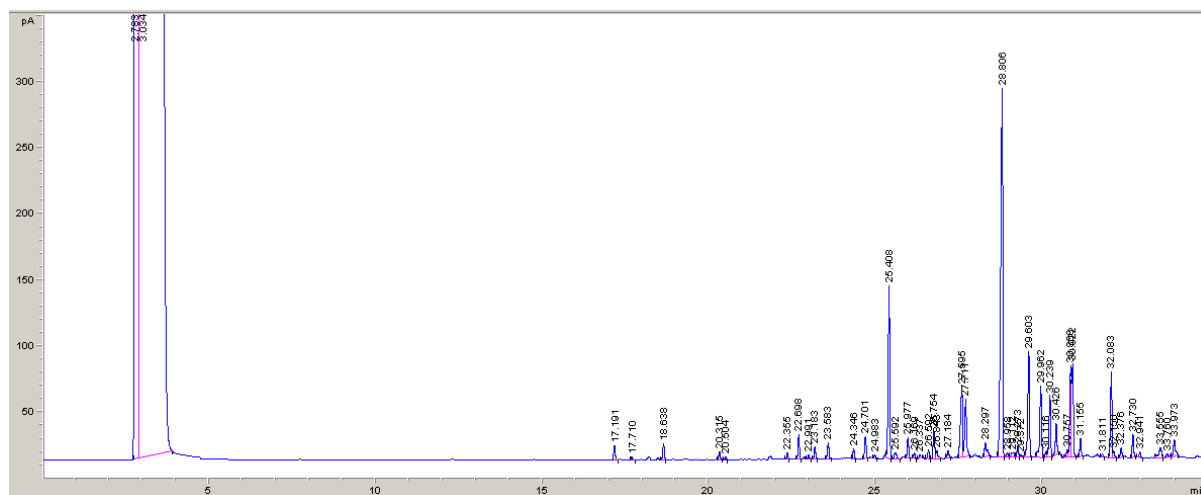
**Figure 5.10** GC trace of D930 (*L. natalensis*) crude extract



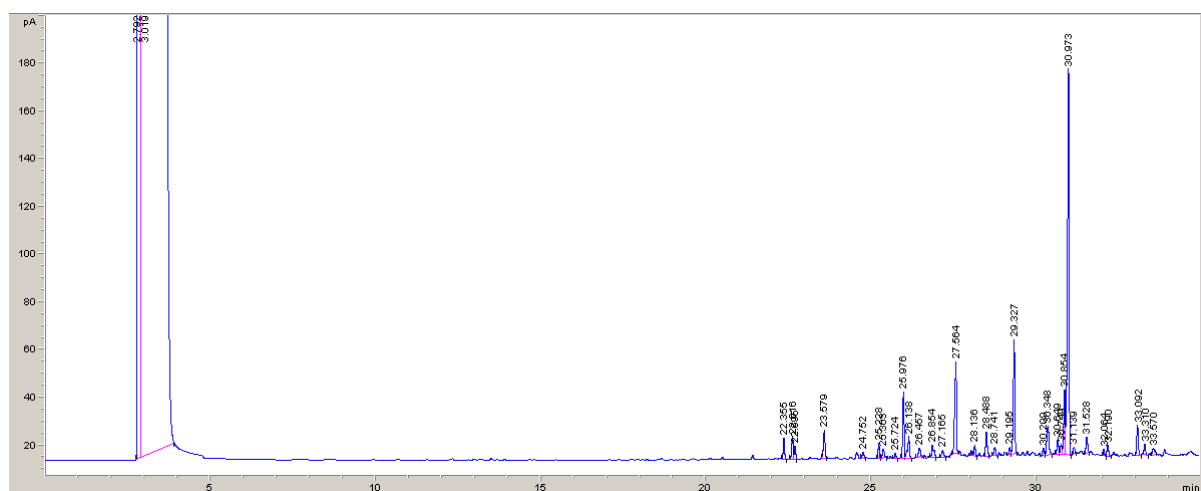
**Figure 5.11** GC trace of D943 (*L. glomerata*) crude extract



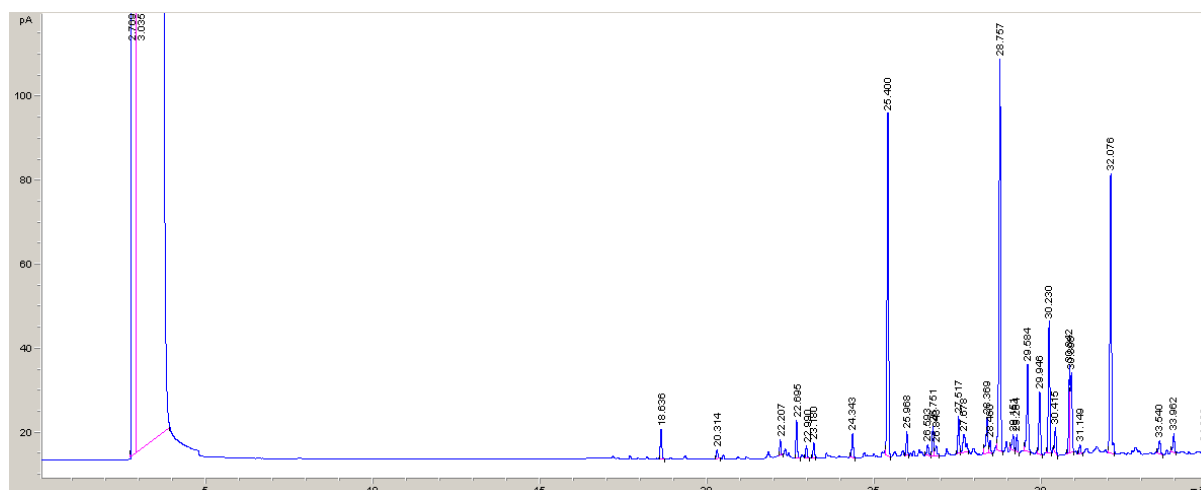
**Figure 5.12** GC trace of D947 (*Laurencia* sp. growing as a clump – perhaps *L. glomerata*) crude extract



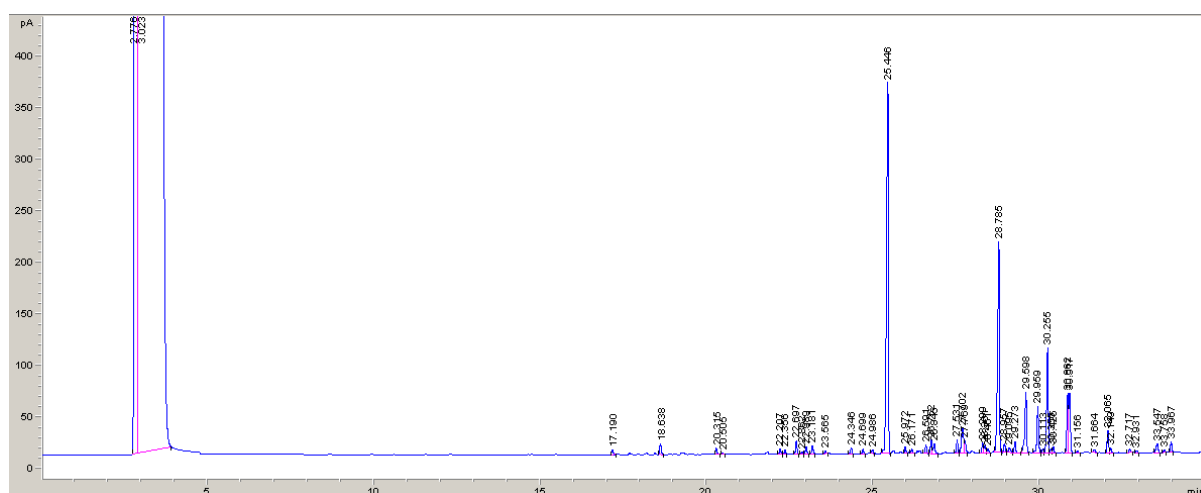
**Figure 5.13** GC trace of D903 (*Laurencia* sp. (attenuated)) crude extract



**Figure 5.14** GC trace of D922 (*Laurencia* sp.) crude extract



**Figure 5.15** GC trace of D926 (*L. glomerata*) crude extract



**Figure 5.16** GC trace of D927 (*Laurencia* sp. (+ parasite – not to be confused with *L. flexuosa*)) crude extract

The GC ‘fingerprints’ above, which were obtained from the same various crude *Laurencia* extracts used for  $^1\text{H}$  NMR analysis, confirmed what had already been concluded using  $^1\text{H}$  NMR data alone. Once again, different *Laurencia* samples showed chemical profiles unique to each species. When comparing the major metabolite profiles of different *Laurencia* species,  $^1\text{H}$  NMR was preferred over GC due to the fact that  $^1\text{H}$  NMR seemed to give a clearer overall ‘chemical picture’ of the major metabolites present in each *Laurencia* species.

These samples were also analysed by Ms Caitlynne Francis at UCT (University of Cape Town) for molecular systematic analysis. Ms Francis's work involved searching for differences between the chloroplast molecular marker (*rbcL*) that occur between different *Laurencia* species. Due to the fact that this is part of Ms Francis's MSc research project, it was not possible to present her results. However, surprisingly, all of Ms Francis's molecular systematics findings correlated very well with what was predicted using  $^1\text{H}$  NMR alone. This was further verified using GC. During the course of much personal communication with Francis (2011), the following was revealed;

Molecular systematic analysis confirmed that D930 (which was originally thought to be *L. natalensis*) was in fact a new species of turf-forming *Laurencia*. D928 was *L. flexuosa* and D943 was indeed *L. glomerata*. From the  $^1\text{H}$  NMR and GC profiles it was noted that the chemistry of D930, D928 and D943 were remarkably different. In addition to this, molecular systematic analysis of D922 (*Laurencia* species) suggested that this was also an entirely new species of *Laurencia*. The chemistry of D922 concurred and showed that it was not related to any of the other samples (i.e. it was neither *L. natalensis*, nor *L. flexuosa* nor *L. glomerata*).

Interestingly, *L. glomerata* (or what was believed to be *L. glomerata*) exhibited two distinctly different chemical profiles. Molecular systematics analysis also confirmed this finding. Unfortunately, Ms Francis was unable to get the sequence of D947, but reported that according to molecular systematic analysis, D943 was the 'true' *L. glomerata*. The  $^1\text{H}$  NMR spectra of D947 (*Laurencia* species) and D943 (*L. glomerata*) is also similar.

Samples D926 (*L. glomerata*) and D903 (*Laurencia* species – attenuated) showed the same  $^1\text{H}$  NMR and GC chemical profile. Ms. Francis confirmed that the molecular systematic analysis of D926 and D903 was indeed the same and that these samples were not *L. glomerata*, but a new species.

As mentioned above, the chemistry of D943 (*L. glomerata*) and D926 (*L. glomerata*) is markedly different. Earlier it was postulated that this data suggested that either the chemistry of *L. glomerata* varies depending on external environmental factors, or that one of the *L. glomerata* could be a different species of *Laurencia*. Based on molecular systematics,  $^1\text{H}$  NMR or GC analysis, it can be concluded that D943 and D926 may appear morphologically the same, but they are in fact two different species of *Laurencia*.



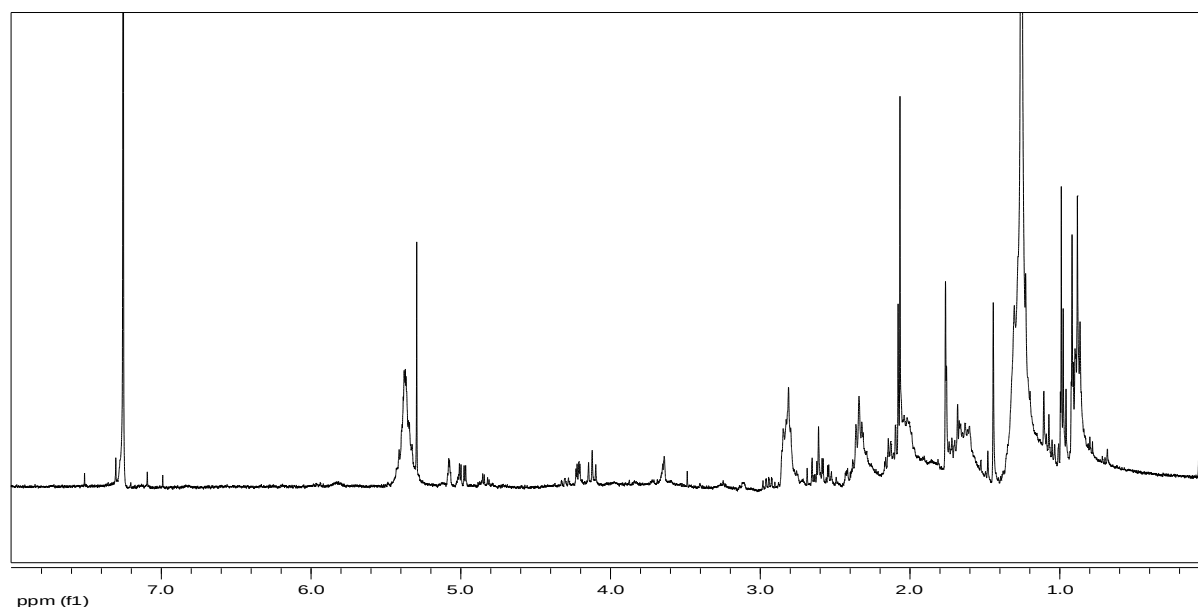
The results of the De Hoop collection suggest that there is a correlation between the molecular systematics of different *Laurencia* species and their unique crude  $^1\text{H}$  NMR chemical profiles.

Pietra's work is largely based on individual compounds which are commonly found in different *Laurencia* species (Pietra, 2002). By taking a look at the bigger picture, for example, the chemistry of crude *Laurencia* extracts from different *Laurencia* species, a different perspective becomes apparent. Surprisingly, the findings presented above are in contrast to some of Francesco Pietra's assumptions. Several different South African species of *Laurencia* were collected from the same location. The chemistry and molecular findings showed a *trait d'union* in each *Laurencia* species that was investigated. Each different *Laurencia* species had its own unique  $^1\text{H}$  NMR and GC fingerprint. Although *Laurencia* samples from the same species seemed to contain the similar major compounds (in the broad sense of the word), the only major aspect of the chemistry that differed, was, in some instances, the relative amounts of individual compounds concerned, which could be a result of external environmental factors.

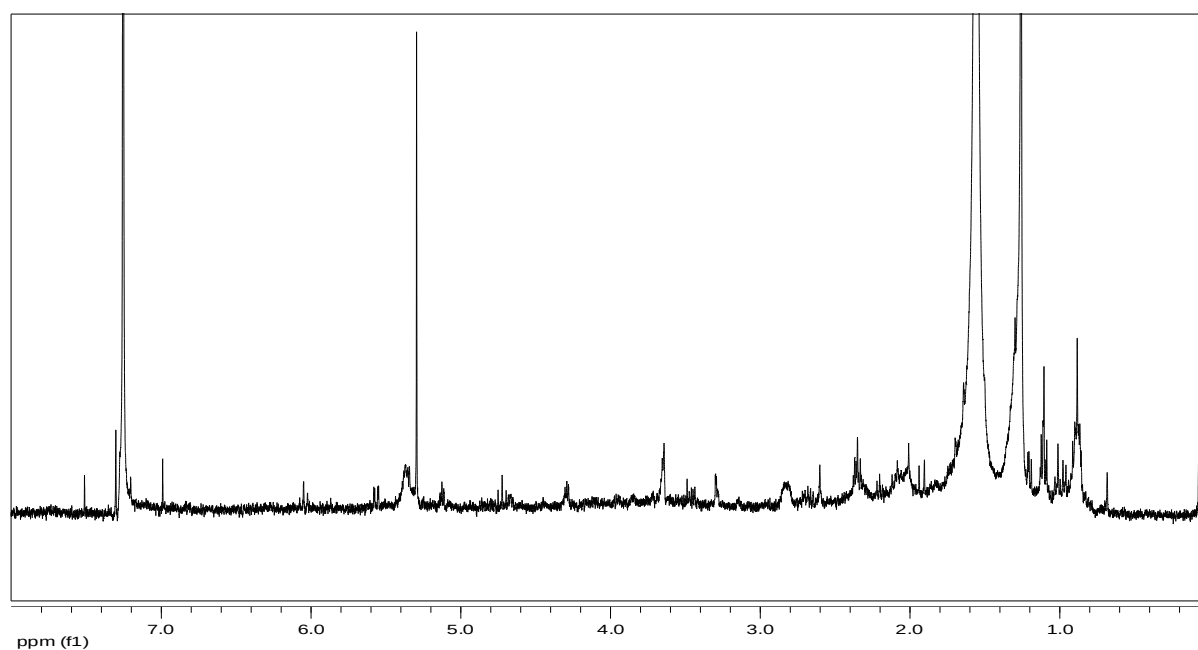
The *trait d'union* that exists between the chemical and molecular findings, suggested that chemical methods such as  $^1\text{H}$  NMR or GC might be used to assist in distinguishing between different *Laurencia* species. Interestingly, most species of *Laurencia* can be characterised by at least one specific compound not found in the others (Fenical and Norris, 1975). This idea was supported by Howard *et al.* (1980) who showed that the chemistry of several *Laurencia* species remained the same under varying field and culture conditions. However, it should be noted that this is only a preliminarily pilot study, and that a number of other factors still need to be taken into account. For example, intra-species chemical variation is well documented in some *Laurencia* species such as *L. obtusa* (Caccamese *et al.*, 1981). Assuming intra-species variation is a factor to consider with regards to South African *Laurencia* species, this does not take away the fact that different *Laurencia* species appear to have unique  $^1\text{H}$  NMR and GC spectra, and that being able to recognise these 'chemical fingerprints' is of taxonomic value. For example, say hypothetically that intra-species variation was a factor to consider in the case of *L. glomerata* and that *L. glomerata* had five different kinds of unique  $^1\text{H}$  NMR and GC spectra. These five spectra would still be unique to *L. glomerata*, and that as long as one knew which spectra was unique to which *Laurencia* species (based on *both* molecular systematics and  $^1\text{H}$  NMR analysis), one would still be able to use  $^1\text{H}$  NMR to assist in the

rapid identification of different *Laurencia* species. For this to work the  $^1\text{H}$  NMR spectra for *L. glomerata* must not only be unique, but also reproducible. However, much work still needs to be done in correlating the molecular systematics and  $^1\text{H}$  NMR profiles of different South African *Laurencia* species, before  $^1\text{H}$  NMR profiles alone (of *Laurencia* samples) can be used to rapidly identify *Laurencia* species with speed and accuracy.

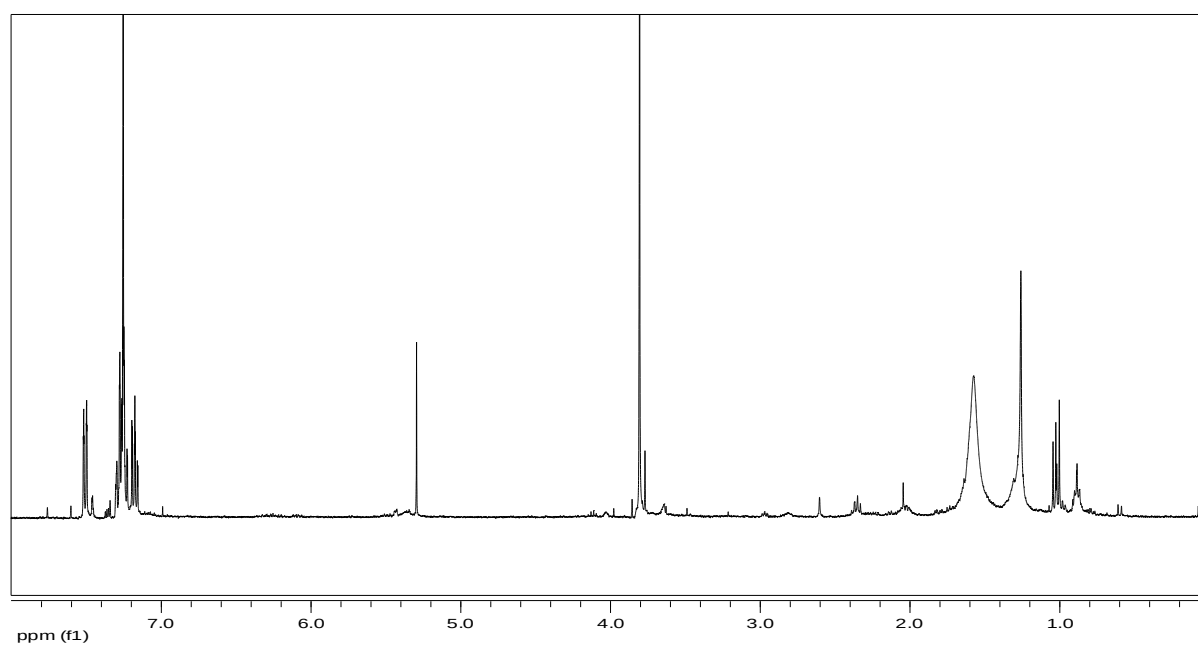
The idea of transferring this concept to identify other South African *Laurencia* species using  $^1\text{H}$  NMR alone was also investigated. Three random samples (although not statistically significant) of *Laurencia* species collected from different parts of South Africa, were obtained from the freezer at Rhodes University, extracted in the standard manner (as mentioned in 5.5.3 Extraction and isolation) and investigated using  $^1\text{H}$  NMR.



**Figure 5.17** Crude extract of an unknown species of *Laurencia* (KOS 110121)



**Figure 5.18** Crude extract of an unknown species of *Laurencia* (D1024)



**Figure 5.19** Crude extract of *L. complanata* (D1053)

These  $^1\text{H}$  NMR results showed that KOS 110121-1 was similar to D930 from De Hoop. D1024 had a number of proton signals which resembled that of D922 from the De Hoop collection, while D1053 (as expected) resembled none of the other  $^1\text{H}$  NMR spectra as it was *L. complanata* which had not yet been investigated. These preliminary results seem to support the idea that South African *Laurencia* species which are the same, have similar crude  $^1\text{H}$  NMR profiles to each other.

It should be noted that this study has many shortcomings. As a result these findings are only preliminary and still need to undergo a number of further studies before conclusive comments can be made. The main issue is the small sampling pool that was used to test the molecular systematics and crude  $^1\text{H}$  NMR chemistry of different *Laurencia* species (i.e. only eight samples from the same geographical location were looked at). For example, sampling from different geographical locations, experimenting with different sexes of the same *Laurencia* species, investigating the impact that light has on the secondary metabolite profile of different *Laurencia* species, as well as considering that seasonal variations might also play a significant role in the production of secondary metabolites from *Laurencia* species. This is yet to be determined, and as a result, the results of this chapter are still very preliminary.

To answer the original questions that were posed (based on these initial results);

- 1) How much of an impact does the environment have on the secondary metabolite production of South African *Laurencia* species? *At the moment it is not possible to answer this question using the current set of data.*
- 2) With regards to South African *Laurencia* species, do the same molecular markers mean similar major secondary metabolite chemistry and vice versa? *From these results, it holds true that samples of Laurencia with the same molecular systematic profile, have a similar major secondary metabolite chemical profile, and vice versa. However, significantly more samples of South African Laurencia species need to be tested and correlated using molecular systematics and  $^1\text{H}$  NMR analysis before these results become more convincing.*

## 5.5 Experimental section

### 5.5.1 General experimental procedures

The  $^1\text{H}$  (400 MHz) spectra were all recorded on a Bruker 400 MHz. Some of the NMR spectra (as cleared identified in the text) were also recorded on a 600 MHz NMR spectrometer using standard pulse sequences. Spectra were referenced to residual protonated solvent resonances ( $\text{CHCl}_3$   $\delta_{\text{H}}$  7.25,  $\delta_{\text{C}}$  77.0). The following NMR acquisition parameters were used on the Bruker 400 MHz: Instrum – spect; Probhd – 5 mm BBO multi; Pulprog – zg30; TD – 65536; Solvent –  $\text{CDCl}_3$ ; NS – 32; DS – 0; SWH – 8278.146 Hz; Fidres – 0.126314 Hz; AQ 3.9584243 sec; RG – variable depending on sample; DW – 60.400  $\mu\text{sec}$ ; DE – 6.50  $\mu\text{sec}$ ; TE 300.2 K; D1 – 1.0 sec; MCREST – 0.0 sec; MCWRK – 0.015 sec. The following NMR acquisition parameters were used on the Bruker 600 MHz: Probhd – 5 mm BBI multi Pulprog – zg30; AQ\_mod – DQD; TD – 49340; Solvent –  $\text{CDCl}_3$ ; NS – 32; DS – 2; TD0 – 1; SWH – 9014.423 Hz; Fidres – 0.182700 Hz; AQ 2.7367754 sec; RG – variable depending on sample; DW – 55.467  $\mu\text{sec}$ ; DE – 6.00  $\mu\text{sec}$ ; DECIM 2218.67.

### 5.5.2 Plant material

On the 18<sup>th</sup> February 2011, several species of *Laurencia* from De Hoop in the Western Cape. These samples were collected, identified (by Prof. John Bolton) and immediately frozen for taxonomic research.

### 5.5.3 Extraction and isolation

#### *Method for the rapid chemical screening of Laurencia species using $^1\text{H}$ NMR*

Frozen samples were washed under de-ionised water and gently dried over absorbing paper. Approximately 2 g of each sample was then placed into a test-tube. To this, 3 ml of MeOH was added and sonicated for 5 minutes (make of model - Soniclean). After this, 6 ml of  $\text{CH}_2\text{Cl}_2$  was added, thereafter it was sonicated for another five minutes. The  $\text{CH}_2\text{Cl}_2$ :MeOH (2:1) mixture was then passed through a cotton-wool plug and dried (using a Buchi rotavapor R-215). The dried extract was then weighted and made up with  $\text{CDCl}_3$  before being placed in the NMR for analysis. This procedure was repeated in triplicate for each *Laurencia* sample.

*Method for the rapid chemical screening of Laurencia species using GC*

<sup>1</sup>H NMR samples (above) were dried and passed through a cotton-wool plug in a pipette. Each sample was then made up to 2.5 ml with CH<sub>2</sub>Cl<sub>2</sub> and 1 µl was then injected onto the GC using the parameters as set out below.

**Table 5.2** GC parameters (Agilent Technologies 7890A, 7693 Auto-sampler)

|                                                                                                                                                                                                                                                                                                         |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Oven</b>                                                                                                                                                                                                                                                                                             |
| Initial temp: 75 °C; Final temp: 325 °C; Ramp 5 °C/minute; Post temp: 250 °C; Run time: 35.00 and 30.00 min                                                                                                                                                                                             |
| <b>Front inlet</b>                                                                                                                                                                                                                                                                                      |
| Mode: Splitless; Initial temp: 280 °C; Pressure: 5.955 psi; Total flow: 9 ml/min; Gas saver: On 20 ml/min after 2 min                                                                                                                                                                                   |
| <b>Column 1</b>                                                                                                                                                                                                                                                                                         |
| Agilent 19001J-413; HP-5 5% Phenyl Methyl Siloxane; Max temp: 325 °C ; Nominal length 30 m; Nominal film thickness: 0.25 µm; Nominal diameter: 320 µm; Mode: constant flow; Initial flow 1 ml/min, Pressure: 5.9552 psi; Average velocity: 19.996 cm/sec; In: Front SS inlet He; Out: Back detector FID |
| <b>Back detector FID</b>                                                                                                                                                                                                                                                                                |
| Heater: 300 °C; Hydrogen flow: 35 ml/min; Air flow: 400 ml/min; Makeup flow: 5 ml/min; Const Col + Makeup flow: 6 ml/min; Flame: On; Electrometer: On                                                                                                                                                   |
| <b>Signals</b>                                                                                                                                                                                                                                                                                          |
| Back Signal: Save On 20 Hz                                                                                                                                                                                                                                                                              |
| <b>Solvent injected</b>                                                                                                                                                                                                                                                                                 |
| Dichloromethane (HPLC grade) 1 µl injection                                                                                                                                                                                                                                                             |

## 5.6 References

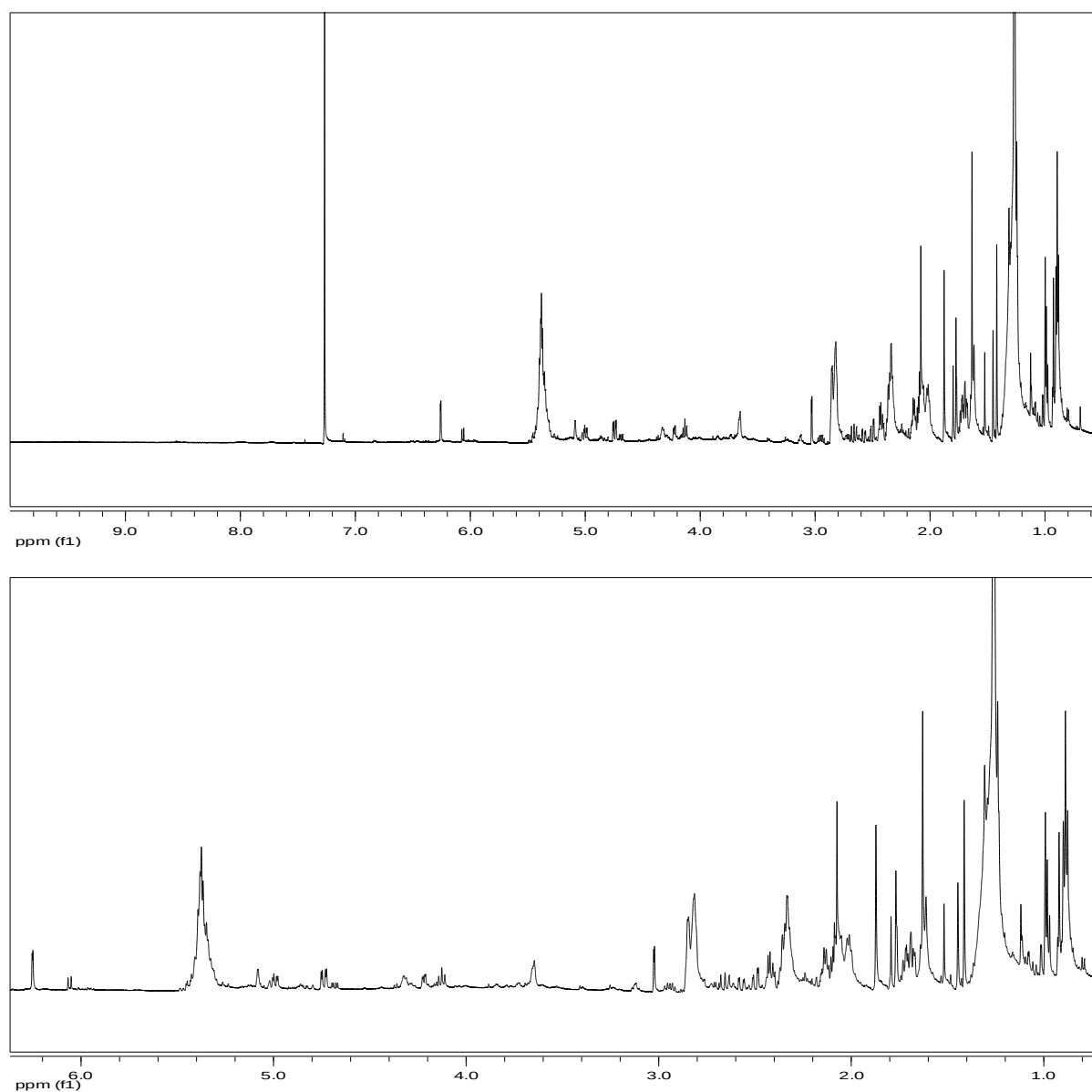
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## 5.7 Appendix 1a

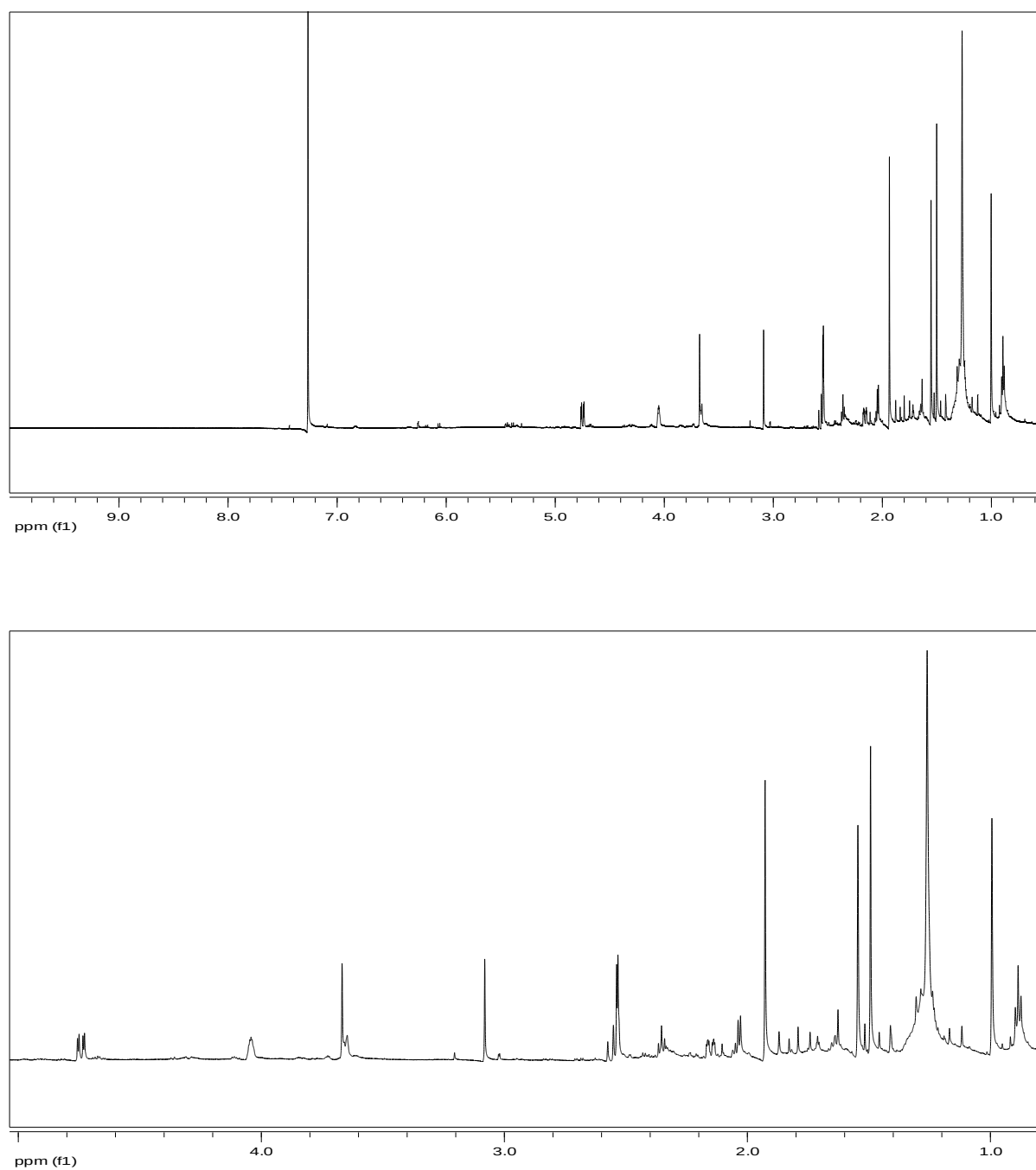
**Table 5.3** Identification number and total extract weight of different *Laurencia* species tested on the 600 MHz Bruker NMR.

| Identification no. | Total extract weight (mg) |
|--------------------|---------------------------|
| D930               | 10.4 mg                   |
| D943               | 6.8 mg                    |
| D947               | 4.9 mg                    |
| D903               | 8.0 mg                    |
| D922               | 3.8 mg                    |
| D926               | 5.0 mg                    |
| D927               | 8.2 mg                    |

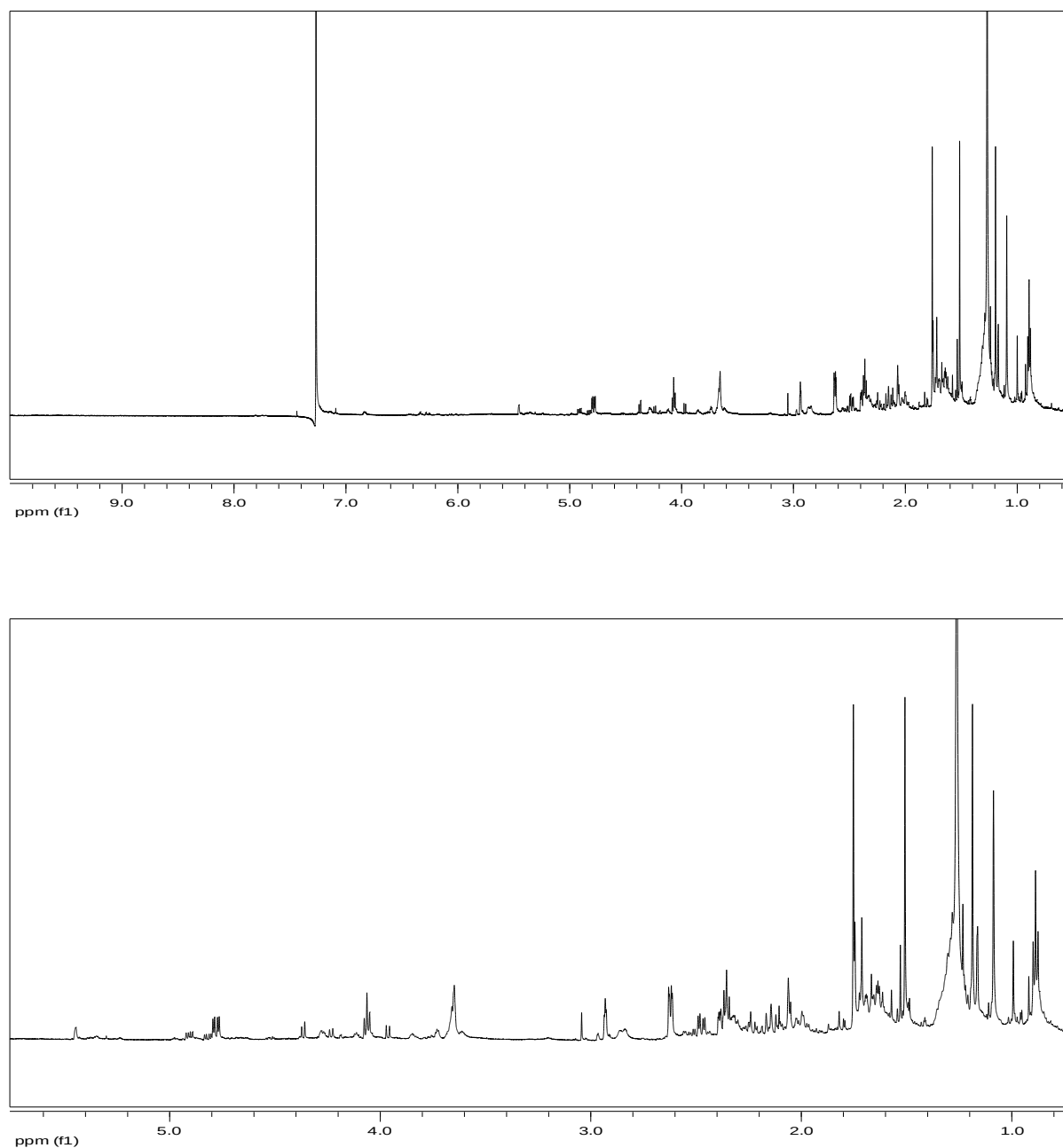




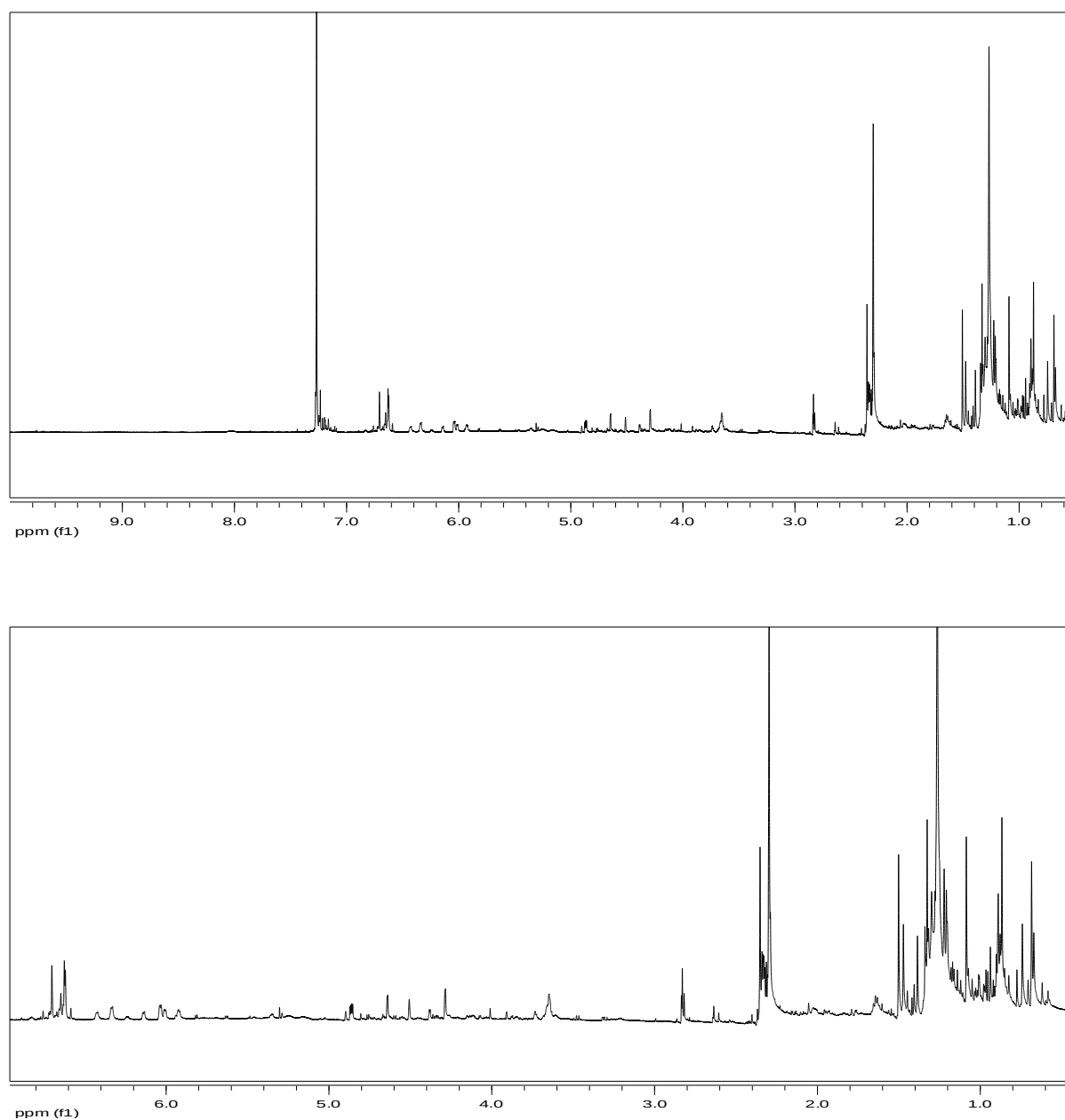
**Figure 5.20**  $^1\text{H}$  NMR data of the D930 (*L. natalensis*) crude extract



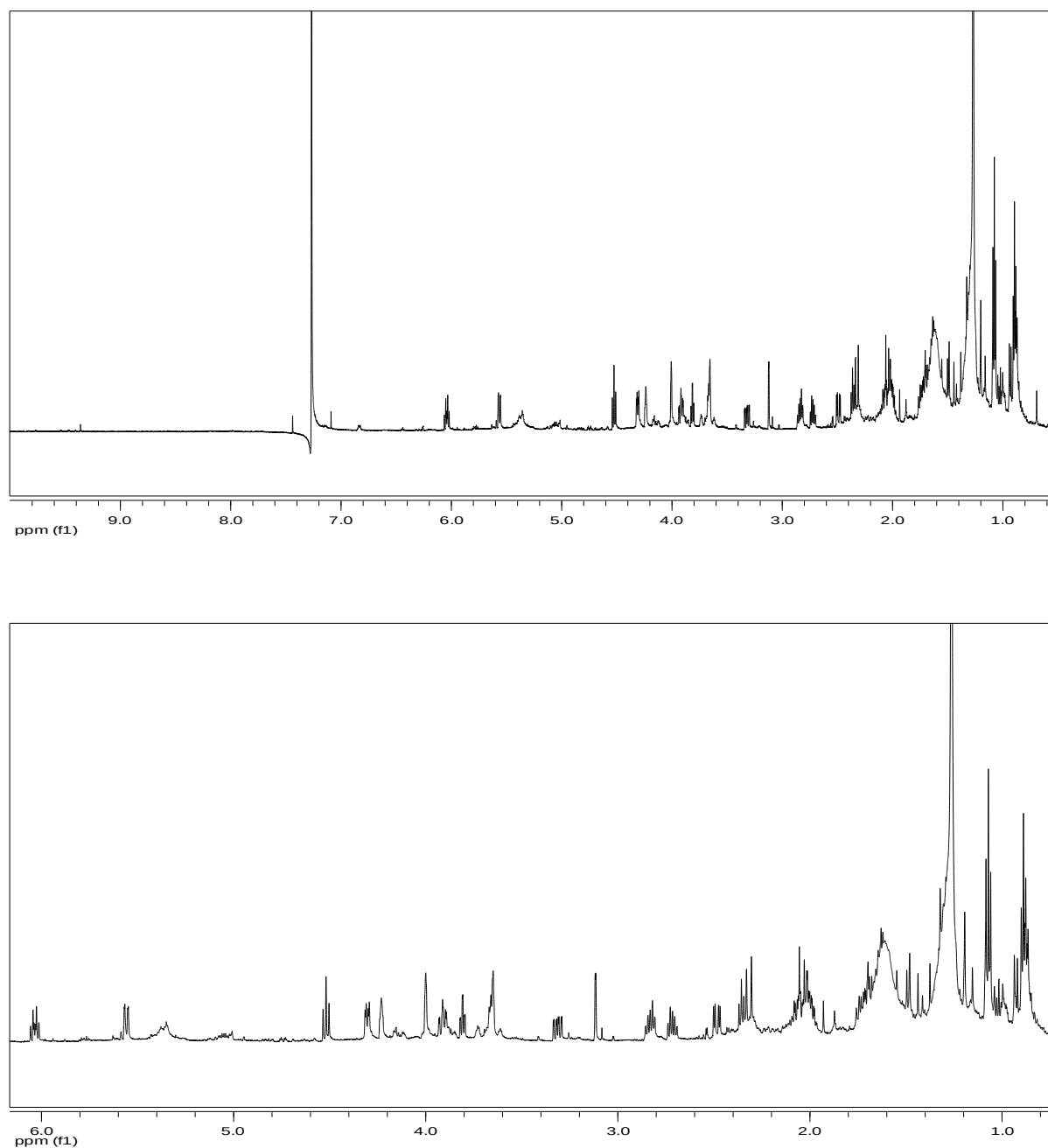
**Figure 5.21**  $^1\text{H}$  NMR data of the D943 (*L. glomerata*) crude extract



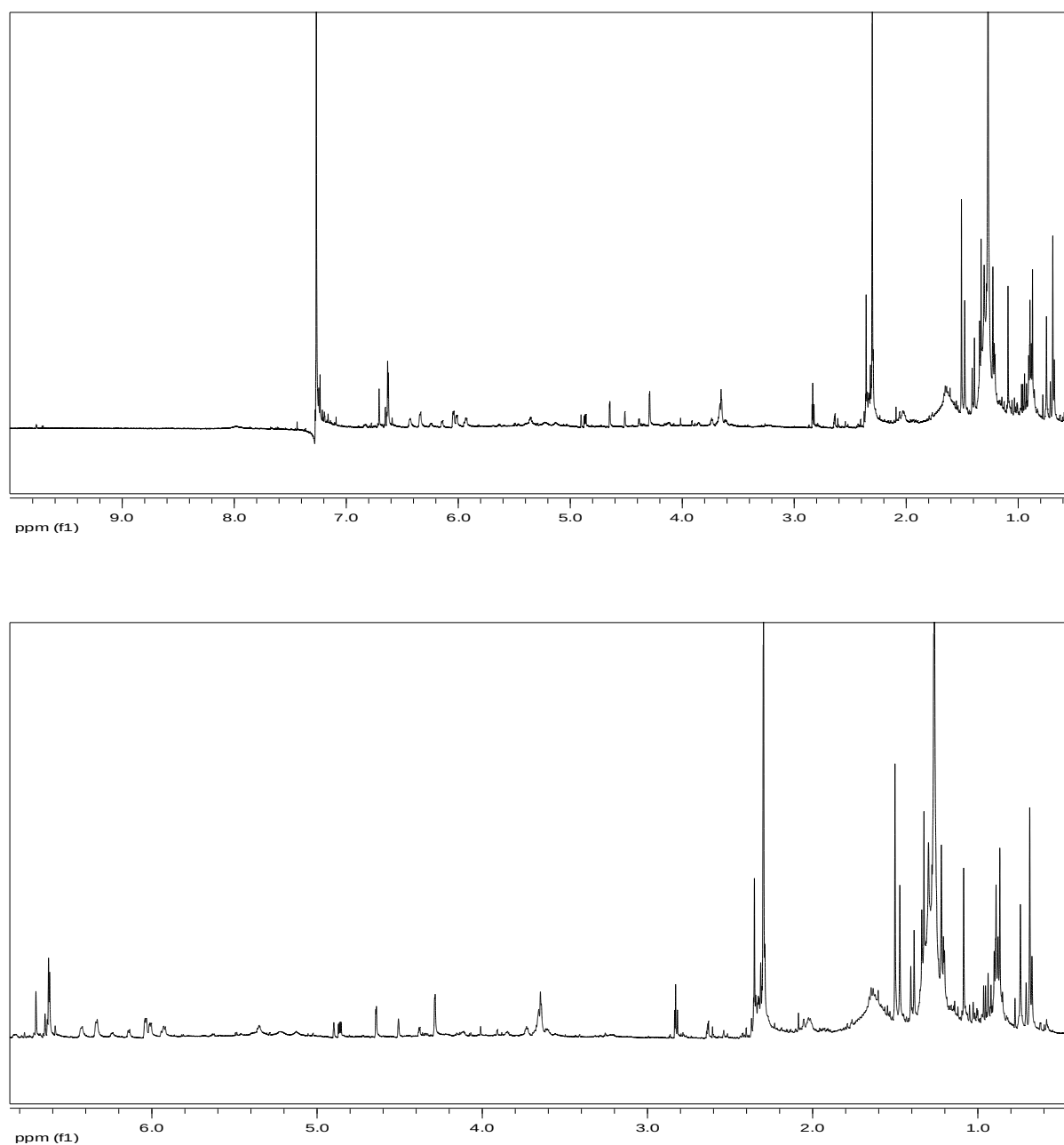
**Figure 5.22**  $^1\text{H}$  NMR data of the D947 (*Laurencia* sp. (growing as a clump) – perhaps *L. glomerata*) crude extract



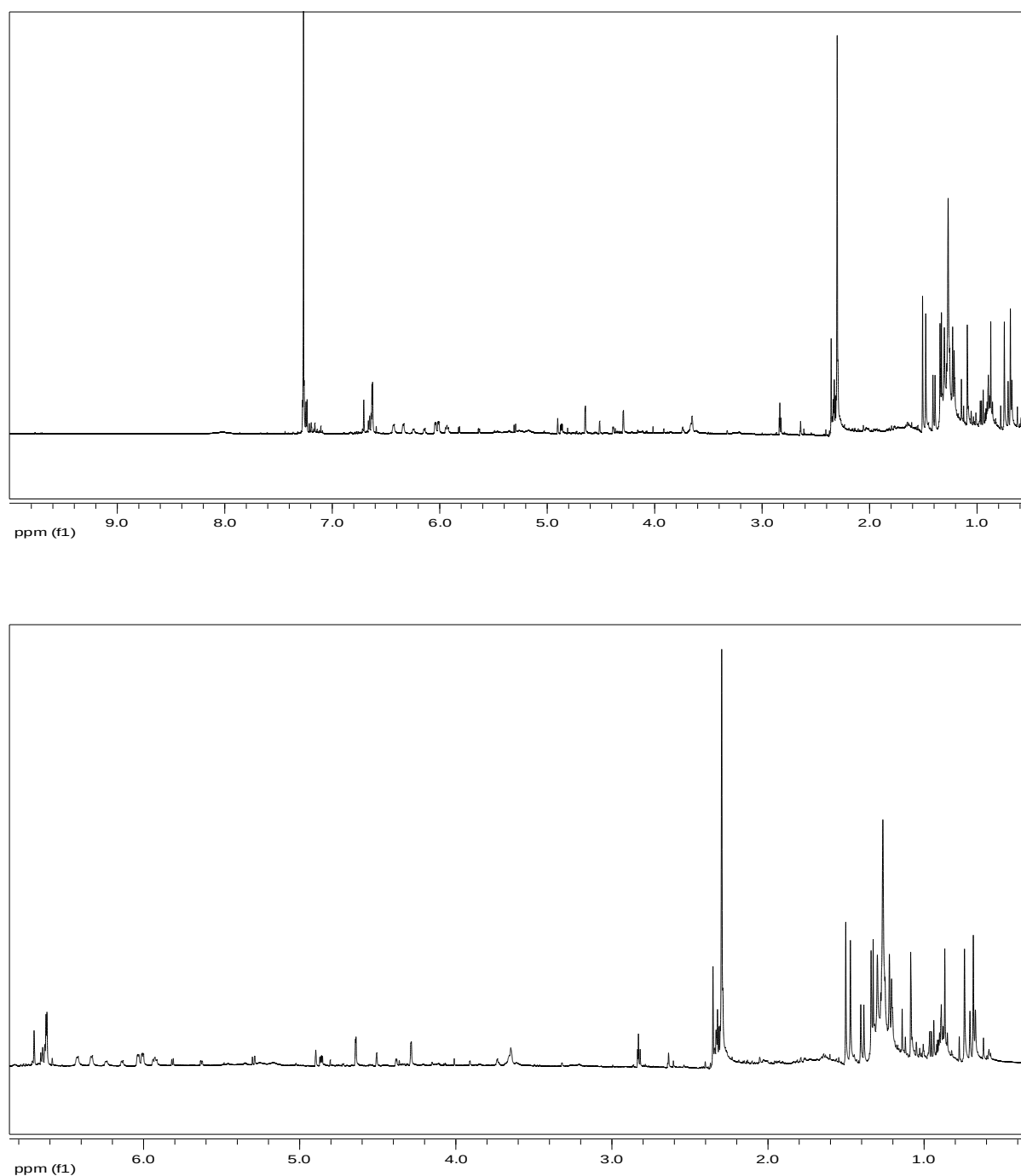
**Figure 5.23**  $^1\text{H}$  NMR data of the D903 (*Laurencia* sp. (attenuated)) crude extract



**Figure 5.24**  $^1\text{H}$  NMR data of the D922 (*Laurencia* sp.) crude extract



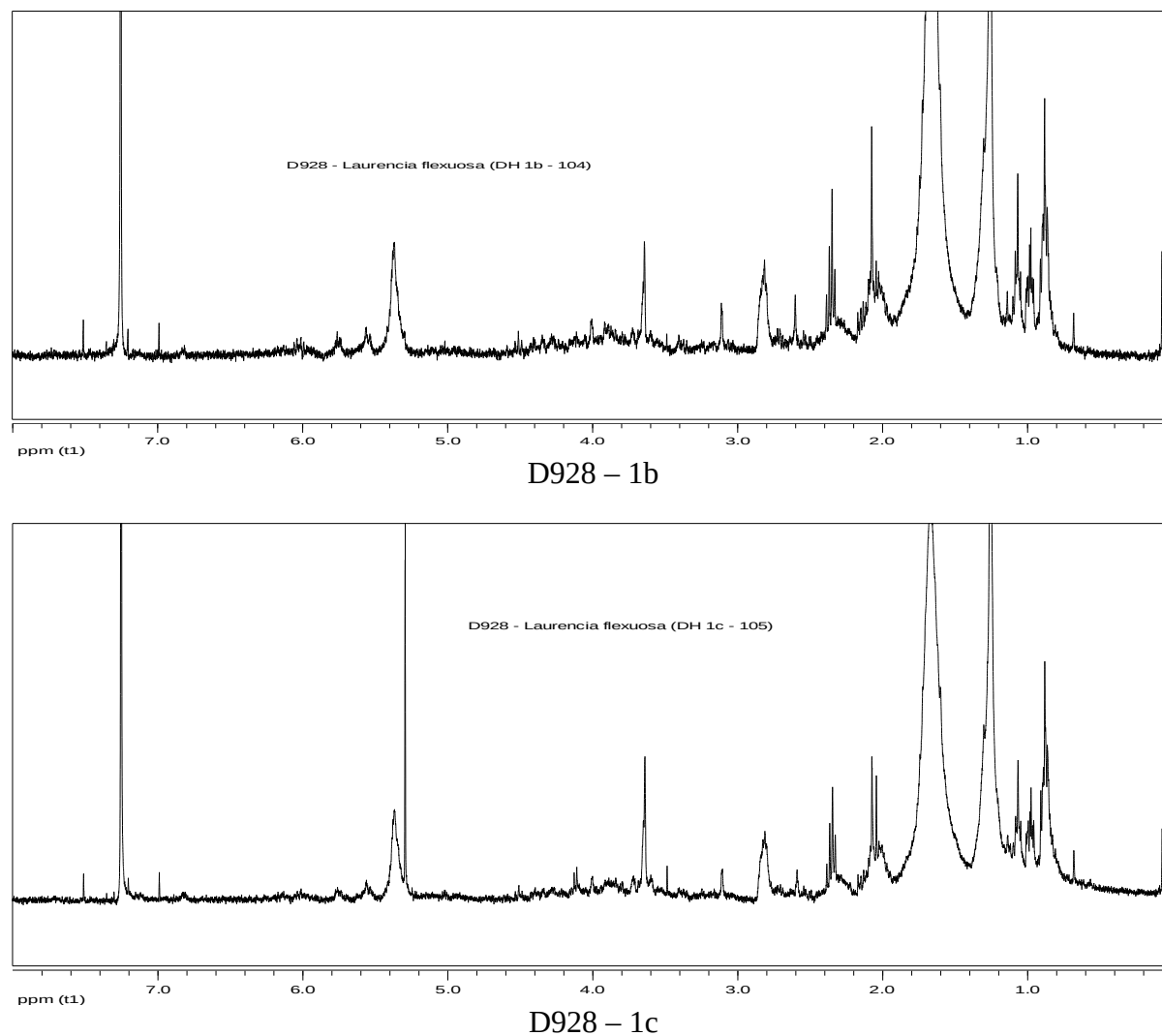
**Figure 5.25**  $^1\text{H}$  NMR data of the D926 (*L. glomerata*) crude extract



**Figure 5.26**  $^1\text{H}$  NMR data of the D927 (*Laurencia* sp. (+ parasite) – not to be confused with *L. flexuosa*) crude extract

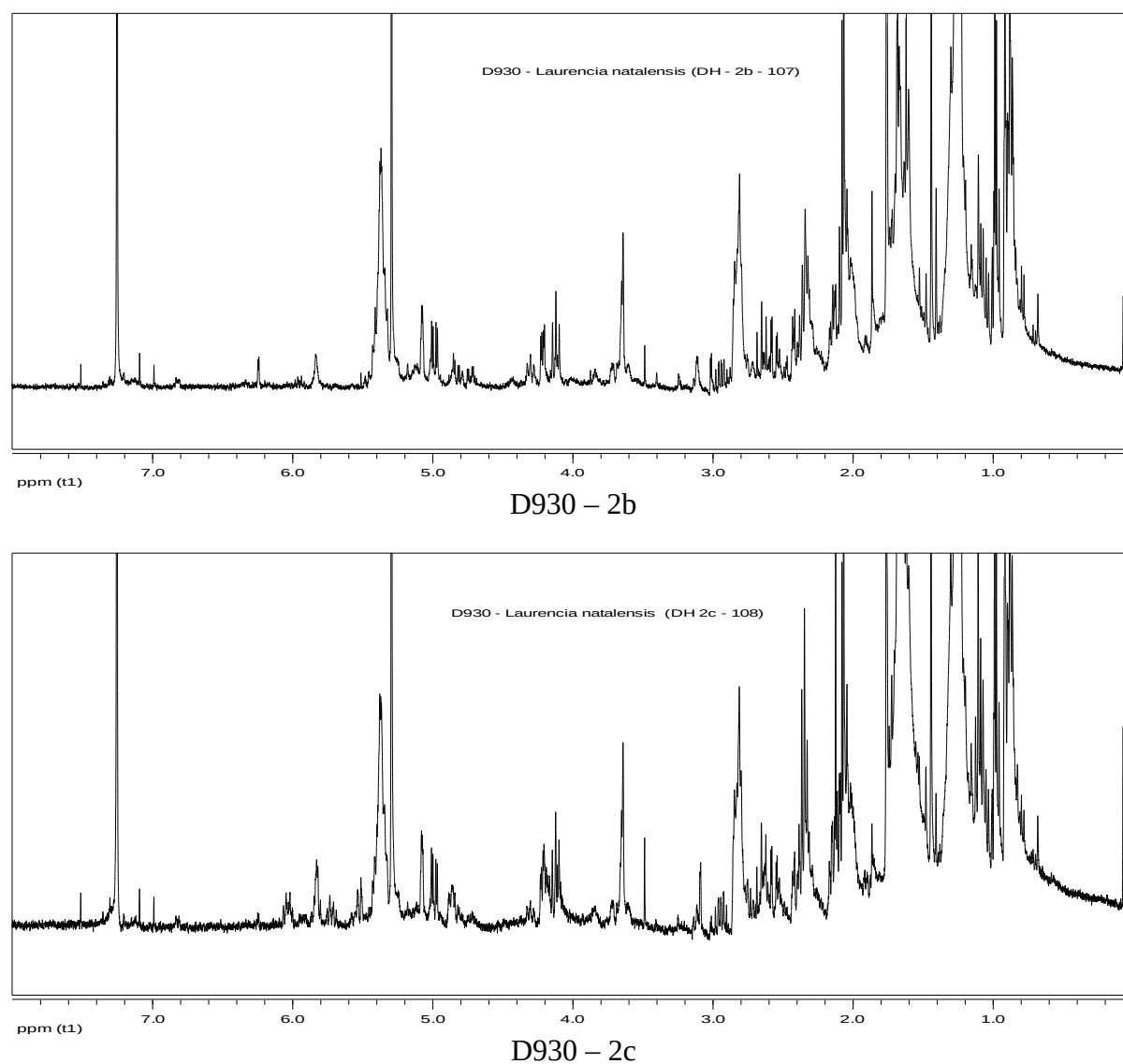
## Appendix 1b

$^1\text{H}$  NMR (400 MHz) data of crude *Laurencia* extracts collected at De Hoop, during February 2011

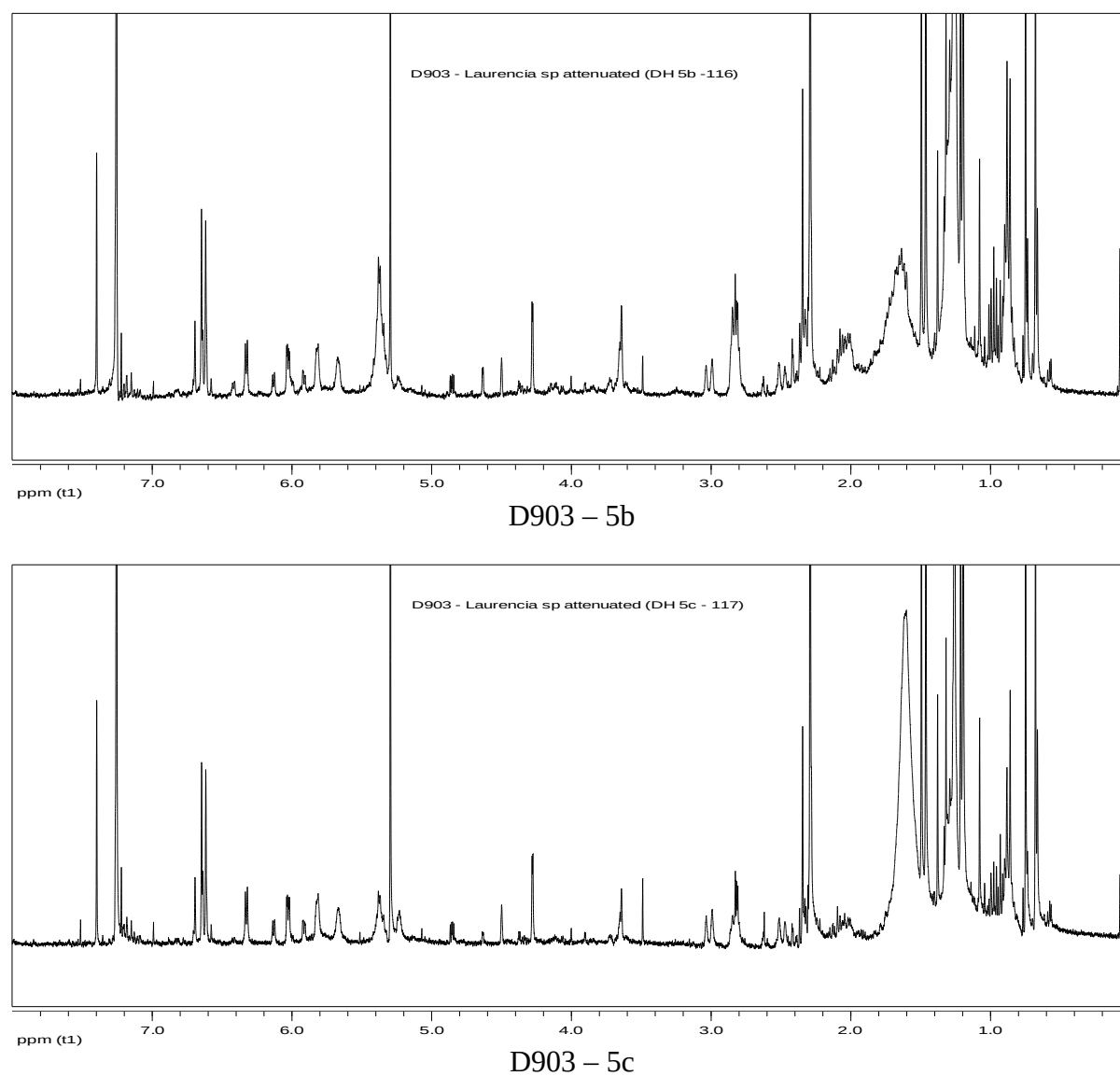


**Figure 5.27**  $^1\text{H}$  NMR spectra of the D928 (*L. flexuosa*) crude extract

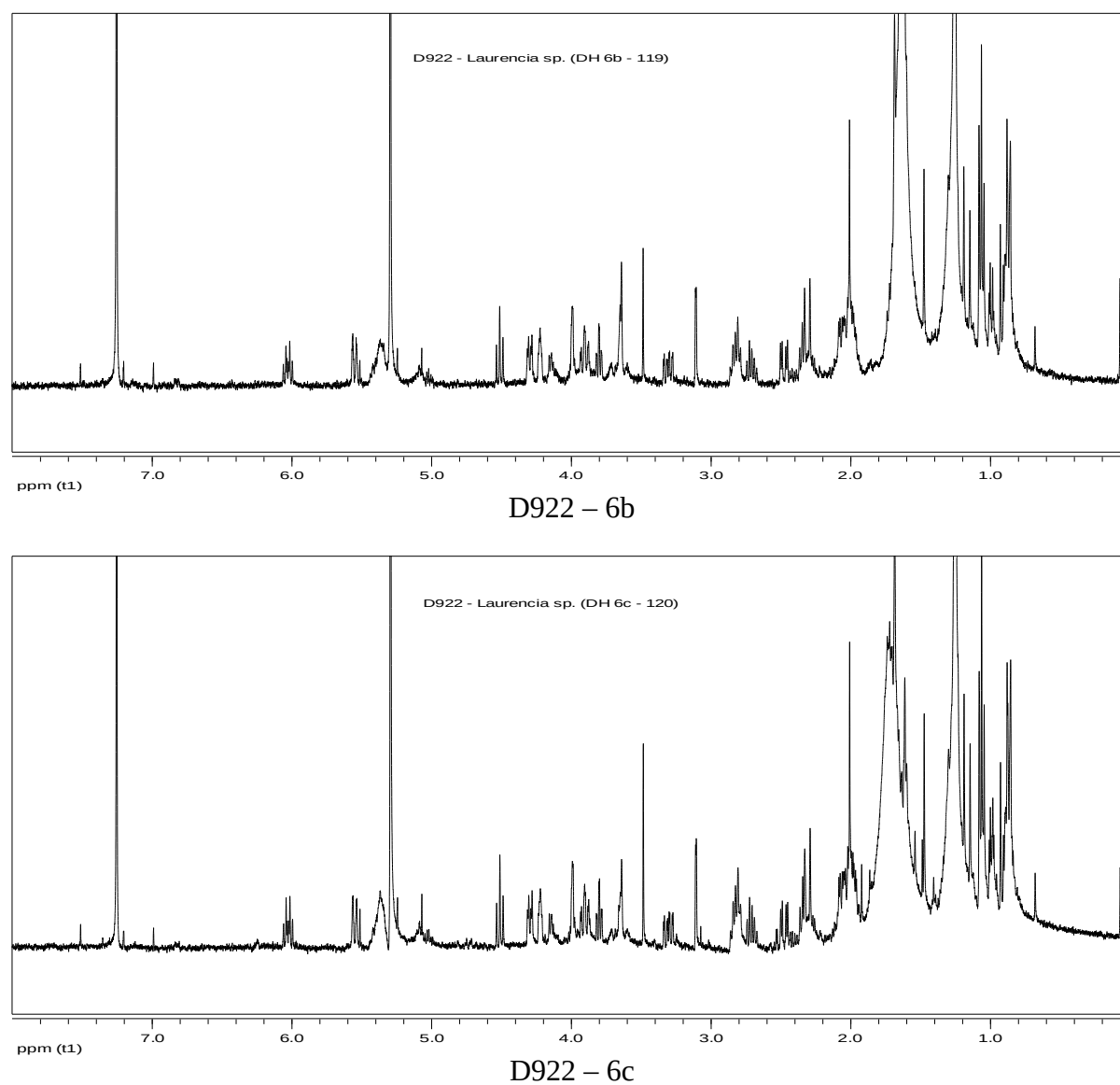




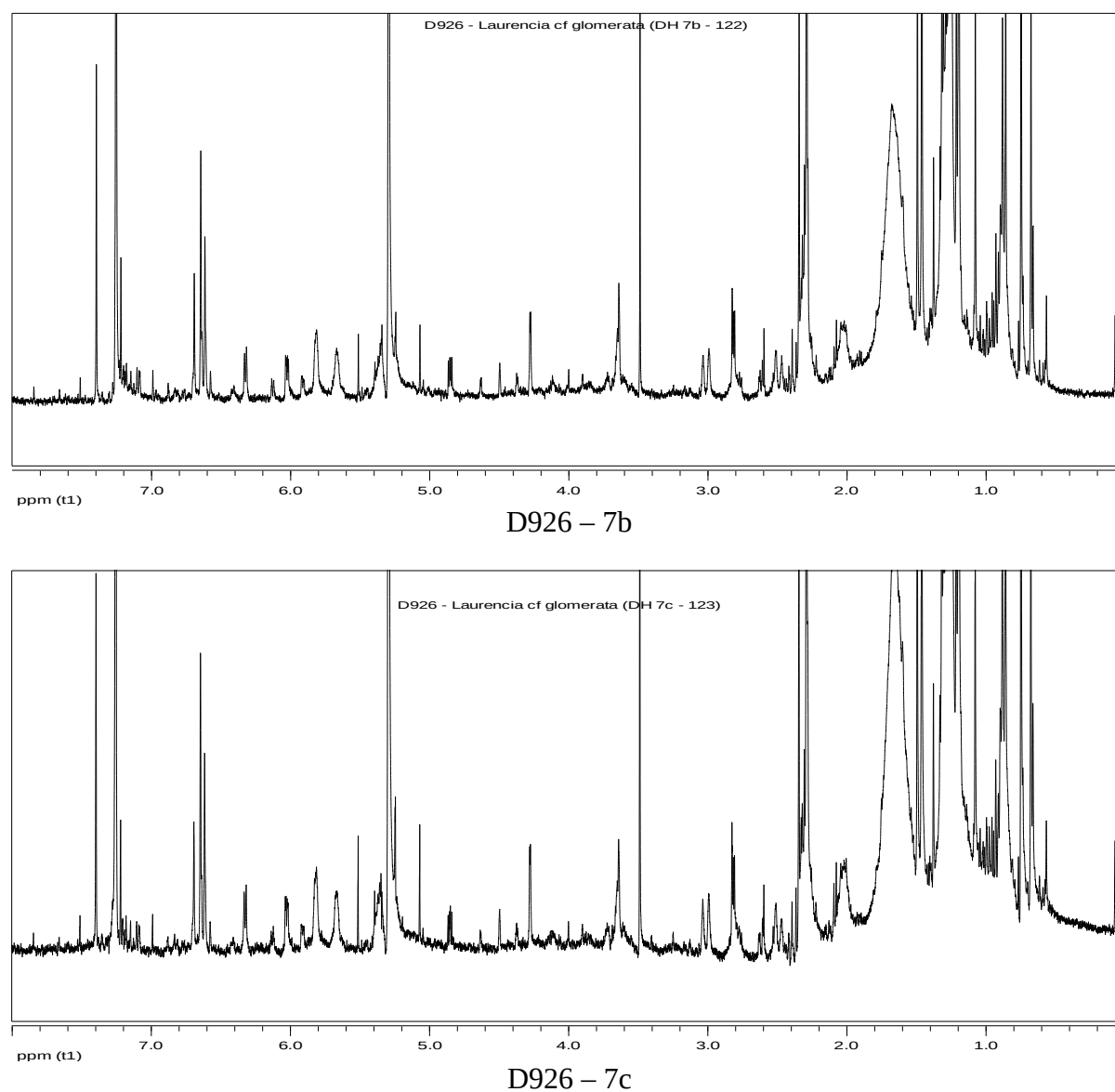
**Figure 5.28**  $^1\text{H}$  NMR spectra of the D930 (*L. natalensis*) crude extract



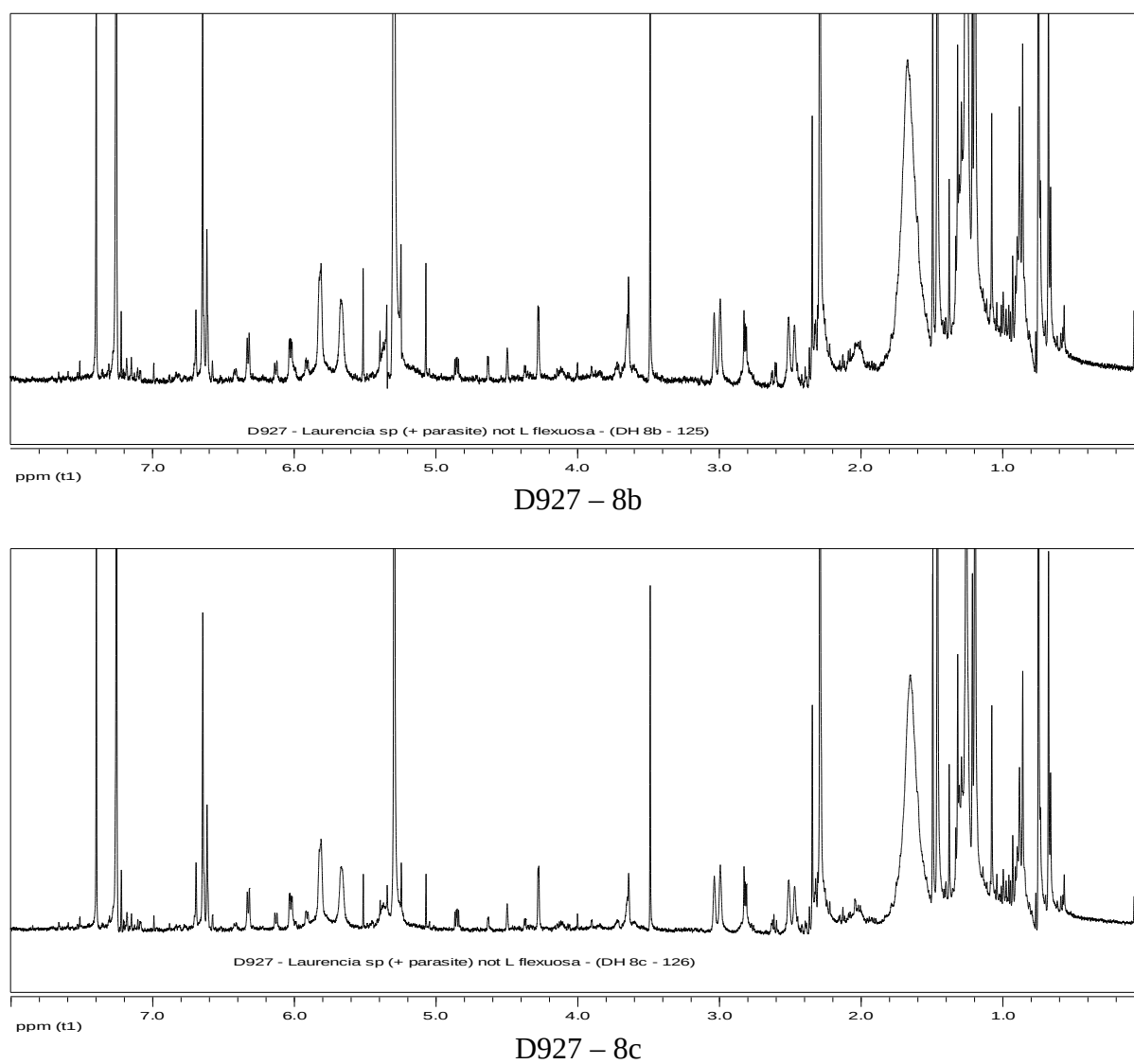
**Figure 5.29**  $^1\text{H}$  NMR spectra of the D903 (*Laurencia* sp. (attenuated)) crude extract



**Figure 5.30**  $^1\text{H}$  NMR spectra of the D922 (*Laurencia* sp.) crude extract



**Figure 5.31**  $^1\text{H}$  NMR spectra of the D926 (*L. glomerata*) crude extract



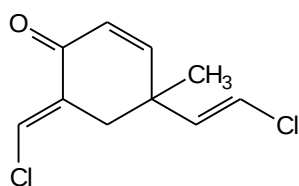
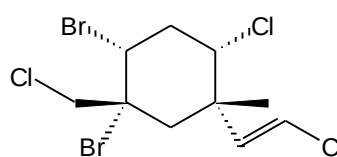
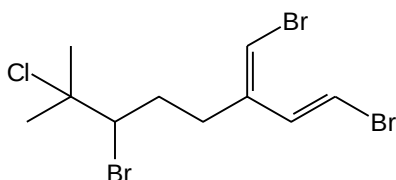
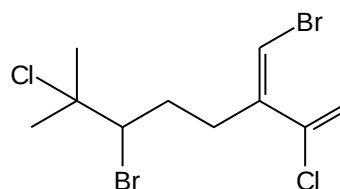
**Figure 5.32**  $^1\text{H}$  NMR spectra of the D927 crude extract

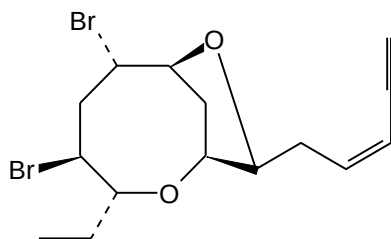
## Chapter Six

## Conclusion

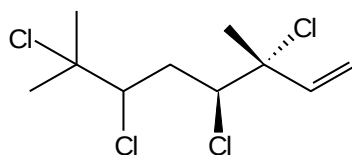
### 6.1 Conclusive remarks

From this research, it appears that the major metabolites isolated from selected South African *Plocamium*, *Portieria* and *Laurencia* species are unique to each species. For example, the major metabolite found in *Plocamium maxillosum* is **2.1**, while in *Plocamium robertiae* it is **2.6**. The major metabolites collect from *Portieria hornemannii* appeared to differ. This might suggest that there are at least two cryptic species of *Portieria hornemannii* in South Africa.  $^1\text{H}$  NMR data suggested that one species of *Portieria hornemannii* has **3.2** as the major metabolite, while the other species has **3.5** as the major metabolite. The major metabolite found in *Laurencia flexuosa* is 3Z-bromofucin (**4.13**). Interestingly, the major compound isolated from *Laurencia glomerata* was *not* the same as those previously isolated by Elsworth and Thomson (1989). This discrepancy could support the idea that there are two different species of *Laurencia glomerata* that look morphologically identical, an idea which was also proposed in chapter five using molecular systematics and  $^1\text{H}$  NMR analysis. However, it should also be noted that since only small samples of *Laurencia glomerata* were investigated, it is possible that other compounds might have been overlooked.

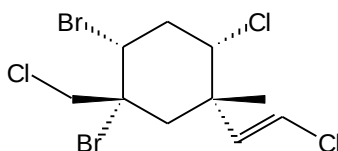
**2.1****2.6****3.2****3.5**

**4.13**

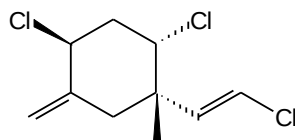
Some degree of overlap appears to occur with regards to some of the minor secondary metabolites isolated in each species of a specific genus, within the South African context. This is most likely due to common biogenic pathways of origin; for example, metabolites from the genus *Plocamium* follow the ocimene biogenesis pathway, while metabolites from the genus *Portieria* follow the mycrene biogenesis pathway (Naylor *et al.*, 1983). Similarly, secondary metabolites from *Laurencia* species have their own pathway for biogenesis. For example, within the *Plocamium* genus; 3,4,6,7-tetrachloro-3,7-dimethyl-1-octene (**1.38**) was isolated from *P. corallorhiza* (Knott, 2003) and *P. Suhrii* (Antunes, 2011).

**1.38**

4,5-dibromo-5-chloromethyl-1-chlorovinyl-2-chloro-methylcyclohexane (**2.6**) had previously been isolated as a minor compound from *P. corallorhiza* (Knott, 2003), but was re-isolated as a major compound in *P. robertiae*.

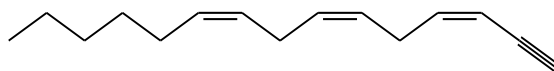
**2.6**

2,4-dichloro-1-chlorovinyl-1-methylcyclohexane-5-ene or Plocamene D (**2.9**) was isolated as a minor compound in *P. corallorhiza* (Mann, 2008) and was then re-isolated as a minor compound in *P. robertiae*.



**2.9**

It was further demonstrated that *neolaurencenyne* (**4.12**) was found as a minor compound in both *L. glomerata* and *L. okamurai*.



**4.12**

These results suggest that understanding the origins of the minor secondary metabolites found in the genus *Plocamium*, *Portieria* and *Laurencia* can be useful in assisting one to define a specific genus.

To the untrained eye, *Plocamium corallorhiza* and *Plocamium robertiae* look very similar to each other. This research also suggested that there are at least two South African cryptic *Portieria hornemannii* species (which appear the same), although their chemistry is differs. Different species of *Laurencia*, while being similar in morphology, have also been well documented. At the risk of stating the obvious, this research highlighted the fact that it is vital to make sure that one is absolutely sure of the identity of a species, before isolating secondary metabolites from it. This is especially true of a place like South Africa where there is an especially high degree of endemic marine algae.

In some cases relative intra-species chemical variation may occur due to geographical and seasonal factors. However, this does not exclude the importance of the fact that the major metabolites reflected by each species (within a genus of either *Plocamium*, *Portieria* or



*Laurencia* in the South African context), appear to be unique to each species, and that these major metabolites (which can be valuable taxonomic indicators) can easily be observed by looking at the crude  $^1\text{H}$  NMR spectra of the species in question. In the future, if the method of correlating molecular systematics with crude  $^1\text{H}$  NMR spectra for the purposes of chemotaxonomy proves to be accurate (as demonstrated in chapter five with several *Laurencia* species), the promising idea of investigating the chemotaxonomy of different *Plocamium* and *Portieria* species in more detail should also be explored in a similar manner.

As was mentioned in chapter one, the principle objective of this thesis was to discover new compounds from South African red marine alga. Exploring the cytotoxic activity of all the new compounds that were isolated, as well as trying to formulate a better understanding of the chemotaxonomic usefulness of these compounds was also key. Thus, the author believes that the data yielded from his research has significantly contributed to this research objective.

Marine algae (and indeed other marine organisms) still have a lot to offer potential bio-prospecting chemists. The number of new compounds that were isolated during this study are a testament to South Africa's unique marine *flora*, which have an unusually high ability to produce original compounds from known marine algae. The good cytotoxic bioactivity profile of some of these compounds, for example compounds **2.1** and **2.2**, further emphasised the pharmacological value of our indigenous marine *flora*. Although there is still much work to be done in the field of South African marine algal bio-prospecting and taxonomy, this thesis will serve as a valuable reference to anyone interested in the chemotaxonomy and cytotoxic potential of South African *Plocamium*, *Portieria* and *Laurencia* species.

## 6.2 References

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