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Chemical Investigation of two Antarctic Invertebrates, Synoicum adareanum (Chordata: Ascidiaceae; Enterogona; Polyclinidae) and Austrodoris kergulenensis (Molusca; Gastropoda; Nudibranchia; Dorididae)

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Chemical Investigation of two Antarctic Invertebrates,

*Synoicum adareanum*

(Chordata: Ascidiaceae: Enterogona: POLYCLINIDAE) and

*Austrodoris kergulenenensis*

(Molusca: Gastropoda: Nudibranchia: Dorididae)

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
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Keywords: Antarctic tunicate, palmerolide macrolide, cytotoxicity, nudibranch, palmadorin

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DEDICATION

This dissertation is dedicated to my family.

To my parents, for their guidance, dedication and wisdom towards my education since my childhood.

To my wife, Dilshani, for her understanding and endurance during a difficult period in a foreign country.

To my daughter Randima, for refreshing my mind with her childhood grace whenever I was tired.

To my late Maternal Grand father Somapala Ratnaweera, who wanted to see me become a medical doctor and help the mankind.

To my late Paternal Grand father Diyabalange Podisinggno, who used to cure patients with snake bites using the herbs grown in our back yard giving me the first lessons of biomolecules.
I wish to express my most sincere gratitude to my major advisor Dr Bill J Baker for his exemplary guidance in last five years. His understanding, sheer dedication and patience have been as invaluable asset to me during this period of extensive research.

I want to thank my committee members Dr Kirpal Bisht, Dr Abdul Malik and Dr Roman Manetsch for their valuable comments and encouragement. I take this opportunity to extend my gratitude to Dr Sridevi Ankisetti and Dr Solomon Weldegirma, post docs at Baker lab. My sincere thanks are also due to all my past and present colleagues at Baker lab for their friendship, encouragements and words of wisdom.

I wish to thank National Cancer Institute and Lee Moffitt Cancer Research Institute for carrying out anticancer activity tests on Palmerolides. I would like to acknowledge US Antarctic Program and National Science Foundation for providing financial assistance for my research.
TABLE OF CONTENTS

LIST OF FIGURES vi
LIST OF TABLES x
LIST OF SCHEMES xi
LIST OF ABBREVIATIONS xii
ABSTRACT xiv

CHAPTER 1. INTRODUCTION

1.1 Drugs from the Sea 1
1.2 Biodiversity of Antarctica 12
1.3 Research Objectives 19
1.4 Summary 20

CHAPTER 2. CHEMICAL INVESTIGATION OF THE ANTARCTIC TUNICATE SYNIOICUM ADAREANUM

2.1 Introduction 21
2.1.1 Tunicates: a rich source of bioactive natural products 21
2.1.2 Chemistry of the genus Synoicum 24
2.1.3 Research Objectives 26

2.2 Results and Discussion 27
2.2.1 Extraction and isolation of secondary metabolites 27
2.2.2 Characterization of palmerolide A 31
2.2.3 Stereochemical determination 41
2.2.3.1 Application of Mosher’s method 41
2.2.3.1.1 R-MTPA monoester of palmerolide A (64) 42
2.2.3.1.2  $R, R$-MTPA diester of palmerolide A (66)  44

2.2.3.1.3  $S$-MTPA monoester of palmerolide A (65)  45

2.2.3.1.4  $S, S$-MTPA diester of palmerolide A (67)  47

2.2.3.1.5  Absolute stereochemistry assignment at C-7 of palmerolide A (58)  48

2.2.3.1.6  Absolute stereochemistry assignment at C-10 of palmerolide A (58)  49

2.2.3.2  Application of Murata’s method  50

2.2.4  Characterization of palmerolide C (59)  55

2.2.5  Characterization of palmerolide D (60)  63

2.2.6  Characterization of palmerolide E (61)  71

2.2.7  Characterization of palmerolide B (62)  78

2.2.8  Characterization of palmerolide H (63)  88

2.2.9  Bioactivity of palmerolides  96

2.2.9.1  $In vitro$ cytotoxicity of palmerolide A (58)  96

2.2.9.2  $In vivo$ cytotoxicity of palmerolide A (58)  97

2.2.9.3  $In vitro$ cytotoxicity of palmerolide C (59)  98

2.2.9.4  $In vitro$ cytotoxicity of palmerolide E (61)  99

2.2.9.5  Mechanism of action of palmerolides  99

2.2.9.6  V-ATPase  99

2.2.9.7  Known V-ATPase inhibitors  100

2.2.9.8  Palmerolides as V-ATPase inhibitors  103

2.3  Summary  103
CHAPTER 3 CHEMICAL INVESTIGATION OF THE ANTARCTIC NUDIBRANCH \textit{Austrodoris kerguelenensis}

3.1 Introduction

3.1.1 Nudibranchs 105

3.1.2 Chemistry of \textit{Austrodoris} 111

3.1.3 Bioactivity 116

3.1.4 Research objectives 116

3.2 Results and Discussion

3.2.1 Extraction and isolation of secondary metabolites 117

3.2.2 Characterization of palmadorin A (110) 118

3.2.3 Stereochemical determination of palmadorin A (110) 125

3.2.3.1 Absolute stereochemistry determination 128

3.2.4 Characterization of palmadorin B (111) 132

3.2.5 Stereochemistry determination of palmadorin B (111) 139

3.2.6 Characterization of palmadorin C (112) 141

3.2.7 Stereochemistry determination of palmadorin C (112) 147

3.2.7.1 Diacetyl derivative of palmadorin C (114) 148

3.2.7.2 \textit{R}-MTPA ester of palmadorin C diacetate (115) 152

3.2.7.3 \textit{S}-MTPA ester of palmadorin C diacetate (116) 155

3.2.7.4 Application of Mosher’s method 160

3.3 Summary 161

CHAPTER 4 EXPERIMENTAL

4.1 General procedure 162
4.2 Isolation of secondary metabolites from *Synoicium adareanum* 163

4.2.1 Palmerolide A (58) 164

4.2.2 Preparation of MTPA esters of palmerolide A 165

4.2.2.1 Palmerolide A 7-(R)-MTPA ester (64) 165

4.2.2.2 Palmerolide A 7,10-(R,R)-MTPA diester (66) 166

4.2.2.3 Palmerolide A 7-(S)-MTPA ester (65) 167

4.2.2.4 Palmerolide A 7,10-(S,S)-MTPA diester (67) 167

4.2.3 Palmerolide C (59) 168

4.2.4 Palmerolide D (60) 169

4.2.5 Palmerolide E (61) 170

4.2.6 Palmerolide B (62) 171

4.2.7 Palmerolide H (63) 172

4.3 Isolation of secondary metabolites from *Austrodoris kerguelenensis* 173

4.3.1 Palmadorin A (110) 174

4.3.2 Ozonolysis of palmadorin A (110) 174

4.3.3 Ozonolyzed product of palmadorin A (113) 175

4.3.4 Palmadorin B (111) 175

4.3.5 Palmadorin C (112) 176

4.3.6 Acetylation of palmadorin C (112) 177

4.3.7 Palmadorin C diacetate (114) 177
4.3.8 Preparation of $R$-MTPA esters of palmadorin C diacetate (114) 178

4.3.9 Palmadorin C diacetate $R$-MTPA (115) 178

4.3.10 Preparation of $S$-MTPA esters of palmadorin C diacetate (114) 179

4.3.11 Palmadorin C diacetate $S$-MTPA (116) 179

REFERENCES 180

APPENDICES 188

Appendix A Cytotoxicity profile of palmerolide A 188

ABOUT THE AUTHOR End Page
LIST OF FIGURES

Figure 1. *Synoicum adareanum* at Palmer Station Antarctica (photograph supplied by Bill J. Baker, University of South Florida) 26

Figure 2. LRFABMS positive mode spectrum of palmerolide A (58) 31

Figure 3. $^{13}$C NMR spectrum of palmerolide A (58) (125 MHz, DMSO-$d_6$) 32

Figure 4. DEPT 135 spectrum of palmerolide A (58) (125 MHz, DMSO-$d_6$) 33

Figure 5. $^1$H NMR spectrum of palmerolide A (58) (500 MHz, DMSO-$d_6$) 34

Figure 6. gHMBC spectrum of palmerolide A (58) (500 MHz, DMSO-$d_6$) 36

Figure 7. gHSQC spectrum of palmerolide A (58) (500 MHz, DMSO-$d_6$) 37

Figure 8. Key gHMBC and gCOSY correlations of palmerolide A (58) 38

Figure 9. gCOSY spectrum of palmerolide A (58) (500 MHz, DMSO-$d_6$) 39

Figure 10. Palmerolide A (58) planer structure 41

Figure 11. $^1$H NMR spectrum of palmerolide A $R$-MTPA monoester (64)

(500 MHz, CD$_3$OD) 43

Figure 12. Key gHMBC correlations demonstrating the attachment of MTPA in compound 64. 44

Figure 13. $^1$H NMR spectrum palmerolide A $R$, $R$-MTPA diester (66)

(500 MHz, CD$_3$OD) 44

Figure 14. Key gHMBC correlations demonstrating the attachment of MTPA moiety in compound 66. 45

Figure 15. $^1$H NMR spectrum of palmerolide A $S$-MTPA monoester (65)

(500 MHz, CD$_3$OD) 46
Figure 16. Key gHMBC correlations demonstrating the attachment of MTPA moiety in compound 65.

Figure 17. $^1$H NMR spectrum of palmerolide A $S, S$-MTPA diester (67)

Figure 18. Key gHMBC correlations demonstrating the attachment of MTPA moiety in compound 67

Figure 19. $\Delta \delta$ Chemical shift differences ($\delta \Delta^* 1000$) of palmerolide A MTPA $R$- and $S$-monoesters

Figure 20. MTPA model for configuration correlations

Figure 21. $\Delta \delta$ Chemical shift values ($\Delta \delta^* 1000$) of palmerolide A MTPA $R, R$- and $S, S$- diesters

Figure 22. gHSQMBC spectrum of palmerolide A (58)

Figure 23. 1D analysis of the gHSQMBC cross peak of the respective slice

Figure 24. Determination of the coupling constant by the subtraction of

$^1$H NMR spectrum;

Figure 25. Coupling constant based configuration analysis

Figure 26. ROESY spectrum of palmerolide A (58) (500 MHz, DMSO-$d_6$)

Figure 27. Key ROESY correlations supporting the stereochemical determination of C-19 and C-20.

Figure 28. Full stereochemical assignment of palmerolide A (58)

Figure 29. Palmerolide C (59) HRESIMS spectrum
Figure 30. $^{13}$C NMR spectrum of palmerolide C (59) (125 MHz, DMSO-$d_6$)

Figure 31. DEPT 135 spectrum of palmerolide C (59) (125 MHz, DMSO-$d_6$)

Figure 32. gHMBC spectrum of palmerolide C (59) (500 MHz, DMSO-$d_6$)

Figure 33. gCOSY spectrum of palmerolide C (59) (500 MHz, DMSO-$d_6$)

Figure 34. Key gHMBC and gCOSY correlations of palmerolide C (59)

Figure 35. $^1$H NMR spectrum of compound 59 (500 MHz, DMSO-$d_6$)

Figure 36. gHMQC spectrum of palmerolide C (59) (500 MHz, DMSO-$d_6$)

Figure 37. HRESIMS spectrum of palmerolide D (60)

Figure 38. $^{13}$C NMR spectrum of palmerolide D (60) (125 MHz, DMSO-$d_6$)

Figure 39. DEPT 135 spectrum of palmerolide D (60) (125 MHz, DMSO-$d_6$)

Figure 40. Key gHMBC and gCOSY correlations of Palmerolide D (60)

Figure 41. gHMBC spectrum of palmerolide D (60) (500 MHz, DMSO-$d_6$)

Figure 42. gCOSY spectrum of palmerolide D (60) (500 MHz, DMSO-$d_6$)

Figure 43. gHMQC spectrum of palmerolide D (60) (500 MHz, DMSO-$d_6$)

Figure 44. $^1$H NMR spectrum of palmerolide D (60) (500 MHz, DMSO-$d_6$)

Figure 45. Planer structure of palmerolide D (60)

Figure 46. Palmerolide E (61) HRESIMS spectrum

Figure 47. Key gHMBC and gCOSY correlations of palmerolide E (61)

Figure 48. $^1$H NMR spectrum of palmerolide E (61) (500 MHz, DMSO-$d_6$)

Figure 49. gHMQC spectrum of palmerolide E (61) (500 MHz, DMSO-$d_6$)

Figure 50. gHMBC spectrum of palmerolide E (61) (500 MHz, DMSO-$d_6$)
Figure 51. gCOSY spectrum of palmerolide E (61) (500 MHz, DMSO-d$_6$)  
Figure 52. $^1$H NMR spectrum of palmerolide B (62) (500 MHz, CD$_3$OD)  
Figure 53. $^{13}$C NMR spectrum of palmerolide B (62) (125 MHz, CD$_3$OD)  
Figure 54. DEPT 135 spectrum of palmerolide B (62) (125 MHz, CD$_3$OD)  
Figure 55. gHSQC spectrum of palmerolide B (62) (500 MHz, CD$_3$OD)  
Figure 56. gHMBC spectrum of palmerolide B (62) (500 MHz, CD$_3$OD)  
Figure 57. Key gCOSY and gHMBC correlations of palmerolide B (62)  
Figure 58. gCOSY spectrum of palmerolide B (62) (500 MHz, CD$_3$OD)  
Figure 59. HRESIMS spectrum of palmerolide B (62)  
Figure 60. Fragmentation of palmerolide B (62) in LR ESIMS positive mode  
Figure 61. $^1$H NMR spectrum of palmerolide H (63) (500 MHz, CD$_3$OD)  
Figure 62. $^{13}$C NMR spectrum of palmerolide H (63) (125 MHz, CD$_3$OD)  
Figure 63. gHSQC spectrum of palmerolide H(63)(500 MHz, CD$_3$OD)  
Figure 64. gHMBC spectrum of palmerolide H(63)(500 MHz, CD$_3$OD)  
Figure 65. gCOSY spectrum of palmerolide H (63) (500 MHz, CD$_3$OD)  
Figure 66. Key gHMBC and gCOSY correlations of palmerolide H (63)  
Figure 67. HRESIMS spectrum of palmerolide H (63)  
Figure 68. *Austrodoris kerguelenensis* at Bonaparte Point Antarctica (photograph supplied by Bill J. Baker, University of South Florida)  
Figure 69. LRFABMS spectrum of palmadorin A (110)  
Figure 70. $^{13}$C NMR spectrum of palmadorin A (110) (125 MHz, CDC$_1$3)  
Figure 71. $^1$H NMR spectrum of palmadorin A (110) (500 MHz, CDC$_1$3)  
Figure 72. gHSQC spectrum of palmadorin A (110) (500 MHz, CDC$_1$3)
Figure 73. gHMBC spectrum of palmadorin A (110) (500 MHz, CDCl₃)  
Figure 74. Key gHMBC and gCOSY correlations of palmadorin A (110)  
Figure 75. ROESY spectrum of palmadorin A (110) (500 MHz, CDCl₃)  
Figure 76. Selective 1D NOE experiments of palmadorin A (500 MHz, CDCl₃)  
Figure 77. Key ROESY correlations of palmadorin A (110)  
Figure 78. Key gHMBC correlations of 113  
Figure 79 Two possible trans decalin enantiomers of palmadorin A  
Figure 80. CD spectrum of compound 113 showing a negative cotton effect  
Figure 81. Absolute stereochemistry of palmadorin A (110)  
Figure 82. LRFABMS spectrum of palmadorin B (111)  
Figure 83 ¹³C NMR spectrum of palmadorin B (111) (125 MHz, CDCl₃,)  
Figure 84. gHMBC spectrum of palmadorin B (111) (500 MHz, CDCl₃)  
Figure 85. gHSQC spectrum of palmadorin B (111) (500 MHz, CDCl₃)  
Figure 86. gCOSY spectrum of palmadorin B (111) (500 MHz, CDCl₃)  
Figure 87. Key gHMBC and COSY correlations of palmadorin B (111)  
Figure 88. ¹H NMR spectrum of palmadorin B (109) (500 MHz, CDCl₃)  
Figure 89. ROESY spectrum of palmadorin B (109) (500 MHz, CDCl₃)  
Figure 90. Palmadorin B (111) ROESY correlations  
Figure 91. Absolute stereochemistry of palmadorin B (111)  
Figure 92. LRFABMS spectrum of palmadorin C (112)  
Figure 93. ¹³C NMR spectrum of palmadorin C (112) (125 MHz, CDCl₃)  
Figure 94. gHSQC spectrum of palmadorin C (112) (500 MHz, CDCl₃)  
Figure 95. ¹H NMR spectrum of palmadorin C (112) (500 MHz, CDCl₃)
Figure 96. gHMBC spectrum of palmadorin C (112) (500 MHz, CDCl$_3$) 144
Figure 97. gCOSY spectrum of palmadorin C (112) (500 MHz, CDCl$_3$) 145
Figure 98. Key HMBC and COSY correlations of palmadorin C (110) 145
Figure 99. Key ROESY correlations of palmadorin C (112) 148
Figure 100. $^1$H NMR spectrum of palmerolide C diacetate (114)
(500MHz, CDCl$_3$) 149
Figure 101. $^{13}$C NMR spectrum of palmadorin C diacetate (114)
(125 MHz, CDCl$_3$) 150
Figure 102. Key HMBC correlations of palmadorin C diacetate (112) 151
Figure 103. LRESIMS spectrum of palmadorin C diacetate (112) 151
Figure 104. LRESIMS spectrum of palmadorin C diacetate
$R$-MTPA ester (115) 152
Figure 105. $^1$H NMR spectrum of palmadorin C diacetate $R$-MTPA ester
(115) (500 MHz, CDCl$_3$) 154
Figure 106. gHSQC spectrum of palmadorin C diacetate $R$-MTPA ester
(115) (500 MHz, CDCl$_3$) 154
Figure 107. gHMBC spectrum of palmadorin C diacetate $R$-MTPA ester
(115) (500 MHz, CDCl$_3$) 155
Figure 108. LRESIMS of palmadorin C diacetate $S$- MTPA (116) 156
Figure 109 $^{13}$C NMR spectrum of palmadorin C diacetate $S$-MTPA ester
(116) (125 MHz, CDCl$_3$) 157
Figure 110. $^1$H NMR spectrum of palmadorin C diacetate $S$-MTPA ester
(116) (500 MHz, CDCl$_3$) 158
Figure 111. gHSQC spectrum of palmadorin C diacetate $S$-MTPA ester
(116) (500 MHz, CDCl$_3$) 158
Figure 112. gHMBC spectrum of palmadorin C diacetate $R$-MTPA ester
(114) (500 MHz, CDCl$_3$) 159
Figure 113. $\Delta \delta$ value assignments for palmadorin C diacetate MTPA esters

Figure 114 Absolute stereochemistry of palmadorin C (112)
# LIST OF TABLES

Table 1. Potential therapeutic compounds isolated from marine sources 3

Table 2. NMR data of palmerolide A (58)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{DMSO-}d_6 \) 35

Table 3. NMR data of palmerolide C (59)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{DMSO-}d_6 \) 61

Table 4. NMR data of palmerolide D (60)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{DMSO-}d_6 \) 70

Table 5. NMR data of palmerolide E (61)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{DMSO-}d_6 \) 76

Table 6. NMR data of palmerolide B (62)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{CD}_3\text{OD} \) 83

Table 7. NMR data of palmerolide H (63)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{CD}_3\text{OD} \) 93

Table 8. Comparison of cytotoxicity and V-ATPase activity 103

Table 9. NMR data of palmadorin A (108)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{CDCl}_3 \) 124

Table 10. NMR data of palmadorin B (109)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{CDCl}_3 \) 137

Table 11. NMR data of palmadorin C (110)  
\( ^1\text{H}, 500 \text{ MHz}, ^{13}\text{C}, 125 \text{ MHz}, \text{CDCl}_3 \) 146
<table>
<thead>
<tr>
<th>Scheme 1. Extraction and purification of palmerolides</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme 2. Extraction and purification of palmadorins</td>
<td>118</td>
</tr>
<tr>
<td>Scheme 3. Ozonolysis of palmadorin A</td>
<td>129</td>
</tr>
<tr>
<td>Scheme 4. Acetylation of palmadorin C</td>
<td>149</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ac$_2$O</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>$[\alpha]$</td>
<td>specific rotation = 100$\alpha$/lc</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>deuterated chloroform</td>
</tr>
<tr>
<td>CD$_3$OD</td>
<td>deuterated methanol</td>
</tr>
<tr>
<td>C18</td>
<td>octadecyl bonded silica</td>
</tr>
<tr>
<td>$\delta$</td>
<td>chemical shifts (NMR)</td>
</tr>
<tr>
<td>DEPT</td>
<td>distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DMAP</td>
<td>dimethyl amino pyridine</td>
</tr>
<tr>
<td>DMSO-$d_6$</td>
<td>deuterated dimethylsulfoxide</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethylacetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>the molar extinction coefficient in UV spectroscopy</td>
</tr>
<tr>
<td>gCOSY</td>
<td>gradient correlation spectroscopy (NMR)</td>
</tr>
<tr>
<td>gHSQC</td>
<td>gradient heteronuclear single quantum correlation (NMR)</td>
</tr>
<tr>
<td>gHMQC</td>
<td>gradient heteronuclear multiple quantum coherence (NMR)</td>
</tr>
<tr>
<td>gHMBC</td>
<td>gradient heteronuclear multiple bond connectivity (NMR)</td>
</tr>
<tr>
<td>HRFABMS</td>
<td>high resolution fast atom bombardment mass spectrometry</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>$J$</td>
<td>coupling constant</td>
</tr>
<tr>
<td>$n_J^{CH}$</td>
<td>n-bond hydrogen to carbon correlation (n = 2,3 or 4)</td>
</tr>
<tr>
<td>$n_J^{HH}$</td>
<td>n-bond hydrogen to hydrogen correlation (n = 2,3 or 4)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LRESIMS</td>
<td>low resolution electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>LRFABMS</td>
<td>low resolution fast atom bombardment mass spectrometry</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>the wavelength at which maximum absorption occurs</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MTPA-Cl</td>
<td>$\alpha$-methoxy-$\alpha$-(trifluoromethyl)phenylacetyl chloride</td>
</tr>
<tr>
<td>$m/z$</td>
<td>mass/charge ratio in mass spectrometry</td>
</tr>
<tr>
<td>ROESY</td>
<td>rotating-frame overhauser enhancement spectroscopy (NMR)</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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</table>
Synoicum adareanum is a colonial tunicate commonly found on the benthos around Palmer Station on Anvers Island, Antarctica. A comprehensive chemical investigation of the lipophilic extract of the frozen tunicates gave a new series of polyketide macrolides, palmerolides A-E and H. The structure elucidation of these compounds was accomplished by extensive NMR and mass spectral studies.

The palmerolides are unusual 20-membered macrolides displaying functionality more commonly found in sponges or cyanobacteria. Palmerolide A, the major member of the group, shows significant and selective in vitro cytotoxicity against melanoma with three orders of greater sensitivity relative to other cell lines tested, in the National Cancer Institute (NCI) 60 human cancer cell-line panel. In addition, it displays potent cytostatic activity against several other cancer cell lines. Based on NCI’s COMPARE analysis, palmerolide A was investigated as a V-ATPase inhibitor and shown to bind the V₀ subunit with 2 nM inhibition.

Austrodoris kerguelenensis is a common Antarctic nudibranch widely distributed in the High Antarctic and Sub Antarctic Zone. It is characterized by the presence of terpenoid glyceryl esters which are supposed to be involved in defense. Chemical investigations of several specimens of A. kerguelenensis collected near Palmer station Antarctica afforded
hitherto undescribed series of clerodane diterpenoid glycerides. The structure elucidation of three major compounds of this series, palmadorin A, B and C was accomplished.
Chapter 1. INTRODUCTION

1.1 Drugs from the Sea

The oceans compose more than 70% of the earth’s surface and over 90% of its volume of its crust.\textsuperscript{1,2} The marine environment has been an exceptional reservoir of bioactive natural products.\textsuperscript{3} Due to the physical and chemical features of oceans, such as extreme variation of temperature, pressure and salinity, marine organisms have evolved biochemical and physiological mechanisms that give rise to bioactive compounds with unique structural features, often not found among terrestrial natural products. These biomolecules play a pivotal role in reproduction, communication and protection against predation, infection and competition in the oceanic environment.\textsuperscript{4,5}

Many marine organisms are soft-bodied or move slow whereas some of them have sedentary life styles. However, they often do not have physical armament like hard external shells or spicules for their protection, making them extremely vulnerable to predation and competition.\textsuperscript{4,5} Such organisms need some form of defense. Many marine organisms have developed defenses employing bioactive natural products against their enemies.\textsuperscript{4,5} These compounds, belonging to the category of secondary metabolites, help them to either deter or have a competitive edge over the predators.\textsuperscript{4}

Some of these marine organisms have evolved the ability to synthesize their own secondary metabolite defense chemicals via \textit{de novo} biosynthesis. Other organisms have
been found to derive their defense chemicals by a symbiotic relationship or simply from their diet.\textsuperscript{4}

Beyond chemical diversity, the marine environment is known to hold enormous biological diversity as well. Evidence for this coming from recent research on marine ecosystems like the deep sea floor and coral reefs. In fact, their biodiversity is known to be higher than that of tropical rain forests, a terrestrial ecosystem renown for its enormous biodiversity.\textsuperscript{2} For instance, according to recent estimates, out of the all species living on this planet only 10% are known and the unexplored majority live in the sea\textsuperscript{1,3} Meanwhile, out of the 34 fundamental phyla of life, 17 occur on land whereas 32 occur in the sea (with some overlap). The deep sea floor harbors millions of hither to unknown species including thousands of marine microorganisms.\textsuperscript{1,2}

It has been estimated that more than 10,000 marine natural products have been isolated by 2000.\textsuperscript{6} Among them 25% are from algae, 33% from sponges, 24% from coelenterates and the remaining 24% from representatives of other invertebrate phyla such as ascidians, ophisthoibranch molluscs, echinoderms and bryozoans.\textsuperscript{2} Further analysis of this data highlighted that the search for drugs from the sea progresses at a rate of 10% per year.\textsuperscript{2}

During last few years several such marine natural products have successfully advanced into clinical trials (Table 1). However, none of these discoveries have reached a marketable stage.
Table 1. Potential therapeutic compounds isolated from marine sources (Adapted from reference 3)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Compound</th>
<th>Organism</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cancer</strong></td>
<td>Aplidine\textsuperscript{7}</td>
<td>Tunicate</td>
<td>Mediterranean</td>
</tr>
<tr>
<td>HIV</td>
<td>Bryostatin 1\textsuperscript{8}</td>
<td>Bryozoan</td>
<td>Gulf of California</td>
</tr>
<tr>
<td></td>
<td>Didemnin B\textsuperscript{9}</td>
<td>Tunicate</td>
<td>Caribbean</td>
</tr>
<tr>
<td></td>
<td>Dolastanin 10\textsuperscript{10}</td>
<td>Sea hare</td>
<td>Indian Ocean</td>
</tr>
<tr>
<td></td>
<td>Ecteinascidin 743\textsuperscript{11,12}</td>
<td>Tunicate</td>
<td>Caribbean</td>
</tr>
<tr>
<td></td>
<td>Halichondrin B\textsuperscript{13}</td>
<td>Sponge</td>
<td>Okinawa</td>
</tr>
<tr>
<td></td>
<td>Kahalaide F\textsuperscript{14}</td>
<td>Mollusc</td>
<td>Hawaii</td>
</tr>
<tr>
<td></td>
<td>Mycaperoxide B\textsuperscript{15}</td>
<td>Sponge</td>
<td>Thailand</td>
</tr>
<tr>
<td></td>
<td>Cycloclidemniserol</td>
<td>Tunicate</td>
<td>Palau</td>
</tr>
<tr>
<td></td>
<td>Lamellarin A 20 sulphate\textsuperscript{17}</td>
<td>Tunicate</td>
<td>Australia</td>
</tr>
</tbody>
</table>

The history of marine derived drugs dates as far back as the 1950s when Bergmann \textit{et al.} isolated several nucleoside analogues from the Caribbean sponge \textit{Tethya crypta}.\textsuperscript{18} Two of these compounds, spongorthymidine (1) and spongouridine (2) had a rare arabinose sugar rather than ribose, which is more common in nucleosides. Later, the discovery of their antiviral activity led the researchers to synthesize a wide range of analogues\textsuperscript{19,20} such as Ara-A (3) and Ara-C (4), which have demonstrated significantly improved activity. They
represent the only marine derived drugs in current clinical usage. Bergmann’s initial discovery can be considered a significant breakthrough as it led to the introduction of nucleoside analogues in antiviral and anticancer therapy with profound success (eg. 3’-azido-3’-deoxythymidine (AZT, Zidovudin) (5)).

Isolated from the bryozoan _Bugula neritina_, bryostatin 1\(^8\) (6) is one of the first drug candidates from the ocean to advance into clinical trials. Bryostatin 1, a complex polyketide which inhibits Protein Kinase C and thereby prevents cancer, is in Phase II clinical trials.\(^2\) It has been found that bryostatin 1 is not effective in cancer treatment by itself, but seems to enhance the activity of other anticancer drugs such as taxol and...
cisplatin. Therefore, it may be used in combination with taxol, in the treatment of breast, ovarian and lung cancers which usually respond to taxol.³

![Bryostatin 1 (6)](image)

Discodermolide²³ (7) is a polyketide isolated from the marine sponge *Discodermia dissolute*. It was found to be a potent antitumor agent that inhibited the polymerization of microtubules. Structure-activity studies of discodermolide and its synthetic analogues have shown that it has a greater promise and versatility than taxol. Both, the natural product (+)-discodermolide and its synthetic analogue (-)-discodermolide have been found to be active as inhibitors of cell proliferation despite their mechanism of action differing considerably.²⁴

![(+)-Discodermolide (7)](image)
Eleutherobin (8)\textsuperscript{25} and sarcodictyin A (9) and B (10)\textsuperscript{26} are a group of diterpenes isolated from different sources despite their distinct similarities in structure. Eleutherobin was isolated from \textit{Eleutherobia} species of soft corral found in the Indian Ocean near Western Australia\textsuperscript{25} whereas sarcodictyins were isolated from the Mediterranean soft coral \textit{Sarcodictyon roseum}.\textsuperscript{26} Both compounds showed microtubule stabilization properties.\textsuperscript{24,27}

\[
\text{Eleutherobin (8)} \hspace{2cm} \text{Sarcodictyin A (9) R = Me}
\]
\[
\text{Sarcodictyin B (10) R = Et}
\]

Dolastatin 10\textsuperscript{10} (11), isolated from the sea hare \textit{Dolabella auricularia} collected from the Indian ocean is a short polypeptide containing unique amino acids which showed microtubule stabilization properties.\textsuperscript{6} Later the same compound has been isolated from a nudibranch and a cyanobacterium. Dolastatine 10 is in Phase I clinical trials as anticancer agent for use in the treatment of breast and liver cancer.\textsuperscript{24}
Dolastatin 10 (11)

Ecteinascidins\textsuperscript{11,28} isolated from the colonial ascidian \textit{Ecteinascidia turbinata} found in the Caribbean and Mediterranean, are novel cytotoxins that act via DNA intercalation. Ecteinascidin 743 (12) was selective against breast cancer and melanoma. Currently it is undergoing Phase II clinical trials.\textsuperscript{3}

Ecteinascidin 743 (12)

Recent research on the anticancer activity of ecteinascidin 743 suggestes its potential to prevent tumors from becoming drug resistant.\textsuperscript{29} It was discovered that it can prevent the formation of P-glycoprotein, a membrane protein that transports toxins out of the cancer cell, thereby preventing such agents from destroying the tumor. Once chemotherapy is
administered this protein has the ability of preventing it reaching the intended target. In fact, it was found that when tumors are exposed to chemotherapeutic agents, they quickly boost the activity of the MDR1 gene, that is responsible for the formation of P-glycoprotein, which ultimately results in multidrug resistance. Therefore, ecteinascidin 743 has the potential to increase the susceptibility of the tumor cells to chemotherapy by inhibiting the transcription of this gene, in addition to being a potent chemotherapeutic agent by itself.

Kahalaide F (13), a cyclic depsipeptide isolated from the sea slug *Elysia rufescens*, is another anticancer drug candidate in clinical trails. However, it is believed that actual organism which produces the compound is the green algae *Bryopsis* sp. on which it feeds.
Kahalaide F has a unique pattern of cytotoxicity with selective \textit{in vitro} and \textit{in vivo} cytotoxicity against prostate cancer. In particular, it has been shown to be selective against hormone-independent prostate tumor cells which are aggressive and hard to treat. Phase I clinical trails of kahalaide F in patients with androgen independent prostate cancer have already begun.\textsuperscript{3}

Didemnin B (14), an unusual cyclic depsipeptide isolated from the Caribbean tunicate \textit{Trididemnum solidom} in the 1980s, generated great excitement due to its pronounced antitumor activity.\textsuperscript{7,16,30,31} Later, it was found to show antineoplastic, antiviral and immunosuppressive activities as well. Its mechanism of action was identified as due to the interference in protein synthesis. Despite its toxicity being too high to be useful as an antiviral or immunosuppressive agent, it has been in Phase I clinical trails as an anticancer agent and eventually promoted to Phase II. However, its further development as an anticancer drug was cancelled due to the hepataotoxic side effects.\textsuperscript{6}

Dehydrodidemnin B (15), or aplidine, cyclic depsipeptide isolated from the Mediterranean tunicate \textit{Aplidium albicans}, showed less toxicity and more potency than didemnin B. It has shown a broad spectrum of anticancer activity and has been promoted to Phase II clinical trails.
Didemnin B (14)

Aplidine (15)
Manoalide$^{32}$ (16), a sesquiterpene isolated from the Indo-Pacific sponge *Luffariella variabilis*, is a potent analgesic and antiinflammatory agent. A low concentration of manoalide inhibited calcium channels. It has been undergone Phase I clinical trails for psoriasis. It is commercially available as a standard probe for PLA$_2$ inhibition.$^{3, 33}$

Pseudopterosin A (17) is a tricyclic diterpenoid glycoside isolated from the Caribbean Sea whip (gorgonian) *Pseudopterogorgia elisabethae* (Gorgoniidae). It is a potent antiinflammatory and analgesic agent. Further it was found that it can inhibit eicosoniod biosynthesis by inhibition of both PLA$_2$ and 5-lipoxygenase. It is thought that the cell type selectivity is due to the presence of the glycoside moiety.$^{3, 33}$
1.2 Biodiversity of Antarctica

Research on Antarctic ecosystems has revealed that marine biota have thrived under ice covered seas for 20 million years, providing static environmental conditions for the evolution of broad biodiversity. Moreover, the continent has been isolated from its lower latitude neighbors even longer and the circumpolar current that encircles the land mass of Antarctica has prevented the Antarctic species exchanging genetic information with Northern congenors. The factors such as physical stability and isolation are important criteria for genetic divergence and have given rise to the high levels of endemism in Antarctica.

Predation and competition are the dominant forces that determine the species composition and distribution of such an ecosystem and eventually could facilitate the evolution of novel biogenetic pathways leading to bioactive secondary metabolites with structural diversity. In fact, being a very stable ecosystem operating under very unique set of ecological conditions, the benthic community of Antarctica has evolved many interspecies relationships where secondary metabolites perform an important role assisting defense against predators and competitors.

A few decades back, it was conventional wisdom that competition and predation among marine species are most intense in tropical waters and as a result the chemical ecology of marine organisms dwelling in tropical waters received more attention in drug discovery programs. However, recent research on organisms from the Southern Ocean sea floor suggested that predation and competition similarly drive the chemical defense.
An expedition undertaken to study the benthos of McMurdo Sound, Antarctica, in 1980 revealed that the sea floor under the ice is rich in marine life and is covered with an immense community of sponges, soft corals, molluscs, tunicates and echinoderms. Many of these organisms are immobile and therefore cannot move to less densely populated regions if the area they occupy becomes overgrown with competitors, nor can they escape predators. Nevertheless, they manage to survive. Hence, it is apparent either these sessile organisms do not have predators or they have a defensive strategy.\textsuperscript{4}

Further studies of Antarctic invertebrates showed that they do have many predators. These include swarms of the voracious \textit{Paramoera antarctica}, a one centimeter-long crustacean resembling shrimp and dense populations of sea stars.\textsuperscript{4,35} In addition, it was evident that the benthic invertebrates are pressured by fouling diatoms, invertebrate larvae, algal spores and potentially infectious water column microorganisms. These environmental features suggest the likelihood of the evolution of chemically mediated defensive strategies.\textsuperscript{35} Thus, the attention was focused on the chemistry of these bottom dwellers in search of secondary metabolites that might provide chemical defense. Subsequently, chemical investigations have been performed on Antarctic organisms and numerous bioactive molecules have been characterized.\textsuperscript{35}

Sponges of McMurdo Sound Antarctica are subject to predation by sea stars, and it is considered to be a dominant ecological factor that might drive the production of defense chemicals.\textsuperscript{35} The bright yellow Antarctic sponge \textit{Isodictiya erinacea} which lacks physical defense such as spicules and mucus, is one of several chemically defended sponges in the
region. An investigation of its secondary metabolites showed the presence of purine and nucleoside metabolites including previously unreported erinacean (18) which showed cytotoxicity. The tryptophane derivative erebusinone (19), which is a yellow pigment found in the sponge, showed molt inhibition in crustaceans, a possible strategy of chemical defense.

![Chemical structures](image)

Erinacean (18)  
Erebusinone (19)

The bright yellow Antarctic cactus sponge was observed as extremely slow growing, but never observed to be eaten by the spongivorous sea star *Perknaster fuscus*. It has neither apparent spicules nor mucus suggesting a chemical defense. Chemical investigation of this sponge, *Dendrilla membranosa*, yielded three new diterpenoids, membranolides B (20), C (21), D (22) and membranolide (23) which is reported to have antibiotic activity.
Membranolide (23)

*Suberites* sp. is a common McMurdo Sound sponge that has a muted yellow coloration. Suberitenones A (24) and B (25) are two sesterterpenes that have been described from sponge collected at several sites around Antarctica and are suspected to be involved in chemical defense of the sponge.\(^4\) Suberitenones have shown activity in both sea star tube foot retraction feeding deterrent assay and an antibacterial assay using sympatric bacteria.\(^4\) The former assay was first developed to study the feeding relationships between the sea star *Perkanaster fuscus* and the sponges in McMurdo Sound. The tube feet of the sea star are known to be chemosensory, and when they are in contact with an unsuitable sponge extract, they illicit a retraction, indicative of feeding deterrence.

Another sponge that elicits a significant sea star tube foot retraction is *Latrunculia apicalis*. A subsequent fractionation of the sponge produced discorhabdin alkaloids, a group of pigments first isolated from temperate sponges, including discorhabdin G (26) and C (27), the latter of which was identified as a potent mammalian cytotoxin.\(^3\)\(^,\)\(^4\)
Leucetta leptorhapsis, a calcareous sponge commonly known as the rubber sponge due to its appearance as being stretched, is a common member of the McMurdo Sound benthic community. Comprehensive study of this organism yielded the cytotoxic agent rhapsamine (28).
The pteropod *Clione antarctica* is a shell-less pelagic mollusc which blooms in each austral summer in McMurdo Sound. It has an intriguing relationship with the amphipod *Hyperiella dilatata* where the amphipod positions the mollusc on its dorsum and defends itself from predatory fish utilizing the defense chemicals of the mollusc.\(^47\) A bioassay guided fractionation of the mollusc afforded the feeding deterrent pteroenone (29).\(^48\)

![Pteroenone (29)](image)

It has been observed that among the Antarctic deep water octocorals, some are replete with calcareous spicules and others are devoid of them. The octocorals that have spicules were found to yield chemical extracts palatable to *Odontaster validus*, a common predaceous sea star in Antarctica, whereas octocorals that did not have spicules produced extracts that deterred predation, suggesting the presence of a chemical defense. Chemical investigation of one such horny corals, *Ainigmaptilon antarcticus*, afforded two bioactive sesquiterpenoids, ainigmaptilone A (30) and B (31) based on the eudesmane carbon skeleton, which is fairly uncommon in corals.\(^49\)

![Ainigmaptilone A (30)](image) ![Ainigmaptilone B (31)](image)
Macroalgae are known to pay a role in structuring the near shore Antarctic benthos. *Delisea pulchra*, a marine red algae collected near Palmer Station, yielded three new dimeric halogenated furanones, pulchralides A (32), B (33), C (34) along with fimbrolide (35), acetoxyfimbrolide (36) and hydroxyfimbrolide (37). The latter two compounds have shown significant antibacterial properties.\(^5\)

![Structural formulas of compounds](image)

Pulchraide A (32) \( R = R_1 = \text{OAc}, R_2 = R_3 = \text{H} \)

Pulchraide B (33) \( R = R_1 = R_2 = R_3 = \text{H} \)

Pulchraide C (34) \( R = \text{OAc}, R_1 = R_2 = R_3 = \text{H} \)

Fimbrolide (35) \( R = \text{H} \)

Acetoxyfimbrolide (36) \( R = \text{OAc} \)

Hydroxyfimbrolide (37) \( R = \text{OH} \)

*Plocamium cartilagineum*, a common red alga found in shallow-water Antarctic environments produces halogenated monoterpenes including anverene (38), epiplocamene D (39) and pyranoid 40.\(^5\) Anverene, which induces feeding deterrence in the amphipod *Gondogeneia antarctica*, displayed modest but selective antibiotic activity whereas 40 showed selective antifungal activity. Epiplocamene D exhibited a greater degree of feeding deterrence by the amphipod than the other two compounds. However all three compounds had no effect on the sea star *Odontaster validus*.\(^5\)
1.3 Research Objectives

The benthos of Antarctica harbors many organisms that have not been investigated for their chemistry and bioactivity. The unique features of its ecosystem and dynamic relationship between the predators and prey could lead to the evolution of biosynthetic pathways leading to molecules with interesting biological activity. Therefore, in terms of drug discovery a comprehensive chemical investigation of Antarctic species is very important.

In addition, there are many parts of the Antarctic Peninsula that have never been explored. Considering the significant variations in chemistry of the species that have been studied from the regions of McMurdo Sound and Palmer Station\textsuperscript{35} a comprehensive investigation of the chemistry and the bioactivity is of considerable interest from a chemical ecological standpoint.
1.4 Summary

Due to the ecological features of their habitat, many marine organisms have adopted chemical defense strategies. As a result, the marine realm has been an exceptional reservoir of bioactive natural products with a great structural diversity. Numbers of secondary metabolites isolated from marine organisms have advanced into clinical trials for drug development. The research on Antarctic marine biota emphasizes its potential to give rise to novel biogenetic pathways leading to secondary metabolites with attractive biological activities.
Chapter 2. CHEMICAL INVESTIGATION OF ANTARCTIC TUNICATE

SYNOICUM ADAREANUM

2.1. INTRODUCTION

2.1.1 Tunicates: a rich source of bioactive natural products

The tunicates, classified in the phylum Chordata under the subphylum Urochordata, represent one of the most evolved groups of animals that are known to produce secondary metabolites. They are commonly referred to as tunicates, due to the sac like covering or tunic made of cellulose that covers their body. The subphylum Urochordata consists of three classes, Ascidiaceae, Larvacae and Thaliaceae. The members of class ascidiaceae are referred to as ascidians. They are also known as sea squirts, as many species expel water through a siphon when disturbed. The adult ascidians are exclusively marine, and share no resemblance with other Chordates. Their larvae are similar to amphibian tadpoles, they have notochords, pharyngeal slits and dorsal hollow nerve chords, the features that make them classify as Chordates which are ultimately lost during the course of development.

Adult ascidians are sessile filter feeders that have either colonial or solitary lifestyles. They prefer the regions where there is minimal effect from the shock created by wave action, but have a considerable free flow of seawater. The ascidian morphology is diverse. A solitary tunicate can grow as long as 15 cm or as small as 1 cm, where as a colonial tunicates found encrusted in rocks, can be extremely thin and delicate.
The first interest on the chemistry of tunicates was ignited by the scientist’s curiosity to unfold the mysteries behind the color changes observed in the blood samples of tunicates as early as in 1847.\textsuperscript{54} Subsequent research led to the discovery of a series of pigments called tunichromes (41) and vanadium sequestration.\textsuperscript{55, 56} The focus on other bioactive secondary metabolites from tunicates originated in the early 1970s when the first ascidian metabolite, geranyl hydroquinone (42), was isolated. It was found to be active against leukemia, Rous sarcoma and mammary carcinoma in animal tests.\textsuperscript{51}

Since then, especially during the last two decades, tunicates have emerged as a rich source of bioactive natural products bearing unique structural features. Several such compounds have already advanced into clinical stages of drug development. The majority of bioactive compounds described from ascidians are nitrogen containing metabolites and fall into the categories of peptides or amino acid derived alkaloids. However, they have given rise to a smaller number of non-nitrogenous metabolites derived through diverse biogenetic pathways as well.\textsuperscript{51}

![Tunichrome (41)](image1)

![Geranyl hydroquinone (42)](image2)
The tunicates have yielded a broad array of compounds with potent cytotoxicity. The ecteinascidins (11)\textsuperscript{11, 12} and didemmins\textsuperscript{9} (13) have already advanced into clinical trials as prospective anticancer drugs. Eudistomin, a group of $\beta$-carboline alkaloids isolated from the colonial ascidian \textit{Eudistoma olivaceum}, are potent antiviral compounds.\textsuperscript{57-59} These compounds fall into five different general classes (Group 1: simple $\beta$-carbolines such as: eudistomin D (43), Group 2: pyrrolyl-$\beta$-carbolines such as eudistomin A (44), Group 3: pyrrolinyl-$\beta$-carbolines such as eudistomin G (45), Group 4: 2-phenylacetyl-$\beta$-carbolines such as eudistomin R (46) and Group 5: tetrahydro-$\beta$-carbolines such as eudistomin C (47)).
2.12 Chemistry of the genus *Synoicum*

Despite the fact that there is a growing interest in the chemistry of tunicates, out of many species of ascidians in the genus *Synoicum* only few have been subjected to chemical investigation. Studies on *Synoicum prunum*, a colonial ascidian collected from North Stradbroke Island in Queensland, Australia, have yielded a series of tetraphenolic, bis-spiroketalts, prunolides A-C (**48-50**),\(^6^0\) which showed weak cytotoxicity, along with rubrolide A (**51**).\(^6^1\) It is believed that prunolides arise from oxidative dimerization of a rubrolide precursor.

![Chemical structures](image)

Prunolide A (**48**) \(R = H, \quad X = Br, \quad Y = Br\)

Prunolide B (**49**) \(R = H, \quad X = Br, \quad Y = H\)

Prunolide C (**50**) \(R = H, \quad X = H, \quad Y = H\)

Rubrolide A (**51**)

**Eudistomin C** (**47**)
The red colonial tunicate *Synoicum blochmanni* collected from Tarifa Island, Spain, afforded six new nitrogenous metabolites, rubrolides I - N (51-56) along with four related known compounds. Rubrolides I, L, and M showed weak cytotoxicity.\(^{61}\)

![Chemical structures](image)

Rubrolide I (51) \(X = \text{Cl}\)  
Rubrolide J (52) \(X = \text{H}\)  
Rubrolide L (54) \(X = \text{Cl}\)  
Rubrolide K (53) \(X = \text{Br}, \ Y = \text{Cl}\)  
Rubrolide M (55) \(X = \text{Cl}, \ Y = \text{H}\)  
Rubrolide N (56) \(X = \text{Br}, \ Y = \text{Cl}\)

Chemical investigation of the ascidian *Synoicum macroglossum* collected from the Indian Ocean near Tamilnadu, India, found the guanidino alkaloid, tiruchanduramine (57), which showed potent \(\alpha\)-glucocidase activity.\(^{62}\)

![Tiruchanduramine structure](image)

Tiruchanduramine (57)
2.1.3 Research Objectives

Despite the enormous promise that the tunicates have shown as a rich source of bioactive secondary metabolites, the chemistry of Antarctic tunicates have not been completely explored. The unique ecological features found in Antarctica, and the dynamic interaction between these tunicates and other benthic organisms, could facilitate the development of biogenetic pathways leading to secondary metabolites with novel structural features. Therefore, it is of interest to study the chemistry of tunicates found on the benthos of Antarctica.

Figure 1. *Synoicum adareanum* at Palmer Station, Antarctica (Photograph supplied by Bill J. Baker, University of South Florida)
**Synoicum adareanum** is a colonial ascidian that is commonly found in the shallow water around Palmer Station, Antarctica. Its liphophilic extract showed feeding deterrence towards the Antarctic sea star *Odontaster validus*, indicating the presence of defense chemicals. Therefore, as part of our comprehensive chemical investigation of Antarctic tunicates, *S. adareanum* was studied to identify the secondary metabolites responsible for this activity.

### 2.2 RESULTS AND DISCUSSION

#### 2.2.1 Extraction and isolation of secondary metabolites

*Synoicum adareanum* tunicates were collected from the ocean near Anvers Island (64° 46' S, 64° 03'W), Antarctica, in 2003. The freeze dried tunicates were extracted in CH$_2$Cl$_2$/MeOH for three days. After evaporating the solvent the resultant reddish-brown semi-solid was partitioned with EtOAc and water. The EtOAc layer was washed, dried and the solvent removed. The resultant EtOAc extract of the tunicates was fractionated by flash chromatography to obtain 18 fractions (Scheme 1).

Further separation of these fractions with flash chromatography and subsequent purification by HPLC on C-18 afforded palmerolide A ([58](#)) (200 mg, 0.02% dry wt), palmerolide C ([59](#)) (4 mg, 0.0004% dry wt), palmerolide D ([60](#)) (2 mg, 0.0002% dry wt) and palmerolide E ([61](#)) (4 mg, 0.0004% dry wt) (Scheme 1). Fractions 8, 9 and 10, upon further purification by HPLC using 50% water in MeCN (isocratic elution, 2 mL per min) yielded palmerolide B ([62](#)) (2 mg, 0.0002% dry wt) and palmerolide H ([63](#)) (1 mg, 0.0001% dry wt).
Freeze dried Tunicates (520 g)

1. Extraction with CH$_2$Cl$_2$/MeOH 1:1 3 X 24 hrs
2. Evaporation of solvent
3. Partition with EtOAc/MeOH
4. EtOAc layer separated, washed and dried (anhydrous MgSO$_4$)

EtOAc Extract (3.1 g)

6. Gradiant flash chromatography over silica
Hexane/ EtOAc / MeOH

1. Extraction with CH$_2$Cl$_2$/MeOH 1:1 3 X 24 hrs
2. Evaporation of solvent
3. Partition with EtOAc/MeOH
4. EtOAc layer separated, washed and dried (anhydrous MgSO$_4$)

Gradiant elution over silica gel
1-6% MeOH/CHCl$_3$

Fr 40-48

- Palmerolide A (58) (200 mg)
- Palmerolide C (59) (1.2 mg)

Gradiant elution over silica gel
1-10% MeOH/CHCl$_3$

Fr 33-37 (9 mg)

- Palmerolide D (60) (3.5 mg)

HPLC ODS
H$_2$O : MeCN 4:6
Isochratic elution

Fr 40-48

- Palmerolide B (62) (2 mg)
- Palmerolide H (63) (1 mg)

Gradiant elution over silica gel
1-20% MeOH/CHCl$_3$

Fr 30-32 (20 mg)

- Palmerolide C (59) (3.5 mg)
- Palmerolide E (61) (3.5 mg)

HPLC ODS
H$_2$O : MeCN 4:6
Isochratic elution

Fr 33-37 (9 mg)

HPLC ODS
H$_2$O : MeCN 1:1
Isochratic elution

Fr 54-60

- Palmerolide C (59) (3.5 mg)

Scheme 1. Extraction and purification of the palmerolides
Palmerolide A (58)

Palmerolide C (59)

Palmerolide D (60)
Palmerolide E (61)

Palmerolide B (62)

Palmerolide H (63)
Compound 62 was first isolated from a 2001 collection of Synoicum adareanum. However it was found to be unstable in DMSO as it decomposed in the NMR tube while the 2D NMR data were being obtained. Therefore, all the NMR experiments of fractions leading to palmerolides B and H were performed in CD$_3$OD.

### 2.2.2 Characterization of Palmerolide A (58)

Palmerolide A (58) was isolated as a white solid. The low resolution FABMS analysis displayed a prominent peak at $m/z$ 585, which was assigned as $[M + 1]^+$ (Figure 2). The HRFABMS provided a molecular formula of $C_{33}H_{48}N_2O_7$ (HRFABMS $m/z$ 585.3539, $\Delta$ 0.1 mmu for $[M + 1]^+$).

![Figure 2. LRFABMS spectrum of palmerolide A (58)](image)

The $^{13}$C NMR spectrum of compound 58 (Figure 3) displayed 33 carbon signals. These signals were further edited by DEPT 135 (Figure 4) and DEPT 90 experiments which...
established that palmerolide A is composed of five methyls, six methylenes, sixteen methines and six quaternary carbon signals. The quaternary signals at $\delta$ 165.3, 156.6 and 163.1 were assigned as carbons at the oxidation state of a carboxylic acid, whereas the signals at $\delta$ 73.7, 72.5, 69.0 and $\delta$ 75.3 indicated hydroxymethines.

Figure 3. $^{13}$C Spectrum of palmerolide A (58) (125 MHz, DMSO-$d_6$)
Analysis of the $^1$H NMR spectrum of palmerolide A (58) (Figure 5a), showed a series of proton signals in the region of 5-7 ppm (Figure 5b), characteristic of olefins, confirming a high degree of unsaturation. The singlets (3H) at $\delta$ 1.61, 1.70, 1.83, 2.12 and the doublet $\delta$ 0.90 indicated five methyls (Figure 5c). A comprehensive investigation of the structure of palmerolide A was undertaken using gHMQC and gHMBC techniques. The C-1 to C-24 carbon backbone of palmerolide A (58) could be unambiguously assigned based on $^1$H-$^{13}$C assignments (Table 2) from the gHMBC spectrum (Figure 6), to establish a 20 member macrocycle. The ester carbonyl $\delta$ 166.1 (C-1) correlated with the H-2 and H-3 olefins, the latter of which must be disposed trans ($J = 15.2$ Hz).
Figure 5. $^1$H NMR spectrum of palmerolide A (58) (500 MHz, DMSO-$d_6$)

Three methylene carbons ($\delta$ 32.6, 24.8 and 38.3) were observed by both gCOSY (Figures 8, 9) and gHMBC between the C-2/C-3 olefin and a hydroxymethine at $\delta$ 3.82 (H-7). A trans-disubstituted olefin ($J = 15$ Hz) could be positioned between that hydroxymethine and another at $\delta$ 4.14 (H-10). While H-10 showed no HMBC correlations, H-8, -9 and -11 all correlated to C-10. H-11 was found correlated not only with C-10 and C-12/13 (C-12 and -13 were coincident in the $^{13}$C NMR spectrum) but also with an ester-type carbonyl (O\_COX) which displayed no further connectivity using these techniques.
Table 2. NMR data of palmerolide A (58) (1H, 500 MHz, 13C, 125 MHz, DMSO-d$_6$)\textsuperscript{a}

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<tr>
<th></th>
<th>(\delta^{13}C)</th>
<th>(\delta^{1}H) (ppm, mult, (J) (Hz))</th>
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<td>1</td>
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<td>24.8</td>
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<td>7</td>
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<td>6</td>
<td>38.0</td>
<td>1.05 (1H, m)</td>
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<td>4.14 (1H, br s)</td>
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<td></td>
<td>1’ , 3’, 4’, 5’</td>
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<td>2’</td>
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<td>5.69 (1H, br s, 1.0)</td>
<td>1’ , 3’, 4’, 5’</td>
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<td>3’</td>
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<td>4’</td>
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<td>1.83 (3H, s)</td>
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<td>4.72 (1H, d, 3.9)</td>
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\textsuperscript{a}Proton carbon assignments based on gHSQC spectrum (Figure 7)
Figure 6. gHMBC spectrum of palmerolide A (58) (500 MHz, DMSO-$d_6$)
In the gHMBC spectrum, H-13 was coupled back in to the olefinic region to C’s -14 and -15. The C-14/15 trans-olefin ($J = 14.6$ Hz) was shown to be conjugated to a trisubstituted olefin in positions C-16 and C-17 by gHMBC correlations of H-14, -15, -18 and -19 as well as H$_3$-25. The C-16/C-17 olefin must be trans based on the correlations of the ROESY spectrum for H$_3$-25 and H-15. The methylene group at C-18 ($\delta$ 43.9) intervenes between the C-16/C-17 olefin and an oxygen bearing methine (C-19, $\delta$ 75.9), based on gHMBC correlations of H-16 and H$_3$-25 to C-18 as well as H-19 and H-20 correlations to C-18. The macrocycle was completed by observation of correlation between H-19 and the C-1 ester carbonyl.
The features of the C\textsubscript{20} macrocycle were established by extensive analysis of 2D NMR data. In addition to the four \textit{trans} olefins described above, one methyl group and three oxygen atoms were pendant on the macrocycle. Hydroxymethine protons at H-7 and H-10 were conclusively assigned based on the observation of coupling of hydroxyl protons in both the gHMBC and gCOSY spectra; in the gHMBC spectrum, the hydroxyl protons correlated to the respective $\alpha$- and $\beta$-carbons, while the gHMBC correlations were observed between the hydroxyl protons and their respective hydroxymethines. The third oxygen bearing carbon (C-11) as described earlier correlates with an ester-type carbonyl (OC\textsubscript{OX}).

Figure 8. Key gHMBC and gCOSY correlations of palmerolide A
Also pendant on the macrocycle was the C-19 side chain. gHMBC and gCOSY correlations of the multiplet at δ 2.68 (H-20), which was clearly coupled (gCOSY) to a methyl group (C-26, δ 0.90), could be extended to a conjugated diene system based on gHMBC correlations of H-19 and H-20 to olefin C-21 (δ 129.7). Both the C-21 / C-22 and C-23 / C-24 olefins were determined to be trans based, in the former case of by ROESY correlation between H3-27 and H-19, and in the latter case, $J = 14.2$ Hz.

Connectivity of the C-23/ C-24 olefin could be established based on gHMBC correlations of H-23 to C-21, -22, -24 and -27. C-24 marked the terminus of the continuous carbon
chain and could be shown to bear an –NH group due to gHMBC correlations of a amide proton at δ 9.84 to carbons C-23, C-24 and an amide carbonyl, C-1′ (δ 163.1). The presence of amide group was confirmed by an 15N gHSQC experiment.

The isopentenoyl substructure (C-1′ to C-5′) was unusual in displaying 4J CH coupling in the gHMBC spectrum between the amide carbonyl (C-1′) and both vinyl methyl groups (C-4′ and C-5′). Only one vinyl methyl can be placed within the 3J CH reach of the typical HMBC experiment optimized for 8 Hz. Presence of another alternative structure, the 2-methyl-2-butenoyl isomer, wherein one vinyl methyl resides three bonds away from the carbonyl and the second resides four bonds distant, is consistent with these HMBC correlations. However, it was rules out on chemical shift grounds and by the observations of gHMBC correlations between both vinyl methyls. The substructure was secured as the isopentenoyl group by observation of very small coupling constants (J = 1.0 Hz) of the vinyl proton (H-2′) to both vinyl methyl groups (C-4′ and C-5′), excluding a vicinal relationship between the vinyl proton and the vinyl methyl required by the 2-methyl-2 butenoyl isomer. The isopentenoyl structure could be connected to the vinyl proton at δ 6.82 (C-24) based on gHMBC cross peak between amide carbonyl (C-1′) and H-24.

The above described connectivity established the full planer structure of palmerolide A (Figure 10) with the exception of a single open valence, the ester-type carbonyl attached to the macrolide at C-11. Remaining to be accounted from the known molecular formula was –NH2. That the C-11 functional group was a carbamate is supported by the precedence of that functional group on other polyketides, most notably the anticancer agent discodermolide (7).23
2.2.3 Stereochemical determination

Stereochemical assignment of the five stereocenters of palmerolide A was established by application of the modified Mosher’s\textsuperscript{63} and Murata\textsuperscript{64} methods. Further support for stereochemistry assignment was obtained by ROESY and selective 1D NOESY experiments.

2.2.3.1 Application of Mosher’s method

Mosher’s method has been a very successful method to determine the absolute stereochemistry of chiral centers associated with secondary alcohols.\textsuperscript{64} Out of the five stereocenters in palmerolide A (58), C-7 and C-10 both have secondary alcohols making them suitable candidates to employ Mosher’s method.

Palmerolide A (58) was treated with both R- and S-MTPACl separately and the reaction was monitored by TLC. It was observed that in each case two products were formed. In each case, the formation of the first product, which was the major product, was observed.
after one hour and the formation of the second product was noticed after 16 hours. This can be explained as due to the substitution of one secondary hydroxyl in palmerolide A giving rise to $R$ and $S$ monoesters (64 and 65) initially, and then subsequently substitution of the other secondary hydroxyl giving rise to $R$ and $S$ diesters (66 and 67). The products 64 and 65 were less polar than 66 and 67 in agreement with above explanation.

![Chemical structure of palmerolide A](image)

64. $R_1 = R$-MTPA, $R_2 = H$
65. $R_1 = S$-MTPA, $R_2 = H$
66. $R_1 = R$-MTPA, $R_2 = R$-MTPA
67. $R_1 = S$-MTPA, $R_2 = S$-MTPA

Each product was isolated by chromatography on silica gel and purified by HPLC. A comprehensive structure elucidation was undertaken of each of these products using 2D NMR and mass spectroscopy.

### 2.2.3.1.1 $R$-MTPA monoester of palmerolide A (64)

Analysis of the more polar product 64 by ESIMS showed a mass peak at $m/z = 823.1$ representing [M + Na]$^+$. The HRESIMS experiment performed on this ion showed a molecular weight of 823.3776 amu consistent with molecular formulae of
C$_{43}$H$_{55}$N$_2$O$_9$F$_3$Na suggesting the incorporation of one MTPA group. This explains the more polar nature and the faster rate of formation of 64 as compared to the disubstituted product 66. The $^1$H NMR spectrum (Figure 11) indicated significant chemical shift changes around C-7 (Figure 12). The multiplets at $\delta$ 7.51 and 7.43 represented the MTPA phenyl group whereas the singlet at $\delta$ 3.63 indicated the OCH$_3$ group. The multiplet $\delta$ 5.47 assigned for H-7, show a significant deshielding accounting for an esterification at C-7. The observation of a gHMBC correlation between H-7 and $\delta$ 165.9 assigned as the carbonyl carbon of the MTPA group, confirmed the attachment of MTPA at C-7, giving rise to palmerolide A R-MTPA monoester (64).

Figure 11. $^1$H NMR spectrum palmerolide A R-MTPA monoester (64) (500 MHz, CD$_3$OD)
Figure 12. Key gHMBC correlations demonstrating the attachment of the MTPA moiety in compound (64)

2.2.3.1.2 \textit{R, R}-MTPA diester of palmerolide A (66)

The less polar product (66) from the reaction of palmerolide A (58) with MTPA-Cl showed a \( m/z = 1039.1 \) in the ESIMS, consistent with \([M + Na]^+\). HRESIMS indicated a molecular weight of 1039.4145 amu, consistent with molecular formula of C\textsubscript{53}H\textsubscript{62}N\textsubscript{2}O\textsubscript{11}F\textsubscript{6}Na and indicative of incorporation of two MTPA moieties. The \(^1\)H NMR spectrum (Figure 13) indicated significant chemical shift changes around C-7 and C-10 (Figure 14).

![Figure 13. \(^1\)H NMR spectrum of palmerolide A \( R, R \)-MTPA diester (66) (500 MHz, CD\textsubscript{3}OD)](image)
The multiplets at $\delta$ 7.46 and 7.35 could be assigned as the MTPA phenyl group whereas the two singlets at $\delta$ 3.47 (3H) and 3.56 (3H) were due to the two OCH$_3$ groups of two MTPA moieties. H-7 and H-10 both displayed significant deshielding demonstrating the formation of ester bonds. A spectroscopic investigation using gHMBC COSY and HSQC experiments around C-7 and C-10 confirmed the attachment of two MTPA molecules at C-7 and C-10 to give rise to palmerolide A R, R-MTPA diester (66).

![Diagram of palmerolide A]

Figure 14. Key gHMBC correlations demonstrating the attachment of MTPA in compound (66)

2.2.3.1.3 $S$-MTPA monoester of palmerolide A (65)

The HRESIMS of 65 showed a molecular weight of 823.3747 amu consistent with molecular formula of C$_{43}$H$_{55}$N$_2$O$_9$F$_3$Na, affirming the substitution of one MTPA moiety. In addition, it explained the more polar nature and the faster rate of formation of 65 as compared to the disubstituted product 67. The $^1$H NMR spectrum (Figure 15) indicated significant chemical shift changes around C-7 (Figure 16). The multiplets at $\delta$ 7.41, 7.44, 7.50 and 7.60 can account for the aromatic protons of the MTPA moieties and the singlet
at $\delta$ 3.55 was due to the OCH$_3$ group of MTPA moiety. H-7 showed a significant deshielding demonstrating the formation of an ester linkage. In addition it displayed gHMBC correlation with $\delta$ 165.8 assigned for the ester carbonyl of MTPA moiety, further accounting for the attachment of a MTPA molecule at C-7.

Figure 15. $^1$H NMR spectrum of palmerolide A $S$-MTPA monoester (65) (500 MHz, CD$_3$OD)

Figure 16. Key gHMBC correlations demonstrating the attachment of the MTPA moiety in compound 65.
2.2.3.1.4 $S, S$-MTPA diester of palmerolide A (67)

The less polar product from esterification of palmerolide A (58), 67, showed a prominent mass peak at $m/z = 1039.1$ in the ESIMS indicative of $[M + Na]^+$. HRESIMS indicated a molecular weight of 1039.4151 amu that was consistent with molecular formula of $C_{53}H_{62}N_2O_{11}F_3Na$, indicative of incorporation of two MTPA moieties. The $^1$H NMR spectrums (Figure 17) indicated significant chemical shift changes around C-7 and C-10 (Figure 18). The multiplets at $\delta$ 7.61, 7.50, 7.43 and 7.39 represented the aromatic protons of the MTPA moieties whereas the two singlets (3H) at $\delta$ 3.41, 3.57 indicated the two OCH$_3$ groups of two MTPA groups. H-7 and H-10 both displayed significant deshielding due to the formation of ester bonds that attach two MTPA groups at C-7 and C-10 in order to form palmerolide A $S, S$-MTPA diester (67).

Figure 17. $^1$H NMR spectrum of palmerolide A $S, S$-MTPA diester (67) (500 MHz, CD$_3$OD)
Figure 18. Key gHMBC correlations demonstrating the attachment of the MTPA in compound (67)

2.2.3.1.5 Absolute stereochemistry assignment at C-7 of palmerolide A (58)

According to Mosher’s method, the chemical shifts of all the neighboring protons in the either side of the desired chiral center of both R- and S-MTPA monoesters of palmerolide A (58) were assigned as shown in previous sections. In the next step, for each proton, the value of the ∆δ chemical shifts were calculated employing following formula:

$$\delta S - \delta R = \Delta \delta$$

Δδ values were placed on the model designed as described in Mosher’s method (Figure 19) so that all positive Δδ chemical shift differences are assigned to the right hand side of the MTPA plane, whereas all negative Δδ chemical shift difference are assigned to the left hand side.

Figure 19. Δδ Chemical shift differences (Δδ * 1000) of palmerolide A MTPA R- and S-monoesters
Once a model is built satisfying all requirements described in Mosher’s method, the model (Figure 20) should have the absolute stereochemistry of the desired chiral center involving a secondary hydroxyl group. Therefore, in the case of palmerolide A the absolute stereochemistry at C-7 was assigned as R.

![Figure 20. MTPA model for configuration correlations](image)

**2.2.3.1.6 Absolute stereochemistry assignment at C-10 of palmerolide A (58)**

Similarly the Δδ chemical shift values were calculated for the MTPA diesters of palmerolide A and applied to a Mosher’s model as shown in Figure 21. Application of the Mosher’s method indicated that the absolute stereochemistry of C-10 of palmerolide A as R.

![Figure 21. Δδ Chemical shift values (Δδ * 1000) of palmerolide A MTPA R, R- and S, S-diesters](image)
2.2.3.2 Application of Murata’s method

Matura’s method is based on configuration analysis using heteronuclear coupling constants. It has been successfully applied to the stereochemical elucidation of number of natural products. The gHSQMBC experiment based on this principle has proven as a very successful method to determine stereochemistry of macrolides. Hence, a gHSQMBC experiment was acquired using a concentrated sample of palmerolide A (58) (Figure 22). Heteronuclear coupling constants were obtained by analyzing the coupled proton and carbon signals in the corresponding 1D spectrum (Figure 23). Based on the magnitude of the coupling constants the stereochemical relationships between the respective atoms are determined.

Figure 22. gHSQMBC spectrum of palmerolide A (58) (500 MHz, DMSO-$d_6$)
In order to determine the heteronuclear coupling constants, for each gHSQMBC cross peak, the 1D gHSQMBC slice was extracted. The heteronuclear coupling constant was derived by the subtraction of the $^1$H NMR spectrum from itself, offset by the equivalent magnitude of the coupling constant, to produce a difference spectrum which matched the gHSQMBC slice.

![Figure 23. 1D analysis of the gHSQMBC cross peak of the respective slice](image)

Figure 23. 1D analysis of the gHSQMBC cross peak of the respective slice

![Figure 24. Determination of the coupling constant by the subtraction of $^1$H NMR spectrum; Offset = $2J_{C-12/H-10}$](image)

Figure 24. Determination of the coupling constant by the subtraction of $^1$H NMR spectrum; Offset = $2J_{C-12/H-10}$
Configuration analysis of fragment C-10/C-11 showed a gauche relationship between H-10 and H-11, based on the small $^3J_{H-10/H-11}$ observed between the vicinal protons and the large $^3J_{CH}$ for both the H-10/C-12 and H-11/C-9 relationships. Further support for the conformation was found in $^2J_{C-11/H-10}$ and $^2J_{C-10/H-11}$ both of which are large and negative defining the absolute stereochemistry of C-11 as $R$.\textsuperscript{64}

Similarly, configurational analysis of C-19/C-20 system suggested an anti relationship for the respective protons, based on the large $^3J_{H-19/H-20}$, small $^3J_{C-21/H-19}$ and $^3J_{C-18/H-20}$ as well as large $^2J_{C-19/H-20}$. The relative position of C-18 was secured by an observation of ROESY correlations (Figures 25, 26) between H-18 and H-20 while no ROESY correlation was observed between H-21 and H-21 requiring the relative configuration 19$R$, 20$S$.

![ROESY correlations](image)

Figure 25. Coupling constant based configuration analysis\textsuperscript{64}

$^3J_{C-12/H-10} = 6.9$ Hz Large

$^3J_{C-9/H-11} = 5.2$ Hz Large

$^2J_{C-11/H-10} = -6.2$ Hz Large

$^2J_{C-10/H-11} = -4.8$ Hz Large

$^3J_{C-21/H-19} = 3.4$ Hz Small

$^3J_{C-18/H-20} = 2.9$ Hz Small

$^3J_{C-26/H-19} = 3.9$ Hz Small

$^2J_{C-19/H-20} = -5.9$ Hz large
Figure 26. ROESY spectrum of palmerolide A (58) (500 MHz, DMSO-\textit{d}_6)

The four olefins in the macrocycle constrain the flexibility often found in macrolides, facilitating stereochemical analysis by NOE studies. Further analysis of the ROESY spectrum (Figure 26) revealed the macrolide to adopt two largely planer sides of a tear drop shaped cycle, one side consisting of C-1 through C-6, the other C-11 through C-19, with C-7 through C-10 providing a curvilinear connection. In particular, H-9, H$_3$-27, H-15 and H$_2$-13 are sequentially correlated in the ROESY Spectrum, as are H$_3$-26, H$_2$-18, H-16, H-14 and H-12, defining the periphery of the top and bottom face of the western
hemisphere (Figure 27). H-11 correlates only to the top series of protons, a result consistent only with C-19 and C-11 both adopting the $R$ configuration. The absolute stereochemistry of the C-19/C-20 fragment is therefore assigned as 19$R$, 20$S$ (Figure 28).

![Figure 27. Key ROESY correlations supporting the stereochemistry determination of C-19 and C-20.](image)

The presence of type 1 polyketides like palmerolide A ($\text{58}$), is not a common feature among tunicates. Palmerolide A, bearing unusual 20-membered macrolide, display functionality more commonly found in sponges or cyanobacteria than the secondary metabolites generally found in tunicates. For instance, the vinyl amide is a feature very commonly associated with cyanophyte derived macrolides such as tolytoxin.

![Figure 28. Full stereochemical assignment of palmerolide A ($\text{58}$).](image)
2.2.4. Characterization of palmerolide C (59)

Palmerolide C (59), was isolated as a yellow solid and showed a molecular ion at $m/z$ 585.3 in the low resolution LRESIMS. The HRESIMS (Figure 28) showed that it was consistent with the molecular formula of C$_{33}$H$_{49}$N$_2$O$_7$, identical to that of palmerolide A (58).

Analysis of the $^{13}$C NMR spectrum (Figure 29) with DEPT 135 (Figure 30) suggested that palmerolide C (59) resemble palmerolide A further by having five methyls, six methylenes, sixteen methines and six quaternary carbon signals. In fact, out of the six methines, four were assigned to hydroxymethines as found in palmerolide A (58).
Figure 30. $^{13}$C NMR spectrum of palmerolide C (59) (125 MHz, DMSO-$d_6$)

![Figure 30. $^{13}$C NMR spectrum of palmerolide C (59) (125 MHz, DMSO-$d_6$)](image)

Figure 31. DEPT 135 spectrum of palmerolide C (59) (125 MHz, DMSO-$d_6$)

![Figure 31. DEPT 135 spectrum of palmerolide C (59) (125 MHz, DMSO-$d_6$)](image)
Interpretation of HMBC (Figure 32) and COSY (Figure 33) spectrum indicated that palmerolide C (59) is also composed of a 20 member macrocyclic ring with a side chain (Figure 34). The structure of the side chain was the same as that of palmerolide A (58), however, there were significant rearrangements of the structural features in C-3 through C-13 in the macrocyclic ring.

Figure 32. gHMBC spectrum of palmerolide C (59) (500 MHz, DMSO-$d_6$)
The doublet $\delta$ 5.73 (1H, $J = 15.5$ Hz) assigned to H-2 (Figure 35) showed HMBC coupling to ester carbonyl (C-1) $\delta$ 166.9 and methylene at $\delta$ 31.7 (C-4). The multiplet at $\delta$ 6.77 (H-3) correlated to C-1 and a methine at $\delta$ 149.8 (C-2). All these observations were consistent with a trans olefin conjugated to an ester carbonyl as found in palmerolide A. H-3 showed further HMBC correlation to two methylenes, C-4 and C-5 (Table 3), indicating the extension of the carbon backbone by two methylenes after the double bond. The COSY correlations of H-5 and H-6 and HMBC correlation of H-6 to C-5 and -7 confirmed this observation.

Figure 33. gCOSY spectrum of palmerolide C (59) (500 MHz, DMSO-$d_6$)
Figure 34. Key gHMBC and gCOSY correlations of palmerolide C (59)

Figure 35. $^1$H NMR spectrum of compound (59) (500 MHz, DMSO-$d_6$)
Based on the gCOSY and gHMBC correlations of H-8, H-9 and H-10, the three hydroxymethines C-8, C-9 and C-10 were positioned between the C-7 and C-11. Further H-10 was correlated to an ester-type carbonyl (OCOX). COSY correlations of H-11a, and H-11b with H\textsubscript{2}-12 and that of H\textsubscript{2}-12 with H-13a and H-13b established the connectivity between methylenes C-11, C-12 and C-13. The latter two showed overlapping carbon signals in the carbon spectrum.
Table 3. NMR data of palmerolide C (59) ($^1$H, 500 MHz, $^{13}$C, 125 MHz, DMSO-$d_6$)

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The remaining portion of the carbon backbone of the macrocycle was identical to that of palmerolide A (58). Connectivity of double bond C-14/C-15 to C-13 and was established by COSY correlation between H-13b and H-14 and the coupling observed in the HMBC between H-14 and H-15 with C-13. A large coupling constant ($J = 15$ Hz) indicated that H-14 and H-15 have a *trans* relationship. This olefin was conjugated to a trisubstituted double bond based on HMBC correlation of H-16 with carbons C-14, C-15 and C-25. H$_3$-25 showed HMBC correlations with C-16, C-17 and C-18, consistent with this assignment. H-19 demonstrated HMBC correlations with C-1, C-17 and C-18 completing the 20 membered macrocycle.

The side chain of palmerolide C (59) showed features similar to that of palmerolide A (58). The doublet at $\delta$ 0.90 (H$_3$-26) showed coupling in the COSY spectrum with H-20. In addition, this methyl group displayed gHMBC correlation to C-19, C-20 and C-21, confirming, that it is located between C-19 and C-21 and attached to C-20. Meanwhile, olefinic methine H-21 was correlated to another olefinic methine C-23 and C-27 in the gHMBC spectrum. However, evidence from the gHSQC spectrum showed that the latter carbon belongs to the vinylic methyl H$_3$-27. Based on the coupling constant ($J = 14$ Hz) the relationship between olefinic protons H-23 and H-24 was *trans*. These correlations are consistent with a trisubstituted double bond conjugated to a *trans* olefin at C-23/C-24. The doublet $\delta$ 9.85 representing N-H, which was coupled to H-24 as established by gCOSY, displayed HMBC correlations to C-23, C-24 and C-1'. The latter assigned to a carbonyl. The gHMBC relationship of broad singlet H-2' with C-1' and the unusual $^4$J$_{CH}$
HMBC coupling of two vinyl methyl groups C-4′ and C-5′ as observed in palmerolide A suggested an isopentenoyl connected to the amide described earlier.

These data established the full planer structure of palmerolide C (59) with the exception of a single open valence, the ester carbonyl attached to the macrolide at C-10. Remaining to be accounted from the established molecular formula was an -NH₂, suggesting the presence of a carbamate group as it was observed in the case of palmerolide A (58).

2.2.5 Characterization of palmerolide D (60)

Palmerolide D (60) which was separated as a yellow solid, showed a [M + 1]⁺ peak at m/z 625.6 in the low resonance ESIMS spectrum. Analysis by HREISMS (Figure 37) indicated a molecular formula of C₃₆H₅₃N₂O₇ (625.3864, calc 625.3853).

The ¹³C NMR spectrum of (60) (Figure 38), taken with the DEPT 135 spectrum (Figure 39) showed the presence of five methyls, eights methylenes, sixteen methines and seven quaternary carbon signals. 2D NMR investigation (Figure 40) of palmerolide D (60) indicated that it has a macrocyclic ring similar to that of palmerolide A (58). The quaternary carbon at δ 166.1 was assigned as an ester carbonyl (C-1) and was correlated to olefinic protons H-2 and H-3 by gHMBC (Figure 41). These two protons showed a coupling constant of 15.8 Hz indicating a trans double bond.
Figure 37. HRESIMS spectrum of palmerolide D (60)

Figure 38. $^{13}$C NMR spectrum of palmerolide D (60) (125 MHz, DMSO-$d_6$)
Figure 39. DEPT 135 spectrum of palmerolide D (60) (125 MHz, DMSO-\(d_6\))

Figure 40. Key gHMBC and gCOSY correlations of palmerolide D (60)
Three methylenes were positioned between C-3 and hydroxymethine C-7 based on gHMBC correlation of H-4 with C-5, H-5 with C-3 and that of H-5 and H-7 with C-6. In addition, H-7 displayed HMBC resonance with C-8 and C-9, assigned as olefinic methines (Figure 43) indicating the presence of a double bond attached to C-7. The location of two hydroxymethines C-10 and C-11, adjacent to each other was established.
by the COSY correlations (Figure 42) of H-10 and H-11 and further confirmed by HMBC correlations observed for H-11 with C-9 and C-10. In addition, H-11 showed HMBC correlations to the quaternary carbon at δ 156.4 which was assigned as an ester-type carbonyl carbon.

Figure 42. gCOSY spectrum of palmerolide D (60) (500 MHz, DMSO-\textit{d}_6)
The two methylenes C-12 and C-13, appeared overlapped with each other as a one carbon signal in the $^{13}$C NMR spectrum. Their linkage with hydroxymethine C-11 and olefinic methine C-14 was established by gHMBC correlations with H-10, H-11 and H-14. Based on the HMBC spectrum it was evident that the trans ($J = 14.6$ Hz) double bond (C-14 and C-15) is conjugated to a trisubstituted double bond (C-16 and -17). The methylene C-18 was positioned between the hydroxymethine C-19 and quaternary carbon C-17 based on gHMBC correlation of H-19, observed with C-18 and C-17. The 20 member macrocycle was completed by the observation of HMBC cross peak identified for H-19 and C-1.
The doublet at δ 0.89 in the $^1$H NMR spectrum (Figure 44) was assigned as a methyl (H$_3$-26) and showed COSY coupling to H-20. In addition, this methyl group displayed gHMBC correlation to C-19, C-20 and C-21 confirming that it is located between C-19 and C-21 attached to C-20. Meanwhile, H-21 representing an olefinic methine was correlated to another olefinic methine C-23 and C-25 by HMBC. However, HSQC evidence showed that the latter carbon belong to the vinylic methyl H$_3$-27. Based on the coupling constant ($J = 14.6$ Hz) the realtionship between olefinic protons H-23 and H-24 was identified as trans. These correlations are consistent with a trisubstituted double bond conjugated with a trans olefin at C-23/C-24. The doublet δ 9.85 representing N-H, which was coupled to H-24 by gCOSY, displayed gHMBC resonance with C-23, C-24 and C-1'.

![Figure 44. $^1$H NMR spectrum of palmerolide D (60) (500 MHz, DMSO-$d_6$)](image-url)
Table 4. NMR data of palmerolide D (60) (\textsuperscript{1}H, 500 MHz, \textsuperscript{13}C, 125 MHz, DMSO-\textit{d}_6)

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24-NH \textsuperscript{1}H, 9.94 (1H, d, 10.3)
7-O\textsubscript{H} \textsuperscript{1}H, 4.53 (1H, m)
10-O\textsubscript{H} \textsuperscript{1}H, 5.19 (1H, m)
From C-1’ onwards the side chain showed further modification by the elongation of the C-1’ - C-5’ moiety in palmerolide A (58). The quaternary carbon at $\delta$ 153.2 (C-3’) and methine at $\delta$ 120.3 assigned for C-2’ showed distinct gHMBC correlation with the proton signal at $\delta$ 3.34 a methylene, by the analysis of gHMOC and DEPT 135 spectra. This peak further displays gHMBC correlation to quaternary carbons at $\delta$ 143.6 (C-5’) and $\delta$ 112.6 (C-6’), further defining an extended side chain. The carbon signal at $\delta$ 112.6 (C-6’) was found to be an exomethylene based on its HMQC correlation to the proton signal at $\delta$ 4.72, which integrates for two hydrogens. Attachment of the vinylic methyl at $\delta$ 1.61 to C-5’ was based on gHMBC correlations with C-4’, C-5’ and C-6’.

The above discussed correlations established the full planer structure of palmerolide D (Figure 45) with the exception of a single open valence, the ester type carbonyl attached to the macrolide at C-11. As it was previously observed in respect of palmerolides A and C, to be accounted from molecular formula obtained by HREIMS, was an –NH$_2$, suggesting the presence of a carbamate group.

![Figure 45. Planer structure of palmerolide D (60)](image-url)
2.2.6 Characterization of palmerolide E (61)

Palmerolide E (61), isolated as a pale yellow solid, displayed a [M + Na]$^+$ peak at \( m/z \) 512 in the LRESIMS spectrum. Analysis by HRESIMS (Figure 46) afforded the molecular formula of C$_{27}$H$_{39}$NO$_7$Na (\( m/z \) 512.2634, calc 512.2624).

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2D NMR study (Figure 47) of palmerolide E (61) indicated that it has a 20 member macrocyclic ring similar to that of palmerolide A (58). However, the side chain attached to C-19 was shorter with only one olefinic group. The sharp singlet at \( \delta \) 9.41 in the $^1$H NMR spectrum (Figure 48) displayed gHMQC correlation (Figure 49) with a quaternary
carbon resonating at $\delta$ 196.3 suggesting the presence of an aldehyde. The aldehyde was further correlated to olefins C-21 and C-22 in the gHMBC spectrum (Figure 50), confirming the presence of an aldehyde carbonyl conjugated to the C-21/C-22 double bond at the terminus of the side chain.

![Chemical structure](image)

Figure 47. Key gHMBC and gCOSY correlations of palmerolide E (61)

![NMR spectrum](image)

Figure 48. $^1$H NMR spectrum of palmerolide E (61) (500 MHz, DMSO-$d_6$)
The quaternary carbon at $\delta$ 166.1 was assigned as an ester carbonyl (C-1), which correlated to olefinic protons H-2 and H-3 in the gHMBC. These two protons showed a coupling constant of 15.7 Hz indicating a \textit{trans} double bond. Three methylenes were positioned between C-3 and hydroxymethine C-7, based on gHMBC correlations of H-2 with C-4, H-5 with C-6 and H-8 with C-6. This was further supported by the observation of gCOSY correlations (Figure 51) between H-4a and H-5b, H-5a and H-6b and H-7 with H-6a and H-6b. In addition, H-7 displayed a HMBC correlation to olefinic methines C-8 and C-9 (Table 5). Location of two hydroxymethines, C-10 and C-11 adjacent to each
other was established by the COSY correlations of H-9, H-10 and H-11 and further confirmed by HMBC correlation observed for H-11 with C-9 and C-10. In addition, H-11 showed HMBC correlation to the quaternary carbon at $\delta$ 156.4 which was assigned as the carbonyl carbon of the carbamate group.

Figure 50. gHMBC spectrum of palmerolide E (61) (500 MHz, DMSO-$d_6$)
Table 5. NMR data of palmerolide E (61) (\(^{1}H\), 500 MHz, \(^{13}C\), 125 MHz, DMSO-\(d_6\))

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\(\text{OCONH}_2\) 6.48 (2H, br)
The two methylenes, C-12 and C-13, appeared overlapped with each other as a one carbon signal in the $^{13}$C NMR spectrum. Their linkage with hydroxymethine C-11 and olefinic methine C-14 was established by HMBC correlations of H-10, H-11 and H-14. Based on the HMBC relations of H-14, H-15 and H$_3$-25, it was evident that the trans ($J = 14.8$ Hz) double bond (C-14 and C-15) is conjugated to a trisubstituted double bond (C-16 and 17). Methylene C-18 was positioned between the hydroxymethine C-19 and quaternary carbon (C-17) based on gHMBC relationships of H-19, observed with C-18 and C-17. The 20 member macrocycle was completed by the observation of the HMBC cross peak identified for H-19 and C-1.
2.2.7 Characterization of palmerolide B (62).

Palmerolide B (62), was isolated as a colorless, sticky solid. Its $^1$H NMR spectrum (Figure 52) showed signals characteristic of palmerolides despite that it was isolated form a more polar fraction than the fractions that contained other palmerolides. Further, palmerolide B showed a distinctly low $R_f$ value when compared with other palmerolides in thin layer chromatography in normal phase, affirming its higher polarity.

![Figure 52. 1H NMR spectrum of palmerolide B (62) (500 MHz, CD$_3$OD)](image)

The $^{13}$C NMR spectrum (Figure 53) showed that compound 62 had 33 carbon as observed in palmerolide A (58). Further analysis of DEPT 135 (Figure 54) and gHSQC (Figure 55) spectra suggested that it is composed of five methyls, six methylenes, sixteen methines and six quaternary carbon signals. Out of the six methines, four were indicative of hydroxymethines. All these observations were also consistent with the structural features of palmerolide A.
Figure 53. $^{13}$C NMR spectrum of palmerolide B (62) (125 MHz, CD$_3$OD)

Figure 54. DEPT 135 spectrum of palmerolide B (62) (125 MHz, CD$_3$OD)
The interpretation of gHMBC (Figure 56) and gCOSY (Figures 57, 58) data of palmerolide B (62) revealed that it has a 20 member macrocyclic ring with a significant rearrangement of the structural features in C-3 through C-12. Nevertheless, structure of the side chain was identified as identical to that of palmerolide A (58). A quaternary carbon δ 168.2 which was assigned as an ester carbonyl (C-1), showed gHMBC correlation to H-3, which in turn displayed HMBC correlation to two adjacent methylenes, C-4 and C-5. The linkage between the nest two methylenes, C-5 and C-6, was established by observation of COSY correlations between them. These spectral evidence was consistent with an ester carbonyl with a conjugated trans olefin ($J = 15.3$ Hz) attached to these methylenes.
Figure 56. gHMBC spectrum of palmerolide B (62) (500 MHz, CD$_3$OD)

Figure 57. Key gCOSY and gHMBC correlations of palmerolide B (62)
The hydroxymethine $\delta$ 77.4 can be positioned at C-7, between the methylene C-5 and another hydroxymethine $\delta$ 72.1 (C-8) based on gHMBC correlations of H-7 with C-6 and the COSY correlations of H-7 with H-6a and H-8. The presence of another olefin at C-9/C-10 attached to the latter hydroxymethine was supported by gHMBC correlations of H-8 with C-9 and that of H-9 with C-8. This assignment was consistent with the observation of COSY correlations between H-7 and H-8 and H-8 and H-9. In addition, H-7 displayed further HMBC correlations with the quaternary carbon $\delta$ 159.8 characteristic of the carbonyl attached to carbamate group present in all palmerolides.
Table 6. NMR data of palmerolide B (62) (\textsuperscript{1}H, 500 MHz, \textsuperscript{13}C, 125 MHz, CD\textsubscript{3}OD)

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Another hydroxymethine (C-11) could be placed between the former olefin and another methylene (C-12) based on HMBC correlations of H-10, H-12a and H-12b with C-11. The connectivity between the methylenes C-12 and C-13 was confirmed by gCOSY data. The latter methine protons displayed COSY coupling with proton signal $\delta$ 5.38 indicating the presence of two methylenes between hydroxymethine C-11 and olefinic methine C-14. The stereochemistry of this olefin was determined as trans based on the coupling constant between H-14 and H-15. Considering the gHMBC correlations of olefinic protons H-15 to C-14 and the quaternary carbon C-17 and that of vinylic methyl at $\delta$ 1.58 (H$_3$-25) to C-18, C-17 and C-15 a trisubstituted double bond was positioned at C-17/C-16 conjugated to the former olefin.

The hydroxymethine at $\delta$ 76.1 was assigned to C-19, based on HMBC correlations between H-18a and H-18b and COSY correlations of the latter protons to $\delta$ 4.82, representing H-19. The 20 macrolide was completed by the observations of gHMBC correlation between H-19 and C-1.

The side chain of palmerolide B showed features similar to that of palmerolide A. The doublet at $\delta$ 0.88 in the $^1$H NMR spectrum assigned for the methyl (H$_3$-26) showed COSY correlation to H-20. In addition this methyl group displayed gHMBC correlation to C-19, C-20 and C-21 confirming that its located between C-19 and C-21, and attached to C-20. Meanwhile, H-21, an olefinic methine, was correlated to olefinic methines C-23 and C-25 in the gHMBC spectrum. However, gHSQC evidence showed that the latter carbon belong to the vinylic methyl H$_3$-27. Based on the coupling constant ($J = 15$ Hz),
the stereochemistry of olefinic protons H-23 and H-24 was identified as *trans*. These correlations are consistent with a trisubstituted double bond conjugated with a *trans* olefin at C-23/C-24. H-24 displayed gHMBC correlation to C-23, C-24 and C-1’ assigned for an ester carbonyl. The HMBC correlation of broad singlet H-2’ with C-1’ and the unusual $^4J$HMBC coupling of two vinyl methyl groups C-4’ and C-5’ as observed in palmerolide A suggested an isopentenoyl connected to C-24 via an ester linkage.

When the compound 62 was analyzed by LRESIMS it displayed a major peak at $m/z = 663.2$ indicative of $[M - H]^+$. However, in LRESIMS it afforded a major peak at $m/z = 567.4$. This was explained as due to $[M + H - H_2SO_4]^+$, suggesting the presence of a sulphate group attached to one of the secondary hydroxyl groups of palmerolide B. These observations were further confirmed by performing HRESIMS experiments on both above peaks using respective modes of ionization. The HREIMS negative mode (Figure 59) gave a molecular mass of 663.2957 consistent with a formula of $C_{33}H_{48}N_2O_{10}S$. In addition this data indicates the presence of two nitrogens confirming the presence of a carbamate attached to C-7 and an amide between C-24 and C-1’ as was observed in other palmerolides.

The site of attachment of the sulphate group was assigned as C-11 by the observation of relatively high proton and carbon chemical shifts for H-11 and C-11 as compared to corresponding chemicals shifts in other palmerolides. In addition, generation of a stable mass peak at $m/z = 567.3$ in ESIMS can be explained as due to formation of a double bond between C-11 and C-12, after the loss of a sulphuric acid (Figure 60).
Figure 59. HRESIMS spectrum of palmerolide B (62)
Figure 60. Fragmentation of palmerolide B (62) in ESIMS (positive mode)
2.2.8 Characterization of palmerolide H (63)

Further purification of the HPLC fractions containing palmerolide B (62) afforded another compound with $^1$H NMR signals (Figure 61) indicative of palmerolides, which was identified as palmerolide H (63), after a comprehensive structure elucidation with 2D NMR spectroscopy and mass spectrometry.

![Figure 61. $^1$H NMR spectrum of palmerolide H (63) (500 MHz, CD$_3$OD)](image)

The $^{13}$C NMR spectrum (125 MHz) (Figure 62) showed that palmerolide H has 36 carbon signals. Further analysis of gHSQC spectra (500 MHz) (Figure 63) accounted for five methyls, eight methylenes, sixteen methines and six quaternary carbon signals. Out of these methines, four represented hydroxymethines.
Figure 62. $^{13}$C NMR spectrum of palmerolide H (63) (125 MHz, CD$_3$OD)

Figure 63. gHSQC spectrum of palmerolide H (63) (500 MHz, CD$_3$OD)
Extensive study of the gHMBC (Figure 64) and gCOSY spectra (Figure 65) of palmerolide H (63) indicated that it has a 20 member macrocyclic ring similar to that of palmerolide B, in planer structure (Figure 66). The quaternary carbon at $\delta$ 168.2 could be assigned to an ester carbonyl (C-1) based on analogy with other palmerolides, and showed HMBC correlation to H-3, which in turn displayed gHMBC correlation with two adjacent methylenes, C-4 and C-5. The connectivity between two methylenes, C-5 and C-6, was established by gCOSY correlations. These spectral data were consistent with an ester carbonyl bearing a conjugated trans olefin ($J = 15.5$ Hz) followed by C-4 and C-5.

Figure 64. gHMBC Spectrum of palmerolide H (63) (500 MHz,CD$_3$OD)
Figure 65. gCOSY spectrum of palmerolide H (63) (500 MHz, CD3OD).

Figure 66. Key gHMBC and gCOSY correlations of palmerolide H (63).
The hydroxymethine at $\delta$ 77.5 can be positioned between the methine at C-6 and another hydroxymethine $\delta$ 72.2 (C-8) based on the gHMBC correlation of H-8 to C-6 and C-7. This assignment was confirmed by respective gCOSY correlations. H-7 was correlated to an olefinic methine $\delta$ 133.4 (C-9) by HMBC suggesting the presence of an olefin at C-9/C-10, which was further supported by gCOSY correlations between H-8 and H-9. H-7 displayed further gHMBC correlation with the quaternary carbon at $\delta$ 159.9 indicative of carbonyl that was characteristic of the carbamate group present in other palmerolides.

Another hydroxymethine (C-11) could be placed between the former olefin and another methylene $\delta$ 35.5 (C-12) based on gHMBC correlation of H-10, H-12a and H-12b with C-11. The connectivity between the methylenes C-12 and C-13 was confirmed by gCOSY data. The latter methine protons displayed COSY correlation with a proton signal at $\delta$ 5.38 indicating the presence of two methyls between hydroxymethine C-11 and olefinic methine C-14. The stereochemistry of this olefin was determined to be trans based on the coupling constant between H-14 and H-15. Considering the gHMBC correlation of olefinic proton H-15 with C-14 and the quaternary carbon C-17 and that of vinylic methyl at $\delta$ 1.58 (H$_3$-25) to C-18, C-17 and C-15, a trisubstituted double bond was positioned at C-17/C-16, conjugated to the former olefin. The hydroxymethine $\delta$ 76.2 was assigned to C-19 based on HMBC correlations between H-18a and H-18b and COSY correlations of the latter protons with and that of $\delta$ 4.82, H-19. The 20 membered macrolide was completed by the observation of gHMBC correlation between H-19 and C-1 (Table 7).
Table 7. NMR data of palmerolide H (63) ($^1$H, 500 MHz, $^{13}$C, 125 MHz, CD$_3$OD)

<table>
<thead>
<tr>
<th></th>
<th>$\delta$ $^{13}$C</th>
<th>$\delta$ $^1$H (ppm, mult, $J$ (Hz))</th>
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<tr>
<td>1</td>
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<td>2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2’</td>
<td>120.5</td>
<td>5.76 (1H, s)</td>
<td></td>
</tr>
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<td>OCONH$_2$</td>
<td>159.9</td>
<td></td>
<td>2’, 3’, 4’</td>
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The side chain of palmerolide H (63) showed features similar to that of palmerolide D (60). The doublet at $\delta$ 0.89 in the proton NMR spectrum assigned for the methyl (H$_3$-26), showed COSY correlation to H-20. In addition, this methyl group displayed gHMBC correlations with C-19, C-20 and C-21 confirming that it is positioned between C-19 and C-21, and attached to C-20. Meanwhile, H-21, an olefinic methine, was correlated to another olefinic methine (C-23) and C-25 by observation of a cross peak in the gHMBC spectrum. However, gHSQC data showed that the latter carbon belong to the vinylic methyl H$_3$-27. Considering the coupling constant ($J = 14.5$ Hz), the orientation of olefinic protons H-23 and H-24 was assigned as \textit{trans}. These correlations are consistent with a trisubstituted double bond conjugated with a \textit{trans} olefin at C-23/C-24.

The doublet at $\delta$ 6.87 (H-24) displayed gHMBC correlation to the quaternary carbon at $\delta$ 166.0 (C-1$'$). The singlet at $\delta$ 5.83 (H-2$'$) was correlated to C-1$'$, the methyl at $\delta$ 23.8 (C-8$'$) and a methylene at $\delta$ 42.1 (C-4$'$) in the HMBC spectrum. The sharp singlet observed at $\delta$ 3.54, (H-4$'$) in turn showed HMBC correlation with C-3$'$ and C-8$'$ constituting a trisubstituted double bond between the above carbonyl and latter methylene.

The singlet at $\delta$ 3.54 showed further gHMBC correlations with the vinylic methyl C-7$'$, the quaternary carbon at $\delta$ 144.6 (C-5$'$) and an exomethylene carbon at $\delta$ 112.6 (C-6$'$), demonstrating the further elongation of the side chain, giving rise to another disubstituted double bond which defines the terminus in the form of an exomethylene at C-6$'$. 

94
In the LRESIMS spectrum, the compound (63) displayed a major peak at \( m/z = 703.3 \) indicative of \([M - H]^+\). However, the LRESIMS positive mode spectrum demonstrated a major peak at \( m/z = 607.3 \), which was explained as due to \([M + H - H_2SO_4]^+\) suggesting the presence of a sulphate group attached to one of the secondary hydroxyl groups of palmerolide H (63). These observations were further confirmed by performing HRESIMS analysis (Figure 67).

Figure 67. HRESIMS spectrum of palmerolide H (63)
The HRESIMS spectrum gave a molecular mass of 703.3258 consistent with a formula of $C_{36}H_{52}N_2O_{10}S$ (Figure 67). In addition, this mass spectral data indicated the presence of two nitrogens affirming the presence of a carbamate attached to C-7 and an amide between C-24 and C-1′ as was observed in other palmerolides.

2.2.9 Bioactivity of palmerolides

Unique structural features of palmerolide A (58), and the attractive biological activity reported from macrolides with similar appendages, warranted an investigation of its biological activity. Therefore, palmerolide A was tested for anticancer properties at the National Cancer Institute. The very promising anticancer activity displayed by palmerolide A prompted the bioactivity investigation of plamerolide C (59) and D (60) as well.

2.2.9.1 In vitro cytotoxicity of palmerolide A (58)

Palmerolide A displayed reproducible in vitro cytotoxicity towards several melanoma cell lines (Appendix A), UACC-62 (LC$_{50}$ 0.018 µM), MI14 (LC$_{50}$ 0.076 µM), SK-MEL-5 (LC$_{50}$ 6.8 µM) and LOX IMVI (LC$_{50}$ 9.8 µM) in the NCI 60 human cancer cell line panel. Besides melanoma, it showed cytotoxicity against one colon cancer cell line, (HCC-2998, LC$_{50}$ 6.5 µM) and one renal cancer cell line (RFX 393, LC$_{50}$ 6.5 µM). One of the main features of its activity profile was that it was largely devoid of other cytotoxicity (LC$_{50}$>10 µM) making it a promising anticancer drug candidate.
Apart from that, palmerolide A (58) showed potent cytostatic activity against, leukemia (RPMI-8226), colon cancer (HT-116), melanoma cell lines (LOXIMVI, SKMEL-5, UACC-62), ovarian cancer (OVCAR-3) and breast cancer (MDA-MB-231A/ATCC).

2.2.9.2 In vivo cytotoxicity of palmerolide A (58)

Palmerolide A was subjected to the NCI hollow fiber assay, a standard bioassay used by National Cancer Institute to determine in vivo cytotoxicity of anticancer agents. The hollow fiber assay has established as a very efficient bioassay that can provide quantitative information of drug efficacy with minimum expenditures of time and materials. Therefore, it is currently being utilized as the initial in vivo test for agents found to have reproducible activity in the initial in vitro anticancer drug screen.

The hollow fiber assay involves standard panel of 12 tumor cell lines. The cancer cell suspensions are flushed into polyvinylidene fluoride hollow fibers that are heat-sealed at 2 cm intervals. The samples generated from these seals are placed into tissue culture medium and incubated prior to implantation. Each mouse receives three intraperitoneal (Ip) implants (1 of each tumor line) and 3 subcutaneous (Sc) implants (1 of each tumor line). On the day of implantation, samples of each tumor cell line preparation are quantitated for viable cell mass so that the time zero cell mass is known. Mice are treated with experimental agents starting on day 3 or 4 following fiber implantation and continuing daily for 4 days. Each agent is administered by intraperitoneal (Ip) injection at 2 dose levels. The fibers are collected from the mice on the day following the fourth
compound treatment and the viable cell mass in determined again to analyze the efficacy of the drug.\textsuperscript{67}

The percent net growth for each cell line in each treatment group is calculated and compared to the percent net growth in the controls. A 50% or greater reduction in percent net growth in the treated samples compared to the control samples is considered a positive result. Each positive result is given a score of 2 and all of the scores are totaled for a given compound. The maximum possible score for an agent is 96 (12 cell lines X 2 sites X 2 dose levels X 2 [score]). A compound is advanced to the xenograft assay, if it has a combined Ip + Sc score of 20 or greater, a Sc score of 8 or greater, or produces cell kill of any cell line at either dose level evaluated.\textsuperscript{67}

Palmerolide A showed a score of 12 for intraperitoneal implants and a score of 16 of subcutaneous implants giving a total score of 28 which was reproducible, indicating its potent \textit{in vivo} cytotoxicity. Therefore it was advanced to the xenograft assay.

\textbf{2.2.9.3 \textit{In vitro} Cytotoxicity of palmerolide C (59)}

Palmerolide C showed \textit{in vitro} cytotoxicity, and cytostatic activity against leukemia (RPMI-8226, LC\textsubscript{50} 7.33 µM), colon cancer (HCC-2998, LC\textsubscript{50} 7.10 µM and HCT-15, LC\textsubscript{50} 9.87 µM), CNS cancer (SF-295, LC\textsubscript{50} 4.02 µM), melanoma (M-14, LC\textsubscript{50} 1.69 µM), ovarian cancer (OVCAR-3, LC\textsubscript{50} 7.89 µM) and breast cancer (MDM-MB-435, LC\textsubscript{50} 7.75 µM).
2.2.9.4  *In vitro* cytotoxicity of palmerolide E (61)

Palmerolide E showed *in vitro* cytotoxicity against non small cell lung cancer cell line (HOP-62, LC$_{50}$ 9.58 µM), colon cancer (HCC-2998, LC$_{50}$ 7.60 µM and HCT-116 LC$_{50}$ 7.16 µM), CNS cancer (SF-295, LC$_{50}$ 7.35 µM) and melanoma cell line (M-14, LC$_{50}$ 0.56 µM). It also showed cytostatic activity against melanoma.

2.2.9.5 Mechanism of action of palmerolides

By comparison with the activity profiles of the other known anticancer agents, the mode of action of a novel compound can often be predicted.$^{68}$ It was found that the activity profiles of palmerolides are similar to a series of compounds isolated from several other organisms that have been identified as vacuolar ATPase (V-ATPase) inhibitors.

2.2.9.6 V-ATPase

The vacuolar ATPase comprises a class of enzymes that is widely distributed throughout eukaryotes. These enzymes occur in many tissues of multicellular organisms. The major function of V-ATPase is to pump protons from one side of a membrane to the other in order to regulate the pH levels. Hence vacuolar-ATPase performs this function in membranes of vacuoles.$^{69}$

It has been found that V-ATPases are involved in the onset of diseases such as osteoporosis, diabetes, pancreatitis and melanoma. In bones, resorption and remodeling is conducted by osteocasts, specialized cells which possess a V-ATPase on their plasma membrane. Release of V-ATPase acidifies the bone surface thus dissolving the bone
matrix and activating osteoclast secreted acid hydrolases. Defects in osteoclast V-ATPase function have been found to be related to the defects of this enzyme. Involvement of V-ATPase has been proposed in metastatic invasion through degradation of the extra cellular matrix by tumor cells as well. Besides, it has been implicated in the development of melanoma as it was found that organellar pH contributes to the lack of pigmentation in tyrosinase–positive amelanotic melanoma cells. Therefore, V-ATPase inhibitors have emerged as a very attractive target for drug development against osteoporosis and melanoma.69

2.2.9.7 Known V-ATPase inhibitors

Salicylihalamides A (64) and B (65), two macrolides isolated from the marine sponge Halicion sp. showed very selective cytotoxicity against melanoma in the NCI 60 cell line screen.70 However, their activity profile did not match that of any standard clinical agent although a correlation was observed with the patterns of two known V-ATPase inhibitors, concanamycin (66)71 and bafilomycin A (67),72 which were considered unsuitable as anticancer drugs due to high cytotoxicity.

Lobatamide A (68),73,74 belongs to a group of structurally related macrocyclic enamids, isolated from the ascidian Aplidium lobatum and showed a cell growth inhibition profile similar that of salicylihalamides. Later, apicularin A (69),75,76 oximidine I (70) and oximidine II (71),77 which resemble the structure of salicylihalamides more closely, were isolated from marine microbes and also demonstrated similar activity.69
Salicylihalamide A (64)

Salicylihalamide B (65)

Concanamycin (66)

Bafilomycin A (67)
Based on the strong correlations observed between the patterns of cytotoxicity of salicylihalamide A (64) and bafilomycin derivatives, the V-ATPase inhibition activity of salicylihalamides and lobatamides have been tested and they have shown excellent inhibitory activity.69 These observations have simulated interest in these compounds and the functional role of the enamide group, a feature common to most of these compounds in V-ATPase inhibition.68 However, low yields of above compounds from their natural sources have limited the progress of further research.
2.2.9.8 Palmerolides as V-ATPase inhibitor.

Palmerolide A (58) showed inhibition of V-ATPase at 2 nM concentration (Table 8). Further, it was found out that it binds with V-ATPase enzyme at nearly 1:1 molar ratio. Palmerolide C, which has a different ring structure from palmerolide A, displayed lesser activity against V-ATPase at a concentration of 150 nM. Both palmerolides A and C possess an enamide as a part of its side chain and above results are consistent with other macrolides having similar features.68,69 Nevertheless, palmerolide E which does not have an enamide in its structure has only week V-ATPase activity (10 µM) despite submicromolar cytotoxicity against melanoma. These observations suggest the presence of mechanism of action independent of the enamide.

<table>
<thead>
<tr>
<th></th>
<th>Palmerolide A</th>
<th>Palmerolide C</th>
<th>Palmerolide E</th>
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<tbody>
<tr>
<td>V-ATPase activity</td>
<td>2 nM</td>
<td>150 nM</td>
<td>10 µM</td>
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<tr>
<td>Activity against Melanoma</td>
<td>18 nM UACC-62</td>
<td>1.7 µM MI-14</td>
<td>560 nM MI-14</td>
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2.3 Summary

Antarctic tunicate *Synoicum adareanum* elaborates a new series of polyketide macrolides, palmerolides. They are composed of a 20 member macrocycle and a side chain. Characteristic features of the palmerolides include a carbamate group and two secondary hydroxyls attached to the macrolide ring. However, within the series, they show variations in the arrangement of the functional group attached to the ring and side chain.
The structural novelty of palmerolides prompted their bioactivity investigation in NCI 60 cancer cell line panel. The principal member of the series, palmerolide A, showed very potent and selective in vitro cytotoxicity against several melanoma at nanomolar concentrations. In addition, it showed potent cytostatic activity against a number of other cell lines. The in vivo cytotoxicity of palmerolide A was determined by the hollow fiber assay. Palmerolides have been investigated as V-ATPase inhibitors.
3.1 Introduction

3.1.1 Nudibranchs

Nudibranchs are a group of marine molluscs that do not have external shells. They are soft bodied, very often brightly colored and do not have external armaments. The name nudibranchs literally means “naked gills”, as their gills are prominently displayed in the dorsal side in many species. In general, the nudibranchs are carnivores. They feed on variety of other animals, sponges, anemones, tunicates, corals, bryozoans, barnacles and sometimes other nudibranchs.\textsuperscript{5, 78}

Their high visibility, low mobility and lack of physical defense make the nudibranchs extremely vulnerable to predators. Nevertheless, reports of predation are virtually non-existent as they appear to employ chemical defense.\textsuperscript{79} Most nudibranchs obtain their defensive chemicals from their diet, whereas there is a small group of nudibranchs that can synthesize them via \textit{de novo} biosynthesis.\textsuperscript{5}

The secondary metabolite composition of the former category of nudibranchs most often portrays their choice of food. In addition, their chemistry varies according to the location.\textsuperscript{78} Many such sequestered chemicals obtained from other species are toxic and often stored in specialized spherical dorsal glands called mantle dermal formations, a part of the animal that is most susceptible to predation. This further facilitates the usage of a bioactive chemical as a defense weapon with minimal toxic side effects.\textsuperscript{5, 78}
It is understood that nudibranchs can selectively concentrate certain allochemicals from their diet or modify the chemistry of the ingested compounds. For instance, the Mediterranean nudibranch *Hypselodoris orsini* has shown the remarkable ability to convert dietary scalaradial (72) into deoxoscalarin (73) which is concentrated in viscera, and to 6-keto-deoxoscalarin (74), located in mantle dermal formations.5,81

![Scalaradial (72) and Deoxoscalarin (73)](image)

It has been reported that nudibranch *Glossodoris pallida* sequesters scalaradial (72), a sesterterpene identified as a sponge metabolite, and converts it into deoxoscalarin (73). However, it was also noted that it does not sequester scalarin (75), another sesterterpene which is the major sponge metabolite.80 On the other hand, the concentration of deoxyscalarin was found to be significantly higher than the corresponding sponge metabolites. Deoxoscalarin is found in the reproductive system, eggs, and mantle border of *G. pallida*. Although removal of the nudibranch mantle increase susceptibility to predation by reef fish, the specific location of the diet-derived compounds was not significant. It was concluded that the localization of compounds in the mantle tissue, may prevent autotoxicity. However, the egg masses of *G. pallida*, were found to be low.
in the major sesterterpenoid secondary metabolites and are reported to be eaten by a variety of fish.\textsuperscript{5, 80}

![Scalarin (75)](image)

The nudibranchs have also adapted to feed on soft corals, ascidians, bryozoans, or on other molluscs.\textsuperscript{5, 78} The nudibranch \textit{Ovula ovum}, for example, was reported to sequester the toxic terpene sarcophytoxide (76) from soft coral \textit{Sarcophyton} sp. and transform it into the less toxic derivative 77.\textsuperscript{82}

![Sarcophytoxide (76) and 77](image)

A distinct feature of nudibranchs that have the ability to biosynthesize their own defense chemicals was their preference to an invertebrate diet lacking secondary metabolites. In addition, their chemistry is independent of the location that they are collected.
Feeding experiments performed using $^{14}$C-labeled precursors illustrated the ability of the some nudibranchs to undertake *de novo* biosynthesis. Injection of $^{14}$C labeled mevalonate into three species of dorid nudibranchs (*Dendrodoris limbata*, *D. grandiflora*, and *D. arborescens*) demonstrated incorporation of label into three drimane sesquiterpenoids, polygodial (78), 6-β-acetoxyolepupuane (79) and the sesquiterpene ester 80. Incorporation of $^{13}$C-labelled glucose confirmed these terpenes were synthesized *de novo* via the classical mevalonic acid pathway.\(^5\,^{,}{}^{83}\)

![Chemical Structures](image1.png)

Poligodial (78) 6-β-acetoxyolepupuane (79) 80

In biosynthetic studies involving the Canadian nudibranchs *Archidoris montereyensis* and *A. odhneri*, a low rate of incorporation of mevalonic acid into the sesquiterpenoids and diterpenoid acid glyceride metabolites 81, 82 and 83 was reported.\(^5\,^{,}{}^{83}\)

![Chemical Structures](image2.png)

81 82
Use of stable isotopically labeled precursors has led to the convincing experimental evidence for \textit{de novo} biosynthesis in nudibranchs. It was found that effective incorporation levels of respective isotopes can be achieved by simulation of the particular ecological roles of the metabolites. Therefore, handling of the nudibranchs prior to injection leads to release of defensive substances in their mucus and activates the biosynthetic pathways so that the chemical defenses can be unleashed.\textsuperscript{5} Implementation of this strategy demonstrated significantly high incorporations of [1, 2-\textsuperscript{13}C\textsubscript{2}] acetate into the aldehydes nanaimool (84), acanthodoral (85), and isoacanthodoral (86) in \textit{Acanthodoris nanaimoensis}.\textsuperscript{84}

![Nanaimol (84)](image1)

![Acanthodoral (85)](image2)

![Isoacanthodoral (86)](image3)

Subsequent \textsuperscript{13}C studies by the Faulkner and Andersen groups established the mevalonate origin of the terpene portion of glyceride metabolites (81), (83) and tanyolide B (87) in various nudibranchs. Similarly \textit{de novo} biosynthesis of the sesquiterpene metabolites albicanylacetate (88), cadinaldehyde (89), and luteone (90), isolated from \textit{Cadlina}
110

*luteomarginate*, was established. It was further understood that the biosynthesis of above metabolites can be regulated according to the need.\textsuperscript{84,85}

The cadinaldehyde and luteane skeletons were shown to be formed by degradation of a sesterterpenoid precursor. The acetyl residue of albicanylacetate was labeled and its incorporation into the terpene portion was monitored. However, it was only evident during the egg-laying period illustrating that the biosynthesis is regulated.\textsuperscript{85}

It has been found that some nudibranchs utilize both biosynthetic strategies and are able to carry out *de novo* biosynthesis as well as sequestering metabolites. The North American nudibranch *Cadlina luteomarginata* is characterized by the presence number of different sesquiterpenoid and diterpenoid constituents representing 37 carbon skeletons. The origin of most of these have been traced to local sponges. However, the nudibranch
consistently yielded albicanyl acetate (88), a reasonably potent fish deterrent metabolite, from all collection sites. Meanwhile, $1\alpha,2\alpha$-albicanyl acetate (91) was found in egg masses but never found in skin or whole body extracts suggesting that it was produced by the nudibranch. Thus, the nudibranch produces major defense compounds \textit{de novo} in order to ensure reproductive success, but makes use of local sponge toxins for additional protection.\textsuperscript{86,87} Similarly, \textit{Dendrodoris grandiflora} was found to produce drimane sesquiterpenes via \textit{de novo} biosynthesis for egg masses, even though it is known to store some sponge metabolites.\textsuperscript{88}

\begin{center}
\includegraphics[width=0.3\textwidth]{albicanyl_acetate.png}
\end{center}

1$\alpha$, 2$\alpha$-albicanyl acetate (91)

It has been debated as to whether the nudibranchs lost their shell, a major defensive structure, due to the availability of elaborate and efficient defense strategies in the form of chemical defense. On the other hand, the ability to use the toxic substances biosynthesized in other organisms has reduced the metabolic cost to make them on their own.\textsuperscript{78}

\subsection*{3.1.2 Chemistry of \textit{Austrodoris}}

Nudibranchs belonging to the family Doridae are known to employ ichthyotoxic acyl glyceryl esters of diterpenoids or sesquiterpenoids for their protection against predators.\textsuperscript{5} They are only present in the mantle tissues of the animals suggesting their function in
defending the shell-less molluscs. These glycerols were first found in the British Columbian nudibranchs *Archidoris monotereyensis* and *Archidoris odhneri*.\(^8^9-^9^1\) Subsequently, they have been isolated from the Mediterranean nudibranch *Doris verrucosa*, The Antarctic nudibranch *Austrodoris kerguelenensis* and two *Archidoris* species *A. tuberculata* and *A. carvi* which were collected from Northern Spain and Argentina respectively.\(^8^3,^9^2,^9^3\)

*Austrodris kerguelenensis*, a common Antarctic nudibranch widely distributed in the high Antarctic and Subantarctic Zone has been subjected to extensive studies for the presence of defensive chemicals. Studies on different collections of *A. kerguelenensis* have led to a series of *ent*-labdane, halimane and isocopalane diterpenoid glycerols along with some *nor*-sesquiterpenoids.\(^9^1\)

![Austrodoris kerguelenensis at Bonaparte Point, Antarctica (Photograph supplied by Bill J. Baker, University of South Florida)](image)

**Figure 68.** *Austrodoris kerguelenensis* at Bonaparte Point, Antarctica (Photograph supplied by Bill J. Baker, University of South Florida)
Chemical investigation of the mantle tissues of a specimen of *Austrodoris kerguelenensis* collected from McMurdo Sound Antarctica, gave five diterpenoid glyceryl esters 92-96 belonging to labdane family of diterpenoids.\textsuperscript{79} It has been proposed that the oxidative cleavage of the B ring of 92 and 93 between C-8 and C-9 would have given rise to diketones 94 and 95.\textsuperscript{79}

$$\begin{align*}
92 & \quad R_1 = OAc, \quad R_2 = H \\
93 & \quad R_1 = H, \quad R_2 = OAc \\
94 & \quad R_1 = OAc, \quad R_2 = H \\
95 & \quad R_1 = H, \quad R_2 = OAc
\end{align*}$$

Austrodorin (97), a diterpenoid with an unusual halimane skeleton, was reported with its diaceylated derivative from a sample collected from Tethys Bay, of Antarctica.\textsuperscript{93} It may have arisen by a rearrangement of analogous compound having a labdane skeleton. Later, more compounds with this skeleton 98, and 99 were isolated along with labdanes 92 and 93.\textsuperscript{90,94} After synthetic studies to determine the absolute stereochemistry, the absolute
configuration of C-2′ of all these glycerides was established as $S$. Consequently, the stereochemistry of C-2′ of 92 and 93 was assigned as in 100 and 101. Further, reinvestigation of the structures of compounds 99 and 101, led to their revision to 102 and 103.

Another specimen of *Austrodoris kerguelenensis* collected at South Shetland Island Antarctica, yielded two isocopalane diterpenoids austrodorin A (104) and B (105).
The skin extracts of *A. kerguelenensis* nudibranch collected from Terra Nova Bay, Antarctica, gave two *nor*-sesquiterpenoids austrodoral (106) and its oxidized product austrodoric acid (107). In addition, it afforded clerodane diterpenoid acyl glyceride (108) which had the same clerodane diterpenoid residue as archidorin (109) isolated from *Archidoris tuberculata*. 

![Austrodoral (106)](image1.png)  ![Austrodoric acid (107)](image2.png)  ![Archidorin (109)](image3.png)
The observation of the close resemblance of the diterpenoid glycerides of *Austrodris kerguelenensis* to that isolated from *Archidoris montereyensis*, *Archidoris odhnery* and *D. verrucosa*, which are established to be products of *de novo* biosynthesis, led to the suggestion that they also may be biosynthesized *de novo*.\textsuperscript{83, 97}

### 3.1.3 Bioactivity

The biological role of these diterpenoid esters only present in the mantle tissue is linked to the protection of the shell-less molluscs. They have shown toxicity against fresh water fish\textsuperscript{98} and antifeedant activity against marine fish\textsuperscript{99} in different bioassays. The 1,2-diacylglycerols have demonstrated more activity than corresponding 1,3-diacylglycerols. Further, 1,2-diacylglycerols have exhibited potent activity in activation of protein kinase C and in the regenerative tests with fresh water hydrazoan, *Hydra vulgaris*.\textsuperscript{100}

### 3.1.4 Research Objectives

Different collections of *Austrodris kerguelenensis* have shown differences in chemical composition based on the location of the sample collection.\textsuperscript{79, 93} Significant variations of chemistry have been already observed in McMurdo Sound and Palmer Station, in Antarctica.\textsuperscript{35} Therefore, it was of interest to undertake a chemical investigation on the specimens of *A. kerguelenensis* collected from Palmer Station.
3.2 Results and Discussion

3.2.1 Extraction and isolation of secondary metabolites

*P. kerguelenensis* nudibranchs were collected from the ocean near Palmer Station, Antarctica. The CHCl₃ extract of the nudibranchs were fractionated to 12 factions by flash chromatography on silica (Scheme 2). Further purification of fractions 7, 9 and 10 by HPLC on silica gel and C-18 yielded palmadorin A (110) (24 mg, 0.006% dry wt), palmadorin B (111) (7 mg, 0.002% dry wt) and palmadorin C (112) (7 mg 0.002% dry wt).

![Chemical structures](image.png)

palmadorin A (110)  
palmadorin B (111)  
palmadorin C (112)
3.2.2 Characterization of palmadorin A

Palmadorin A, isolated as a viscous oil, showed a \([M + 1]^+\) peak at \(m/z = 379.3\) (Figure 69) in the LRFABMS spectrum. The HRFABMS analysis provided a molecular weight of 379.2842 amu consistent with the molecular formula of \(C_{23}H_{38}O_4\).
The $^{13}$C NMR spectrum of palmadorin A (110) showed 22 carbon signals (Figure 70). The signals at $\delta$ 163.9, 160.7, 114.6 and 102.8 were assigned as olefins whereas the $\delta$ 167.0 was assigned as an ester carbonyl. The carbon resonances $\delta$ 74.6 and 62.9 could be assigned as oxygen bearing carbons. The latter signal exhibited unusually high intensity indicative of coincident signals.
The $^1$H NMR spectrum of compound 110 (Figure 71) showed a distinct sharp doublet at $\delta$ 4.48 (H-18) that integrated for two hydrogens. H-18 exhibited gHSQC correlation (Figure 72) with carbon signal at $\delta$ 102.8, suggesting the presence of an exomethylene. These exomethylene protons displayed strong HMBC (Figures 73, 74) correlations to the quaternary carbon resonances at $\delta$ 160.7 (C-4), 40.2 (C-5) and the methylene carbon signal at $\delta$ 33.2 (C-3). The proton signal $\delta$ 2.09 (H-3a) displayed HMBC correlations with carbons $\delta$ 160.4 (C-4), 40.0 (C-5) and 102.5 (C-18). In addition, H-3a shows HMBC correlation with one carbon resonance at $\delta$ 21.9 (C-1). On the other hand, the proton signal at $\delta$ 2.27 (H-3b) displays HMBC correlations with carbons $\delta$ 160.4 (C-4), 102.5 (C-18) and 28.9 (C-2).
Figure 71. $^1$H NMR spectrum of palmadorin A (110) (500 MHz, CDCl$_3$)

Figure 72. gHSQC spectrum of palmadorin A (110) (500 MHz, CDCl$_3$)
Figure 73. gHMBC spectrum of palmadorin A (110) (500 MHz, CDCl₃)

Figure 74. Key gHMBC and gCOSY correlations of palmadorin A (110)
The singlet at $\delta$ 1.02 (H-19) integrated for three protons and exhibited HMBC correlations to quaternary carbons 160.4 (C-4), C-5 (40.0), methine at $\delta$ 48.9 (C-10) and a methylene at $\delta$ 37.4 (C-6). The proton signal at $\delta$ 1.03 (H-10), in turn shows HMBC correlations to $\delta$ 28.6 (C-2), 40.0 (C-5) and 20.8 (C-19), confirming the structural assignments for the A ring of palmadorin A.

The 3H singlet at $\delta$ 0.71 shows HMBC correlation to $\delta$ 48.7 (C-10), the quaternary carbon at $\delta$ 39.5 (C-9) and a methine at $\delta$ 36.9 (C-8). Meanwhile, the doublet at $\delta$ 0.79, indicative of another methyl, exhibits HMBC correlations to the same carbons suggesting that they are located on adjacent carbons. In addition, it shows further HMBC correlations with the methine at $\delta$ 27.6 (C-7). Both protons attached to C-7, $\delta$ 1.44 (H-7a) and $\delta$ 1.47 (H-7b) exhibit HMBC connectivity to C-8 and C-5 constituting the B ring of palmadorin A (110) (Table 8).

The broad singlet at $\delta$ 5.68 indicative of an olefin displayed HMBC correlation to the quaternary carbon $\delta$ 167.0 (C-15), assigned as an ester carbonyl, as well as methylene $\delta$ 34.8 and methyl $\delta$ 19.7 (C-16). The proton resonance of the latter methyl signal was observed as a doublet at $\delta$ 2.14 in turn displayed HMBC correlations to the former carbonyl, another quaternary carbon signal at $\delta$ 163.9 and the above methylene. These assignments are consistent with a trisubstituted double bond attached to the ester carbonyl and of a methylene group adjacent to the quaternary carbon (C-13). Both protons attached to this methine show distinct HMBC correlations with $\delta$ 163.7 (C-13), 114.4 (C-14), 19.5 (C-16) and 36.1 (C-11). The proton signals at $\delta$ 1.33 and 1.43 (H-11a and H-11b) show
HMBC connectivity to carbons $\delta$ 36.6 (C-8), 39.3 (C-9), 48.7 (C-10), 34.6 (C-12), 163.7 (C-13) and $\delta$ 18.1 (C-20) defining the side chain of the B ring at C-9.

Table 9. NMR data for palmadorin A (108) ($^1$H, 500 MHz, $^{13}$C, 125 MHz, CDCl$_3$)

<table>
<thead>
<tr>
<th></th>
<th>$\delta^{13}$ C</th>
<th>$\delta^1$H (ppm, mult. $J$ (Hz))</th>
<th>gHMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.6</td>
<td>1.43 (1H, m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.48 (1H, m)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>28.6</td>
<td>1.23 (1H, m)</td>
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<td></td>
<td></td>
<td>1.87 (1H, m)</td>
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</tr>
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<td>3</td>
<td>33.0</td>
<td>2.09 (1H, m)</td>
<td>1, 4, 5, 18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.27 (1H, m)</td>
<td>2, 4, 18</td>
</tr>
<tr>
<td>4</td>
<td>160.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>37.2</td>
<td>1.50 (1H, m)</td>
<td>5, 7, 10, 19</td>
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<tr>
<td></td>
<td></td>
<td>1.58 (1H, m)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>27.4</td>
<td>1.44 (1H, m)</td>
<td>5, 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.47 (1H, m)</td>
<td>5, 8</td>
</tr>
<tr>
<td>8</td>
<td>36.7</td>
<td>1.39 (1H, m)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>39.3</td>
<td></td>
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<td>10</td>
<td>48.7</td>
<td>1.03 (1H, m)</td>
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<tr>
<td>11</td>
<td>36.1</td>
<td>1.33 (1H, m)</td>
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<td></td>
<td></td>
<td>1.44 (1H, m)</td>
<td>8, 9, 10, 12, 13, 20</td>
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<td>12</td>
<td>34.6</td>
<td>1.85 (1H, m)</td>
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<td></td>
<td></td>
<td>1.96 (1H, m)</td>
<td>11, 13, 14, 16</td>
</tr>
<tr>
<td>13</td>
<td>163.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>114.3</td>
<td>5.68 (brs s))</td>
<td>12, 15, 16</td>
</tr>
<tr>
<td>15</td>
<td>166.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>19.5</td>
<td>2.14 (3H, s)</td>
<td>12, 13, 14</td>
</tr>
<tr>
<td>17</td>
<td>16.0</td>
<td>0.79 (3H, d, 6)</td>
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<tr>
<td>18</td>
<td>102.5</td>
<td>4.48 (2H, d, 1.5)</td>
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<td>18.0</td>
<td>0.71 (3H, s, 6.0)</td>
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<td>1'</td>
<td>62.7</td>
<td>3.83 (2H, d, 4.5)</td>
<td>2', 3'</td>
</tr>
<tr>
<td>2'</td>
<td>74.4</td>
<td>4.91 (1H, m)</td>
<td>1', 3', 15</td>
</tr>
<tr>
<td>3'</td>
<td>62.7</td>
<td>3.83 (2H, d, 4.5)</td>
<td>1', 2'</td>
</tr>
</tbody>
</table>

124
The C-15 ester carbonyl at $\delta$ 166.7 displays HMBC correlation to the multiplet at $\delta$ 4.91 (H-2'). H-2' in turn displayed COSY correlation to the doublet at $\delta$ 3.83 (H-1'/ H-3') suggesting their presence on neighboring carbons that have downfield shifts due to an oxygen substituent. Meanwhile the latter signal, $\delta$ 3.83 (H-1'/ H-3') integrated for four protons and the carbon attached to it showed significantly increased signal intensity in the $^{13}$C NMR spectrum suggesting they are hydroxymethylene groups with identical carbon ($\delta$ 62.7) and proton ($\delta$ 3.83) chemical shifts. Therefore, a glyceride moiety must be attached to the ester carbonyl at C-2' of the glycerol.

The diterpenoid acid involved in the formation of palmadorin A belongs to the category of clerodane diterpenoids. This carbon skeleton has been reported earlier from tropical plants belonging to the family Annonaceae.$^{101,102}$

3.2.3 Stereochemical determination of palmadorin A (110)

The decalin of palmadorin A (110) can exist in either cis or trans conformations. In order to determine the correct conformation, the ROESY spectrum (Figure 75) of palmadorin A was investigated. In addition, the specific proton signals belonging to the appendages that are most likely to reflect the stereochemistry were irradiated selectively employing a 1D NOESY experiments (Figure 76) to further confirm those observations.
Figure 75 ROESY spectrum of palmadorin A (110) (500 MHz, CDC13)
Selective Irradiation of H-19

![Selective Irradiation of H-19](image)

Selective Irradiation of H-20

![Selective Irradiation of H-20](image)

Figure 76. Selective 1D NOE experiments of palmadorin A \((\textbf{110})\) (500 MHz, CDCl\(_3\))
The protons of the C-19 methyl group of palmadorin A (110) show distinct ROESY correlation to H-3b, H-7b and H-11b defining the top face of the A ring of palmadorin A (Figure 75). The axial orientation of H₃-20 on the top face of the decalin was confirmed by the observation of strong NOE between H₃-20 and H-19, when both peaks were irradiated separately (Figures 76, 77). H-2b and H-10 show ROESY cross peaks demonstrating that they belong on the bottom face of the A ring. The exomethylene protons at C-18 show strong correlations with H-3a and H-6b affirming that the former was equatorial and the latter was axial.

![Figure 77. Key ROESY correlations of palmadorin A (110)](image)

H₃-20 shows further cross peaks with H-11b, H-12b and H₃-17, confirming the axial orientation of C-20. These assignments were consistent with the *trans* decalin conformation for palmadorin A as depicted in Figure 77.

### 3.2.3.1 Absolute stereochemistry determination

Circular dichroism spectroscopy is a very convenient method to determine absolute stereochemistry of organic compounds. In the case of compound 110 this technique can be implemented to determine the absolute stereochemistry of C-5 by converting the A ring that has an exomethylene at C-4 into a cyclohexanone which could give rise to n-π* transitions creating a CD spectrum indicative of its stereochemistry.
Hence, palmadorin A (110) was subjected to ozonolysis under oxidative conditions and the product was separated and purified by silica gel chromatography. Interpretation of the 2D NMR data of the product established its identity as the diketone 111 (Scheme 3).

Scheme 3. Ozonolysis of palmadorin A (110)

The product showed two quaternary carbon signals at $\delta$ 216.3 (C-4) and 209.1 (C-13) indicating the presence of two ketones. The former carbon resonance showed distinct HMBC connectivity (Figure 79) with proton signals $\delta$ 2.58 (H-3a) and 1.15 (H$_3$-15) resulting in its assignment as C-4. The latter quaternary carbon was correlated to proton signals $\delta$ 2.16 (H$_3$-14) and 2.34 (H-12b) providing evidence for the presence of another ketone at C-13. Based on the key HMBC correlations of the methyls H$_3$-15, H$_3$-17 and methine proton H-10 the structure of the product was confirmed as 113. This demonstrated the cleavage of both double bonds in palmadorin A (110) during the ozonolysis to give rise to two ketones.
The ROESY correlations discussed earlier confirmed the *trans* decalin conformation of the A and B rings of palmerolide A. Nevertheless, this *trans* decalin conformation can exist in two possible enantiomeric forms (Figure 78). Identification of the correct enantiomer was accomplished using circular dichroism spectroscopy.

![Figure 78. Key gHMBC correlations of 113](image)

Cotton effect value$^{96} = +0.07$

Figure 79. Two possible *trans* decalin enantiomers for palmadorin A ozonolysis product

According to the principles of CD spectroscopy when the octant rule is applied to the above two enantiomers, 113a should give a positive cotton effect where as 113b should indicate a negative cotton effect.$^{103}$ Therefore, the cotton effect observed for the diketone
113 can be used to distinguish the particular enatiomeric form of palmadorin A. When the CD spectrum of diketone 113 was recorded (Figure 80), it displayed a negative cotton effect confirming that palmadorin A exist as enantiomeric form 113b. Hence the absolute stereochemistry of palmadorin A was established as 5S, 8S, 9R and 10S (110).

Figure 80. CD spectrum of compound 113 showing a negative cotton effect

Figure 81. Absolute stereochemistry of Palmadorin A (108)
3.2.4 Characterization of palmadorin B (111)

Palmadorin B (111) was isolated as colorless, viscous oil and showed an intense FABMS peak at \( m/z = 379.4 \) (Figure 82), indicative of \([M - (CH_3CO)]^+\). It further displayed another prominent peak at \( m/z = 421.4 \), indicative of \([M - H]^+\). Analysis of the HRFABMS data, gave an accurate mass of 421.2974, consistent with the molecular formula \( C_{25}H_{41}O_5 \).

Figure 82. LRFABMS spectrum of palmadorin B (111)
The $^{13}$C NMR spectrum (Figure 83) of palmadorin exhibited 25 carbon signals supporting the HRFBMS data. The carbon signals at $\delta$ 171.2 and 166.3 were assigned as ester carbonyls whereas the signals at $\delta$ 163.6, 160.6, 114.6 and 102.8 appeared to be olefinic carbons. The three carbon signals at $\delta$ 71.6, 62.7 and 61.9 were characteristic of carbons bearing oxygen.

Figure 83. $^{13}$C NMR spectrum of palmadorin B (111) (125 MHz, CDC$_1$)$_3$

A complete structure elucidation of palmadorin B was accomplished by an extensive analysis of its HMBC (Figure 84) and HSQC (Figure 85) data. The COSY spectrum (Figure 86, 87) was used to get supporting information wherever it was appropriate.
Figure 84. gHMBC spectrum of palmadorin B (111) (500 MHz, CDC13)

Figure 85. gHSQC spectrum of palmadorin B (111) (500 MHz, CDC13)
Figure 86. gCOSY spectrum of palmadorin B (111) (500 MHz, CDC$_3$)

Figure 87. Key HMBC and COSY correlations of palmadorin B (111)
The sharp doublet at $\delta$ 4.48 in the $^1$H NMR spectrum (Figure 88) of the compound (111) integrated for two hydrogens and showed HSQC correlations with a single carbon at $\delta$ 102.8 demonstrating that it was an exomethylene. These exomethylene protons showed distinct HMBC correlations to carbons $\delta$ 34.8 (C-3), 160.6 (C-4) and 40.2 (C-5), respectively. The methylene proton H-3a ($\delta$ 2.08) exhibited HMBC correlation to $\delta$ 29.0 (C-2), 40.4 (C-5) and 102.9 (C-18) whereas H-3b ($\delta$ 2.26) is related to only $\delta$ 29.4 (C-2) and 102.9 (C-18) (Table 9).

Figure 88. $^1$H NMR spectrum of palmadorin B (111) (500 MHz, CDC$_3$)
Table 10. NMR data of palmadorin B (111) (1H, 500 MHz, 13C, 125 MHz, CDC13)

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<tr>
<td>_COOCH3</td>
<td>171.2</td>
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The 3H singlet at $\delta$ 1.02 (C-19), exhibited HMBC correlation to the quaternary carbons $\delta$ 160.6 (C-4), 40.4 (C-5), 37.7 (C-6) and 49.1 (C-10), establishing a major portion of the A ring (Figure 87). The rest of the six membered ring was characterized by the COSY correlations between H-2a, H-2b and H-1a, H-1b and H-10.

The sharp 3H singlet at $\delta$ 0.72 (H$_3$-20) displayed HMBC correlations to carbons $\delta$ 37.0 (C-8), 39.7 (C-9), 49.1(C-10), 36.4 (C-11). Meanwhile, the 3H doublet at $\delta$ 0.78 (H$_3$-17) exhibited HMBC cross peaks with carbons $\delta$ 27.8 (C-7), 37.0 (C-8), and 39.7 (C-9).

Unambiguous assignment of the rest of the B ring was accomplished on the basis of HMBC correlation of H-6b ($\delta$ 1.57) with carbons $\delta$ 40.4 (C-5), 37.0 (C-8) and 49.1 (C-10) and that of H-6a ($\delta$ 1.47) with $\delta$ 37.0 (C-8).

The broad singlet observed at $\delta$ 5.64 (H-14) showed HMBC correlation to the ester carbonyl $\delta$ 166.3 (C-15) and the methylene $\delta$ 34.9 (C-12). On the other hand, the 3H doublet at $\delta$ 2.12, indicative of a vinyl methyl, displayed HMBC correlation with $\delta$ 34.9, a quaternary carbon at $\delta$ 163.6 and a methine at $\delta$ 114.6. All these observation were consistent with a trisubstituted double bond conjugated to an ester carbonyl. The connectivity between this side chain into the A and B rings could be established by the HMBC correlations of $\delta$ 1.33 (H-11a) and 1.44 (H-11b) with 39.7 (C-9), 49.1 (C-10) and 34.9 (C-12).

The ester carbonyl at $\delta$ 166.3 (C-15) exhibited HMBC correlation to the proton signal at $\delta$ 5.06, observed as a multiplet. The doublets at $\delta$ 3.73 and 4.26 correlated in the COSY
spectrum with the proton at $\delta$ 5.06 (H-2$^{\prime}$), establishing the presence of the glyceride moiety. The proton resonances at $\delta$ 4.26 (H-1$^{\prime}$) not only shows HMBC coupling with carbon shifts 72.0 (C-2$^{\prime}$) and 62.2 (C-3$^{\prime}$), but also with the ester carbonyl at $\delta$ 171.2. The proton at 4.26 (H-1$^{\prime}$) also correlates to the methyl singlet $\delta$ 2.05 which suggest the presence of an acetyl group. All these observations are consistent with a glyceride moiety attached to a carbonyl via an ester linkage at C-2$^{\prime}$ which is acetylated at C-1$^{\prime}$.

### 3.2.5 Stereochemistry determination of palmadorin B (111)

The stereochemical elucidation of palmadorin B (111) was undertaken by analysis of its ROESY spectrum. Selective one dimensional NOE experiments were also employed to confirm these assignments.

The carbon and proton spectral data of the A and B rings of palmadorin A and B showed significant similarities suggesting that they have similar stereochemistry. As observed in palmadorin A, methyl group C-19 showed ROESY correlations (Figure 89) to H-3b, H-7a indicating that they define the top face of the A ring (Figure 90). In addition, the H$_3$-19 methyl shows a strong ROESY correlation with H$_3$-20 demonstrating that they both are on the top face of the decalin with an axial orientation. Further, the exomethylene protons at C-18 displayed ROSEY cross peaks with H-3a and H-6a, in agreement with this observation.
Figure 89. ROESY spectrum of palmadorin B (111) (500 MHz, CDC13)

Therefore, the stereochemistry of compound 109 can be illustrated as displayed in Figure 91. Hence, by analogy with the stereochemical features of palmadorin A, the absolute stereochemistry of palmadorin B is assigned as 5S, 8S, 9R and 10S.
3.2.6 Characterization of palmadorin C (112)

Palmadorin C (112) was isolated as colorless oil. The LRFABMS (Figure 92) provided a $m/z = 377.3$ indicative of $[M - H_2O]^+$. HRFABMS on this mass peak suggested molecular formulae of $C_{23}H_{38}O_4$.

Figure 92. LRFABMS spectrum of palmadorin C (112)
Analysis of the $^{13}$C NMR spectrum (Figure 93) of palmadorin C (112) showed 23 signals. The carbon resonance at δ 166.9 was assigned as an ester carbonyl whereas the signals at δ 163.5, 163.5, 145.1, 120.0 and 114.8 were indicative of olefins. Meanwhile, the signals δ 74.6, 73.8, 63.0 and 62.9 indicated four carbons bearing oxygen.

![Figure 93. $^{13}$C NMR spectrum of palmadorin C (112) (125 MHz, CDCl$_3$)](image)

According to HSQC spectrum (Figure 94) the $^1$H NMR peak at δ 5.13 (Figure 95) correlated to the carbon resonance at δ 120.0. The carbon signals at δ 145.1 (C-4) and 37.8 (C-5) did not show HSQC correlation suggesting that they were quaternary. The 3H signal at δ 1.60 correlated in the HMBC (Figure 96) spectrum with all above three carbons indicating a trisubstituted double bond.

142
Figure 94. gHSQC spectrum of palmadorin C (112) (500 MHz, CDCl₃)

Figure 95. ¹H NMR spectrum of palmadorin C (112) (500 MHz, CDCl₃)
Figure 96. gHMBC spectrum of palmadorin C (112) (500 MHz, CDCl₃)

The $^1$H NMR signals at $\delta$ 1.27, depicting a singlet and integrating for three protons, displayed HMBC correlation to carbons C-4 ($\delta$ 145.2), C-5 ($\delta$ 37.80), C-6 ($\delta$ 43.14) and C-10 ($\delta$ 38.5). The connectivity from C-3 ($\delta$ 120.0) to C-10 ($\delta$ 46.9) via two methylenes C-1 ($\delta$ 18.2) and C-2 ($\delta$ 27.0) was established by COSY correlations (Figures 97, 98) giving rise to the A ring of palmadorin C.

The 3H doublet at $\delta$ 1.01 displayed HMBC correlation to $\delta$ 73.9 (C-7) and 39.5 (C-8). A 3H singlet at $\delta$ 0.99 correlated in the HMBC with $\delta$ 39.5 (C-8), 38.5 (C-9), 46.9 (C-10) and 37.6 (C-11) (Table 10). In addition, the $^1$H NMR signal at $\delta$ 1.37 (H-10) showed HMBC correlations to $\delta$ 18.2 (C-1), 145.2 (C-4), 37.8 (C-5), 43.1 (C-6), 22.0 (C-19) and
20.3 (C-20). Meanwhile, H-6b (δ 2.10) demonstrated HMBC cross peaks to δ 22.0 (C-19), 73.9 (C-7) and 39.5 (C-8), establishing the B ring.

Figure 97. gCOSY spectrum of palmadorin C (112) (500 MHz, CDCl₃)

Figure 98. Key HMBC and COSY correlations of palmadorin C (112)
Table 11. NMR data of palmadorin C (112) (^1H, 500 MHz, ^13C, 125 MHz, CDCl3)

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<td>13</td>
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<td>3’</td>
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<td>3.82 (2H, d, 4.5)</td>
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The singlet at δ 5.70 (H-14) indicated distinct HMBC correlations to δ 35.10 (C-12), 167.0 (C-15) and 19.7 (C-16). Due to its relatively low field chemical shift, the methyl at δ 2.16 (H3-16) could be assigned as a vinyl methyl. It showed further HMBC correlation to the methine C-12 (δ 35.1), quaternary C-13 (δ 163.4) and C-14 (δ 114.9). These assignments are consistent with a trisubstituted double bond conjugated to an ester.
carbonyl. The connectivity of C-12 (δ 35.10) and C-9 (δ 38.5) via C-11 (δ 37.6) was established by the observed COSY correlations of H-11a (δ 1.39), H-11b (δ 1.51) with H-12a (δ 1.93), H-12b (δ 1.98).

HMBC correlation was observed between the multiplet at δ 4.92 (H-2′), attached to a hydroxymethine δ 74.8 and the ester carbonyl at δ 167.0. In addition, the multiplet at δ 4.92 (H-2′) shows COSY correlation to the 4H doublet at δ 3.82 (H-1′/H-3′) which showed HSQC correlations to two hydroxymethines C-1′ and C-3′ (δ 63.1).

Based on these data palmadorin C (112) was characterized as a diterpenoid glyceride. It shares similar pattern of glyceride formation with palmadorin A (110) and B (111) with the ester linkage established at C-2′ of the glycerol. This is relatively uncommon among diterpenoid glycerides.

3.2.7 Stereochemistry determination of palmadorin C (112)

The relative stereochemistry of palmadorin C was studied by NOE spectroscopy. The olefinic proton H-3 displayed strong ROESY correlations to H-2b and the vinylic methyl at δ 1.60 (H₃-18). A cross peak for the methyls at H₃-19 and H₃-20 indicated that they both are on the same face of the decalin system. Similarly H-7 shows its spatial proximity with H-8 and H-6 by means of ROSEY correlations and H-6a in turn correlated to H-10. H-6a, H-7 and H-8 are therefore all oriented toward the opposite face of the decalin compared to H₃-19 and H₃-20 (Figure 99).
Mosher’s method was chosen to determine the stereochemistry of C-7 taking advantage of the secondary hydroxyl group. However, prior to that the primary hydroxyl groups in the glyceride moiety needed to be protected.

3.2.7.1 Diacetyl derivative of palmadorin C (114)

Palmadorin C (112) was allowed to react with acetic anhydride overnight and the resultant product was separated and purified by chromatography on silica. Two equivalents of acetic anhydride was used to make sure the acetylation takes place only on desired primary hydroxyl groups.

The purified product was identified as palmadorin C diacetate (114) by NMR spectroscopy (Figure 100, 101) and mass spectroscopy.
Scheme 4. Acetylation of palmadorin C (112)

\[
\text{Ac}_2\text{O} \ (2\text{eq}) \\
\text{DMAP} \\
\text{TEA} \\
\text{CH}_2\text{Cl}_2, \ 25^\circ\text{C}, \ 12\text{hr}
\]

Figure 100. \(^1\)H NMR spectrum of palmadorin C diacetate (114) (500 MHz, CDCl\(_3\))

The \(^1\)H NMR spectrum of the diacetate showed a singlet at \(\delta\) 2.06 indicative of a methyl group of an acetyl affirming that acetylation has taken place. In addition, instead of the doublet at \(\delta\) 3.82 representing the H-1\(^\prime\) a, H-1\(^\prime\) b, H-3\(^\prime\) a and H-3\(^\prime\) b, a multiplet was observed at \(\delta\) 4.23. The HSQC correlations confirmed their attached to C-1\(^\prime\)and C-3\(^\prime\). A
HMBC correlation observed between the carbon at $\delta$ 170.8 representing the carbonyl of the acetyl group and the multiplet at $\delta$ 4.23 provided further confirmation for acetylation (Figure 102).

The hydroxymethine proton at C-7 did not show a noticeable difference in its chemical shift affirming that it was not esterified. Further it did not show HMBC correlation to carbonyls. The other proton and carbon signals showed HMBC and COSY correlations in agreement with palmadorin C.

![13C NMR spectrum of palmadorin C diacetate](image)

**Figure 101.** $^{13}$C NMR spectrum of palmadorin C diacetate (**114**) (125 MHz, CDCl$_3$)
Analysis of the compound 114 by FABMS (Figure 103) displayed a prominent peak at $m/z = 477.2$ which was attributed to $[\text{M} - \text{H}]^+$. This indicates a molecular weight of 478, consistent with the addition of two acetyl substituents.
3.2.7.2 $R$-MTPA ester of palmadorin C diacetate (115)

The diacetate of palmadorin C (114) was treated with $R$-MTPACl for 48 hours and the product was separated and purified by chromatography on silica gel. The purified $R$-MTPA ester 115 was analyzed by NMR and mass spectroscopic methods in order to confirm the formation of MTPA derivative.

LRESIMS (Figure 104) of compound 115 produced a peak at $m/z = 717.1$ attributed to $[M + Na]^+$. A HRFABMS produced a molecular mass of 717.3213 consistent with molecular formulae of $C_{37}H_{49}F_{3}O_{9}$ (calc. 717.3219)

![Figure 104. LRESIMS spectrum of palmadorin C diacetate $R$-MTPA ester (115)](image-url)
The $^1$H NMR spectrum (Figure 105) confirmed the attachment of MTPA moiety by displaying two multiplets at $\delta$ 7.35 and 7.52, indicating the aromatic protons of the phenyl ring and the 3H singlet at $\delta$ 3.58 representing the methoxy group. The HSQC spectrum (Figure 106) showed that the proton that gives rise to the signal at $\delta$ 5.35 is attached to C-7. Attachment of the MTPA moiety to C-7 by means of an ester group was further illustrated by the significant decrease of the chemical shift of the neighboring protons.

The sharp singlet at $\delta$ 0.53 was identified as the methyl group at C-19 by HMBC (Figure 107) correlations to the quaternary $\delta$ 144.5 (C-4), methine 46.4 (C-10) and methylene 40.4 (C-6). The 3H doublet at $\delta$ 0.92 (H$_3$-17) and the 3H singlet $\delta$ 0.80 (H$_3$-20), both show HMBC correlations to the carbons at $\delta$ 38.4 and 38.9, assigned to C-9 and C-8 respectively. This assignment was further confirmed by the HMBC cross peaks observed between the singlet $\delta$ 0.80 and the carbon $\delta$ 46.4 (C-10). H-7 exhibited COSY correlation with proton signals $\delta$ 1.92, 1.37 and 1.69 indicating that they represent H-6a, H-6b and H-8. The 3H singlet at $\delta$ 1.43 was identified as the methyl at C-18 based on its HMBC correlations to $\delta$ 120.6 (C-3), 145.2 (C-4) and 37.3 (C-5).
Figure 105. $^1$H NMR spectrum of palmadorin C diacetate R-MTPA ester (115) (500 MHz, CDCl$_3$)

Figure 106. gHSQC spectrum of palmadorin C diacetate R-MTPA ester (115) (500 MHz, CDCl$_3$)
Figure 107. $^1$HMBC spectrum of palmadorin C diacetate $R$-MTPA ester (115) (500 MHz, CDCl$_3$)

The rest of the carbon and proton resonances were not affected by the attachments of the MTPA moiety.

### 3.2.7.3 S-MTPA ester of palmadorin C diacetate (116)

The diacetate of palmadorin C (114) was treated with S-MTPACl for 48 hours and the product was separated and purified by chromatography on silica gel. The purified S-MTPA ester (116) was analysed by NMR and mass spectroscopic methods in order to confirm the formation of the MTPA derivative.
LRESIMS of compound 116 (Figure 108) displayed $m/z = 717.1$ attributed to $[M + Na]^+$. HRFABMS analysis produced a molecular mass of 717.322 consistent with molecular formula of $C_{37}H_{49}F_{3}O_{9}$ (Calc. 717.3219).

Figure 108. LRESIMS of palmadorin C diacetate S-MTPA (116)

The $^{13}$C NMR spectrum (Figure 109) of the product confirmed the formation of the MTPA derivative by demonstrating additional carbon signals at $\delta$ 127.36, 128.47 and 129.65 indicative of the aromatic ring and $\delta$ 166.95 representing the carbonyl involved in the ester group.
The $^1$H NMR spectrum of 116 (Figure 110) provided evidence for the attachment of MTPA moiety by displaying two multiplets at $\delta$ 7.53 and 7.37 indicating the aromatic protons belong to the phenyl ring and the 3H singlet at $\delta$ 3.49 representing the methoxy group. The HSQC correlations (Figure 111) showed that the proton that gives rise to the signal at $\delta$ 5.44 is attached to C-7. Attachment of the MTPA moiety to C-7 by means of an ester group was further illustrated by the significant decrease of the chemical shift of the neighboring protons.

The sharp singlet at $\delta$ 0.93 (3H) displayed HMBC correlations (Figure 112) to the quaternary carbon $\delta$ 143.6 (C-4), the methine $\delta$ 46.6 (C-10) and methylene $\delta$ 40.1 (C-6). The doublet at $\delta$ 0.75 (3H) and the singlet $\delta$ 0.63 (3H) both show HMBC correlations to carbons at $\delta$ 38.4 (C-9) and 39.3 (C-8) indicating that they represent the methyls at C-17.
and C-20 respectively. The latter singlet further displayed HMBC correlations with δ 46.6 (C-10) and 37.2 (C-11) further supporting this assignment. The singlet (3H) at δ 0.93 was assigned as C-19 considering its HMBC cross peaks with δ 143.6 (C-4), 37.4 (C-5) and methine δ 40.06 (C-6).

Figure 110. $^1$H NMR spectrum of palmadorin C diacetate S-MTPA ester (116) (500 MHz, CDCl$_3$)

Figure 111. gHSQC spectrum of palmadorin C diacetate S-MTPA ester (116) (500 MHz, CDCl$_3$)
Figure 112. gHMBC spectrum of palmadorin C diacetate S-MTPA ester (116) (500 MHz, CDCl₃)

Based on COSY correlations exhibited by H-7, the proton signals δ 1.48, 2.06 and 1.65 were identified as H-6a, H-6b and H-8 respectively. The vinyl methyl C-18 appeared at δ 1.54 was assigned based on the HMBC relationships with δ 120.7 (C-3), 144.0 (C-4) and 37.4 (C-5). The rest of the carbon and proton shifts were consistent with that observed for the starting material.
3.2.7.4 Application of Mosher’s method

Once all the signals are assigned for the respective protons in a model according to the Mosher’s method (Figure 113), the stereochemistry of C-7 was found to be R. Considering the ROESY correlations observed, the final structure of palmadorin C was assigned as 5R, 7R, 8S, 9R, and 10R (Figure 114).

Figure 113. Δδ value assignments for palmadorin C diacetate MTPA esters

Figure 114. Absolute stereochemistry of palmadorin C (112)
3.3 Summary

Nudibranches, being shell-less molluscs, are known to employ defensive chemicals in order to survive against predators. A comprehensive chemical investigation of the Antarctic nudibranch *Austrodoris kerguelenensis* afforded a series of new clerodane diterpenoid glyceride esters named palmadorins, which may be involved in its chemical defense. The structure elucidation of palmerodorin A-C was accomplished by spectroscopic methods and semi-synthetic derivatizations.
Chapter 4  EXPERIMENTAL

4.1 General Procedure

Optical rotations were measured on an Autopol IV automatic polarimeter using Na lamp at 25°C. Infrared spectra were obtained with Nicolette Avatar 320FT-IR as films. Ultraviolet-Visible experiments were measured on a Hewlett-Packard 8452A diode array UV-Vis spectrometer. $^1$H and $^{13}$C, gHMQC, gHSQC, gHMBC and $^1$H-$^1$H COSY NMR spectra were obtained on a Varian Inova 500 instrument, operating at 500 MHz for $^1$H and 125 MHz for $^{13}$C, using residual protonated solvent as $^1$H internal standard or $^{13}$C absorption lines of solvents for $^{13}$C internal standard. $^1$H chemical shifts were recorded relative to $\delta$ 7.24 (CDCl$_3$), 4.78 (CD$_3$OD) and 2.50 (DMSO-$d_6$) whereas the $^{13}$C shifts are referenced to $\delta$ 77.2 (CDCl$_3$), 49.2 (CD$_3$OD) and 39.5 (DMSO-$d_6$).

Low resonance mass spectra were recorded on a Aligent Teconologies LC/MSD VL electrospray ionization mass spectrometer. High resonance mass spectra were obtained on an Aligent Teconologies LC/MSD TOF electrospray ionization mass spectrometer. CD spectra were obtained with Aviv Instruments model 215 CD spectrometer. HPLC was performed on with a Shimadzu LC-8A multisolvent delivery system connected to a Shimadzu SPD-10A UV-VIS tunable absorbance detector and using YMC-Pack ODS-AQ C-18 column. EM Science silica gel 60 of 230-400 mesh was used in flash column chromatography. TLC was carried out on Whatman K6F silica gel 60A TLC plates with 0.25 mm thickness. They were visualized by spraying with 5% phosphomolybdic acid in EtOH and heating.
4.2 Isolation of Secondary metabolites from Synoicum adareanum

*Synoicum adareanum* was collected at a depth of 80-100 feet at number of locations near Palmerstation in Antarctica by SCUBA diving, and the animals were frozen immediately after the collection. The samples were identified by Dr. Linda Cole, at the Smithsonian Institute, Washington, D.C.

After freeze drying, 180 g of animals was sequentially extracted 3X with 1:1 CH$_2$Cl$_2$/MeOH for one day each. Upon evaporation of the solvent under reduced pressure, a reddish brown highly viscous oil was obtained. It was partitioned with EtOAc/H$_2$O and the EtOAc layer was separated, washed and dried with anhydrous Na$_2$SO$_4$. The removal of solvent under reduced pressure gave a reddish brown semisolid (2.2 g).

Fractionation of the EtOAc extract was performed by step gradient flash chromatography on silica using 100 mL each of hexane, 2%, 5%, 7%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 80% and 100% EtOAc in hexane followed by 5%, 10%, 15%, 20% MeOH in EtOAc. The factions 3, 5 and 6 were further separated by gradient elusion of 1%, 2%, 5%, 7% and 10% MeOH in CHCl$_3$. Subsequent purification of eluate by HPLC using 40% water in MeCN (isocratic elution, 2 mL per min) afforded compounds 58, 59, 60 and 61 as white solids. The fractions 8, 9 and 10 upon further purification with HPLC using 50% water in MeCN (isocratic elution, 2 mL per min) yielded compounds 62 and 63. Since 62 and 63 found to be unstable in DMSO, the NMR investigations were performed in CD$_3$OD.
4.2.1 Palmerolide A (58)

White solid; \([\alpha]^{D}_D -1.6\) (c 0.5, MeOH); IR (thin film) 3360 (br), 2925, 2856, 1696, 1633, 1517, 1392, 1275, 1190 and 1080. UV (MeOH) \(\lambda_{\text{max}} (\epsilon)\): 224 (2670), 242 (2800), 296 (1775); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) (multiplicity, \(J\) (Hz), assignment): 9.84 (1H, d, 10.1, 24-NH) 6.85 (1H, dd, 10.1, 14.2, H-24) 6.71 (1H, ddd, 5.0, 9.9, 15.2, H-3) 6.49 (2H, br, OCONH$_2$) 6.04 (1H, dd, 11.1, 14.6, H-15) 5.85 (1H, d, 14.2, H-23) 5.77 (1H, d, 15.3, H-2) 5.69 (1H, br s, 1.0, H-2') 5.59 (1H, d, 11.4, H-16) 5.54 (1H, dd, 7.7, 15.0, H-8) 5.48 (1H, dd, 2.9, 15.6, H-9) 5.41 (1H, ddd, 4.7, 10.1, 14.6, H-14) 5.20 (1H, d, 4.9, 10-OH) 5.13 (1H, d, 9.6, H-21) 4.84 (1H, ddd, 1.3, 7.4, 11.2, H-19) 4.72 (1H, d, 3.9, 7-OH) 4.48 (1H, dd, 5.0, 10.5, H-11) 4.14 (1H, br s, H-10) 3.82 (1H, ddd, 4.4, 7.4, 7.6, H-7) 2.68 (1H, qdd, 6.5, 7.4, 9.6, H-20) 2.17 (1H, dd, 1.3, 13.2, H-18) 2.12 (3H, s, H-5') 2.11 (2H, m, H-4) 2.00 (1H, dd, 11.2, 13.2, H-18) 1.96 (2H, m, H-13) 1.83 (3H, s, H$_3$-4') 1.70 (3H, s, H$_3$-27) 1.61 (3H, s, H$_3$-25) 1.59 (1H, m, H-12) 1.50 (1H, ddd, 4.5, 8.2, 11.2, H-6) 1.30 (1H, m, H-6) 1.30 (1H, m, H-5) 1.05 (1H, m, H-5) 0.98 (1H, m, H-12) 0.90 (3H, d, 6.5, H$_3$-26) \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) (assignment): 165.3 (C-1), 163.1 (C-1'), 156.6 (OCONH$_2$), 151.7 (C-3'), 149.2 (C-3), 133.6 (C-8), 132.5 (C-22), 132.0 (C-14), 131.5 (C-17), 129.7 (C-21), 128.9 (C-9), 126.1 (C-15), 125.7 (C-16), 121.8 (C-24), 120.3 (C-2), 117.9 (C-2'), 116.4 (C-23), 73.7 (C-19), 72.5 (C-7), 72.3 (C-11), 69.00 (C-10), 43.6 (C-18), 38.1 (C-6), 36.9 (C-20), 32.6 (C-4), 29.5 (C-13), 29.5 (12), 27.1 (C-4'), 24.8 (C-5), 19.2 (C-5'), 16.9 (C-26), 16.2 (C-25), 12.6 (C-27); FABMS \(m/z\) (%) 737.5 (5, [M$^+$ + dtt]$^+$), 585.5 (10 [M + H]$^+$), 501.4 (200), 309.1 (13), 275.1 (5), 195.1 (15), 155.1 (45); HRFABMS \(m/z\) 585.3539 (C$_{33}$H$_{49}$N$_2$O$_7$ requires 585.3540).
4.2.2 Preparation of MTPA esters of palmerolide A

Palmerolide A (58) (5 mg, 0.0086 mmol) was dissolved in CH$_2$Cl$_2$ and treated with $R$- or $S$- methoxytrifluorophenylacetyl chloride (MTPACl) (10 eq) in the presence of Hunig’s base and DMAP. The reaction was stirred for 48 hrs. Upon concentration of the reaction mixture the two main products were purified by chromatography on silica gel (2% MeOH/CH$_2$Cl$_2$) followed by HPLC (silica gel 2% MeOH:CH$_2$Cl$_2$).

4.2.2.1 Palmerolide A 7-[(R)-MTPA] Ester (64)

Colorless solid; $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ (multiplicity, $J$ (Hz), assignment): 7.92 (s, NH), 7.52 – 7.45 (5H, m, Ph), 6.97 (d, 14.4, H-24), 6.83 (ddd, 5.2, 10.1, 15.5, H-3), 6.12 (dd, 11.1, 14.2, H-15), 5.96 (d, 14.4, H-23) 5.99 (dd, 3.5, 15.5, H-9), 5.80 (d, 15.5, H-2), 5.78 (ddd, 1.8, 8.6, 15.5, H-8), 5.74 (br t, 1.2, H-2'), 5.67 (d, 11.1, H-16), 5.48 (m, H-7), 5.47 (m, H-14), 5.19 (dd, 0.7, 9.9, H-21), 4.95 (m, H-19), 4.68 (ddd, 2.0, 5.3, 10.8, H-11), 4.34 (ddd, 1.9, 3.4, 5.2, H-10), 3.55 (s, OCH$_3$), 2.77 (ddq, 6.6, 8.0, 9.9, H-20), 2.26 (br d, 12.5, H-18a), 2.20 (d, 1.2, H$_3$-5'), 2.18 (m, H-4a), 2.10 (dd, 11.5, 12.8, H-18b), 2.07 (m, H-4b), 2.06 (m, H$_2$-13), 1.92 (d, 1.2, H$_3$-4'), 1.82 (d, 0.7, H$_3$-25), 1.69 (s, H$_3$-27), 1.68 (m, H-6a), 1.65 (m, H-12a), 1.62 (m, H-6b), 1.38 (m, H-5a), 1.24 (m, H-5b), 1.19 (m, H-12b), 0.99 (d, 6.7, H$_3$-26). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ (assignment): 166.8 (C-1), 165.1 (C-1'), 158.3 (OCONH$_2$), 153.7 (C-3'), 149.1 (C-3), 135.0 (C-9), 133.0 (C-22), 132.0 (C-14), 131.8 (C-17), 130.8 (C-21), 129.7 (Ph), 128.4 (Ph), 128.4 (C-16) 127.5 (Ph), 127.5 (C-8), 126.9 (C-15), 121.5 (C-24), 121.2 (C-2), 118.2 (C-23), 117.6 (C-2'), 78.2 (C-7), 76.1 (C-11), 74.9 (C-19), 70.4 (C-10), 54.8 (OCH$_3$), 43.7 (C-18), 37.3 (C-20),
33.9 (C-6), 32.2 (C-4), 30.1 (C-12), 29.6 (C-13), 26.2 (C-4'), 24.4 (C-5) 19.0 (C-5'), 16.2 (C-26), 15.0 (C-27), 11.8 (C-25); LRESIMS m/z 823.1 [M + Na]^+; HRESIMS m/z 823.3779 (C_{43}H_{55}N_{2}O_{9}F_{3}Na requires 823.3757).

4.2.2.2 Palmerolide A 7, 10–[(R, R)-MTPA] diester (66).

$^1$H NMR (500 MHz, CD$_3$OD) δ (multiplicity, $J$ (Hz), assignment): 7.91 (s, NH), 7.36-7.47 (10H, m, Ph), 6.97 (d, 14.4, H-24), 6.78 (ddd, 5.6, 9.1, 15.3, H-3), 6.13 (dd, 10.7, 5.1, 11-15), 5.96 (d, 14.4, H-23), 5.76 (d, 15.5, H-2), 5.74 (br t, 1.1, H-2'), 5.73 (dd, 5.8, 15, 11-9), 5.66 (d, 10.6, H-16), 5.57 (m, H-8), 5.55 (m, H-10), 5.53 (m, H-7), 5.44 (dd, 4.6, 10.0, H-14), 5.19 (d, 9.5, H-21), 4.95 (m, H-19), 4.84 (m, H-11), 3.58 (s, OCH$_3$), 3.49 (s, OCH$_3$), 2.78 (dd, 7.0, 7.1, 10.5, H-20), 2.26 (br d, 13.0, H-18a), 2.19 (d, 1.1, H-5'), 2.16 (m, H-4a), 2.11 (dd, 11.5, 13.1, H-18b), 2.05 (m, H-13), 2.03 (m, H-4b), 1.92 (d, 1.1, H-4'), 1.81 (d, 1.0, H-25), 1.68 (s, H-27), 1.65 (m, H-6a), 1.49 (m, H-6b), 1.43 (m, H-12a), 1.29 (m, H-5a), 1.12 (m, H-5b), 1.10 (m, H-12b), 0.99 (d, 7.0, H-26). $^{13}$C NMR (125 MHz, CD$_3$OD) δ (assignment): 166.7 (C-1), 165.1 (C-1'), 153.9 (C-3'), 148.6 (C-3), 133.3 (C-22), 132.0 (C-17), 131.8 (C-8), 131.0 (C-14), 130.9 (C-21), 130.0 (Ph), 128.7 (Ph), 128.3 (C-16), 128.0 (C-9), 127.5 (C-15), 127.3 (Ph), 121.7 (C-24), 121.6 (C-2), 118.3 (C-23), 117.7 (C-2'), 75.8 (C-10), 75.6 (C-7), 75.1 (C-19), 72.6(C-11), 55.4 (OCH$_3$), 54.8 (OCH$_3$) 43.4 (C-18), 37.2 (C-20), 33.2 (C-6), 31.8 (C-4), 31.2 (C-12), 29.5 (C-5), 28.0 (C-13), 26.1 (C-4'), 19.0 (C-5'), 16.4 (C-26), 14.8 (C-27), 11.5 (C-25). LR ESIMS $m/z$ 1039.1 [M + Na]$^+$. HRFABMS $m/z$ 1039.4145 (C$_{53}$H$_{62}$N$_{2}$O$_{11}$F$_{6}$ Na requires 1039.4155).
4.2.2.3 Palmerolide A 7-[(S)-MTPA ester] (65)

$^1$H NMR (500 MHz, CD$_3$OD) $\delta$ (multiplicity, $J$ (Hz), assignment): 7.92 (s, NH), 7.50 – 7.45 (5H, m, Ph), 6.97 (d, 14.4, H-24), 6.85 (dd, 5.1, 10.0, 15.5, H-3), 6.11 (dd, 11.2, 15.0, H-15), 5.96 (d, 14.7, H-23), 5.92 (dd, 3.4, 15.6, H-9), 5.82 (d, 15.2, H-2), 5.74 (br t, 1.2, H-2'), 5.68 (dd, 1.8, 8.6, 15.2, H-8), 5.67 (d, 15.0, H-16), 5.46 (m, H-7), 5.45 (dd, 4.9, 8.8, H-14), 5.19 (d, 9.8, H-21), 4.95 (m, H-19), 4.32 (ddd, 1.9, 3.2, 5.3, H-10), 4.66 (ddd, 2.0, 5.2, 10.8, H-11), 3.55 (s, OCH$_3$), 2.78 (ddq, 6.8, 7.5, 9.8, H-20), 2.26 (br d, 12.7, H-18a), 2.24 (m, H-4a), 2.19 (d, 0.8, H-5'), 2.16 (m, H-4b), 2.09 (dd, 11.5, 12.4, H-18b), 2.07 (m, H$_2$-13), 1.92 (d, 0.8, H-4'), 1.83 (m, H-6a), 1.82 (s, H$_3$-25), 1.72 (m, H-6b), 1.69 (s, H$_3$-27), 1.64 (m, H-12a), 1.46 (m, H-5a), 1.31 (m, H-5b), 1.17 (m, H-12b), 0.99 (d, 6.9, H$_3$-26). $^{13}$C NMR (125 MHz, CD$_3$OD) (assignment): 167.0 (C-1), 165.2 (C-1'), 158.3 (OCONH$_2$), 153.8(C-3'), 149.2 (C-3), 134.8 (C-9), 133.2 (C-22), 132.0 (C-14), 131.7(C-17), 130.8 (C-21), 129.6 (Ph), 128.5 (C-16), 128.4 (Ph), 127.4 (Ph), 127.3 (C-8), 126.9 (C-15), 121.6 (C-24), 121.3 (C-2), 118.3 (C-23), 117.6 (C-2'), 78.5 (C-7), 76.2 (C-11), 74.8 (C-19), 70.5 (C-10), 54.8 (OCH$_3$) 43.8 (C-18), 37.3 (C-20), 34.2 (C-6) 32.3 (C-4), 30.3 (C-12), 29.7(C-13), 26.3 (C-4'), 24.6 (C-5), 19.1 (C-5), 16.4 (C-26), 15.2 (C-27), 11.9 (C-25). LRESIMS $m/z$ 823.1 [M + Na]$^+$, HRFABMS $m/z$ 823.3747 (C$_{43}$H$_{55}$N$_2$O$_{11}$F$_3$Na requires 823.3757).

4.2.2.4 Palmerolide A 7, 10-[(S, S)-MTPA] diester (67)

$^1$H NMR (500 MHz, CD$_3$OD) $\delta$ (multiplicity, $J$ (Hz), assignment): 7.92 (s, NH), 7.38-7.50 (m, Ph), 6.97 (d, 14.4, H-24), 6.82 (ddd, 6.1, 8.3, 15.5, H-3), 6.10 (dd,11.1, 14.6, H-15), 5.96 (d, 14.5, H-23), 5.80 (d, 15.5, H-2), 5.74 (br t, 1.1, H-2'), 5.70 (m, H-9), 5.64 (d,
11.1, H-16), 5.63 (m, H-10), 5.54 (m, H-7), 5.54 (m, H-8), 5.31 (dt, 4.2, 14.6, H-14), 5.19 (d, 10.0, H-21), 4.96 (m, H-19), 4.71 (dd, 6.5, 12.1, H-19), 3.58 (s, OCH₃), 3.43 (s, OCH₃), 2.79 (ddq, 6.8, 7.5, 10.0, H-20), 2.26 (br d, 13.4, H-18a), 2.22 (m, H-4a), 2.19 (d, 1.0, H-5'), 2.178 (m, H-4b), 2.11 (dd, 11.5, 11.9, H-18b), 1.99 (m, H-13b), 1.92 (d, 1.0, H-4'), 1.90 (m, H-13a), 1.82 (d, 1.0, H-25), 1.78 (m, H-6a), 1.69 (m, H-6b), 1.68 (s, H-27), 1.43 (m, H-12a), 1.41 (m, H-5a), 1.27 (m, H-5b), 1.24 (m, H-12b), 0.99 (d, 6.7, H-26); ¹³C NMR (125 MHz, CD₃OD) δ (assignment): 166.9 (C-1), 165.2 (C-1'), 153.6 (C-3'), 149.1 (C-3), 133.5 (C-22), 132.1 (C-9), 132.0 (C-17), 131.1 (C-14), 130.8 (C-21), 129.8 (C-8). 129.6 (Ph), 128.4 (Ph), 128.3 (C-16), 127.4 (Ph), 127.3 (C-15) 121.6 (C-24), 121.5 (C-2), 118.3 (C-23), 117.6 (C-2'), 76.1 (C-7), 75.0 (C-10), 75.0 (C-19), 72.7 (C-11), 55.2 (OCH₃), 49.3 (OCH₃), 42.6 (C-18), 37.3 (C-20), 34.2 (C-6), 32.1 (C-4), 30.8 (C-12), 26.3 (C-4'), 26.1 (C-13), 24.6 (C-5), 19.2 (C-5'), 16.0 (C-26), 15.3 (C-27), 11.9 (C-25). LRESIMS m/z 1039.1 [M + Na]⁺, HRFABMS m/z 1039.4151 (C₅₃H₆₂N₂O₁₁F₆Na requires 1039.4155).

4.2.3 Palmerolide C (59)

White solid; [α]²⁵_D -27.1 (c 0.1, MeOH); IR (thin film) cm⁻¹: 3364 (br), 2933, 1697, 1637, 1446, 1387, 1274, 1182, 1018, 978; UV (MeOH) λ_max (ε): 216 (1002), 248 (635); ¹H NMR (500 MHz, DMSO-d₆) δ (multiplicity, J (Hz), assignment): 9.85 (1H, d, 10, 24-NH₂), 6.85 (1H, dd, 9, 14.6, H-24), 6.77 (1H, ddd, 7.4, 7.5, 15.4, H-3), 6.37 (2H, br, CONH₂), 6.08 (1H, dd, 12, 15, H-15), 5.85 (1H, d, 14.5, H-23), 5.73 (1H, d, 15.5, H-2), 5.68 (1H, s, H-2'), 5.63 (1H, d, 11, H-16), 5.58 (1H, m, H-7), 5.54 (1H, m, H-6), 5.46 (1H, ddd, 5, 10, 15, H-14), 5.15 (1H, d, 9.5, H-21), 4.85 (1H, ddd, 2.8, 7.8, 10.5, H-19), 168
4.72 (1H, d, 4.5, 9-OH), 4.62 (1H, d, 5, 8-OH), 4.56 (1H, ddd, 3, 7.5, 10.5, H-10), 3.96 (1H, m, H-8), 3.56 (1H, m, H-9), 2.70 (1H, qdd, 6.7, 6.7, 9.8, H-20), 2.07 (1H, m), 2.18 (1H, m, H-18), 2.13 (1H, m, H-4), 2.11 (3H, s, H3-5′), 2.07 (1H, m, H-18), 1.99 (1H, m, H-13), 1.98 (1H, m, H-5), 1.95 (1H, m, H-12), 1.90 (1H, m, H-13), 1.89 (1H, m, H-5), 1.82 (3H, s, H3-4′), 1.69 (3H, s, H3-27), 1.59 (3H, s, H3-25), 1.49 (1H, m, H-11), 1.30 (1H, m, H-11), 1.30 (1H, m, H-4), 0.90 (3H, d, H3-26); 13C NMR (125 MHz, DMSO-d6) δ (assignment): 166.9 (C-1), 164.0 (C-1′), 152.5 (C-3′), 149.8 (C-3), 133.3 (C-22), 132.5 (C-17), 132.3 (C-14), 131.8 (C-6), 131.1 (C-7), 130.5 (C-21), 128.8 (C-16), 127.2 (C-15), 122.8 (C-24), 122.0(C-2), 118.9 (C-2′), 117.2 (C-23), 75.6 (C-9), 74.7 (C-19), 74.2 (C-10), 72.8 (C-8), 44.1(C-18), 37.4 (C-20), 33.3. 32.16 (C-5), 31.7 (C-4), 30.1 (C-14), 30.1 (13), 28.7 (C-12), 28.7 (C-11), 27.4 (C-4′), 20.3 (C-5′), 17.7 (C-26), 16.5 (C-25), 13.3 (C-27); ESIMS m/z (%) 608.3 (37, [M + H + Na]+), 607.3 (38, [M + Na]+), 591.3 (40), 585.3 (15, [M + H]+), 567.3 (15), 503.3 (10), 485.3 (30), 424.2 (25), 389.2 (46), 321 (20). HRESIMS m/z 585.3534 (C33H49N2O7 requires 585.3540).

4.2.4 Palmerolide D (60)
Colorless solid; [α]D25 +67 (c 0.5, MeOH); IR (thin film) cm⁻¹ 3327, 2939, 2829, 2061, 1716, 1558. 1455, 1261, 1025, 975; λmax (ε): 216 (1742), 248 (528); 1H NMR (500 MHz, DMSO-d6) δ (multiplicity, J (Hz), assignment): 9.94 (1H, d , 10.3, 24-NH), 6.85 (1H, dd, 10.4, 15, H-15 ), 6.71(1H, ddd, 4, 11.5, 15.7, H-3), 6.45 (2H, br, OCONH₂), 6.04 (1H, dd, 11.6, 14, H-15), 5.86 (1H, d, 14.6, H-23), 5.81 (1H, s, H-2′), 5.76 (1H, d, 15.8, H-2), 5.59 (1H, d, 12, H-16), 5.53 (1H, m, H-8), 5.49 (1H, m, H-9), 5.41 (1H, ddd, 5, 10, 14.9, H-14), 5.19 (1H, m, 10-OH), 5.14 (1H, d, 9.7, H-21), 4.84 (1H, m, H-19), 4.72 (2H,
d, H-6), 4.53 (1H, m, 7-OH), 4.48 (1H, m, H-11), 4.15 (1H, m, H-10), 3.82 (1H, m, H-7), 2.68 (1H, m, H-20), 2.16 (1H, m, H-18b) 2.15 (1H, m, H-4b), 2.11 (1H, m, H-4a), 2.00 (1H, m, H-18a), 1.98 (1H, m, H-5), 1.94 (2H, m, H-13), 1.94 (2H, m, 12), 1.76 (3H, s, H3-8′) 1.70 (3H, s, H3-27) 1.61 (3H, s, H3-7′) 1.60(3H, s, H3-25), 1.48 (1H, m, H-6b) 1.30 (1H, m, H-6a) 0.89 (3H, d, 6.7, H3-26); 13C NMR (125 MHz, DMSO-d6) δ (assignment): 166.1 (C-1), 163.5 (C-1′), 157.4 (CONH2), 153.2 (C-3′), 150.0 (C-3), 143.6 (C-5′), 134.2 (C-8), 133.6 (C-14), 133.3 (C-22), 132.2 (C-17), 130.7 (C-21), 129.6 (C-9), 128.4 (C-16), 127.0 (C-15), 122.7 (C-24), 121.2 (C-2), 120.3 (C-2′), 117.5 (C-23), 112.6 (C-6′), 75.8 (C-11), 74.5 (C-19), 73.2 (C-7), 69.9 (C-10), 43.9 (C-18), 40.8 (C-4′), 38.4 (C-6), 37.3 (C-20), 30.1 (C-5), 33.0 (C-4), 30.0 (C-13) 30.0 (C-12), 24.8 (C-8′), 22.7 (C-7′), 17.7 (C-26), 16.8 (C-25), 13.3 (C-27). ESIMS m/z (%) 625.6 (45, [M + H]+), 615 (35), 585.3 (30), 520.4 (10), 432.3 (20), 349.4 (50), 305.2 (52); HR ESMS m/z 625.3864 (C36H53N2O7 requires 625.3853)

4.2.5 Palmerolide E (61)

Colorless solid; [α]25°D +17 (c 0.1, MeOH); IR (thin film) cm−1 3635, 2940, 2830, , 1715, 1637, 1540, 1387, 1276, 1194, 1079, 938 ; UV (MeOH) λmax (ε): 216 (1295), 248 (645); 1H NMR (500 MHz, DMSO-d6 ) δ (multiplicity, J (Hz), assignment): 9.41 (1H, s, H-23), 6.74 (1H, ddd, 4.3, 11.5, 15.7, H-3), 6.55 (1H, dd, 1.5, 10.2, H-21), 6.48 (2H, br, CONH2), 6.05 (1H, dd, 10.8, 14.8, H-15), 5.78 (1H, d, 15.7, H-2), 5.61 (1H, d, 10.6, H-16), 5.53 (1H, dd, 1.4, 8, H-8), 5.49 (1H, d, 2.9, H-9), 5.02 (1H, ddd, 2.1, 7.5, 10. H-19), 4.47 (1H, ddd, 1.5, 5.1, 10.7, H-11), 4.12 (1H, m, H-10), 3.81 (1H, m, H-7), 2.94 (1H, qdd, 6.8, 7.1, 9.3, H-20), 2.16 (1H, m, H-18b), 2.14 (1H, m, H-4b), 2.11 (1H, m, H-4a),
2.09 (1H, m, H-18a), 1.95 (1H, m, H-13), 1.67 (3H, s, H3-26), 1.63 (3H, s, H3-24), 1.49 (1H, m, H-6b), 1.30 (1H, m, H-5b), 1.29 (1H, m, H-6a), 1.05 (1H, m, H-5a), 1.01 (3H, d, 6.8, H3-25); 13C NMR (125 MHz, DMSO-d6) δ (assignment): 196.3 (C-23), 166.1 (C-1), 155.8 (C-21), 150.8 (C-3), 139.6 (C-22), 134.4 (C-8), 133.0 (C-17), 132.9 (C-14), 129.6 (C-9), 128.7 (C-16), 127.3 (C-15), 121.2 (C-2), 76.1 (C-11), 73.3 (C-7), 73.0 (C-19), 70.1 (C-10), 43.9 (C-18), 38.5 (C-6), 38.1 (C-20), 33.4 (C-4), 29.8 (C-12), 25.9 (C-5), 17.0 (C-24), 16.5 (C-25), 9.9 (C-26); ESIMS m/z (%) 601.5 (40), 512.3 (30, [M + Na]+), 502.3 (36), 490.3 (80), 472.4 (90), 437.2 (25), 393.3 (50), 349.2 (60), 305.2 (65), 273.1 (80), 195.0 (64), 153 (90); HRESMS m/z 512.2634 (C27H39NO7Na requires 512.2624).

4.2.6 Palmerolide B (62)

White solid; [α]25D +1.6 (c 0.1, MeOH); IR (thin film) cm⁻¹: 3514, 3433 (br), 1648, 1633, 1510, 1392, 1275, 1190; UV (MeOH) λmax (ε): 216 (1756), 240(603); 1H NMR (500 MHz, DMSO-d6) δ (multiplicity, J (Hz), assignment): 6.86 (1H, d, 14.5, H-24), 6.74 (1H, ddd, 5.0, 9.9, 15.2, H-3), 6.01 (1H, dd, 10.5, 14.5, H-15), 5.85 (1H, d, 15, H-23), 5.72 (1H, m, H-9), 5.70 (1H, d, 13, H-2), 5.64 (1H, m, H-10), 5.64 (1H, br s, H-2′), 5.57 (1H, d, 10.9, H-16), 5.38 (1H, m, H-14), 5.08 (1H, d, 10, H-21), 4.82 (1H, m, H-19), 4.64 (1H, m, H-11), 4.57 (1H, m, H-7), 4.20 (1H, m, H-8), 2.68 (1H, qdd, 6.5, 7.4, 9.6, H-20), 2.15 (1H, m, H-18b), 2.14 (2H, m, H-4b), 2.10 (1H, m, H-4a), 2.09 (3H, s, H3-5′), 1.99 (1H, m, H-18a), 1.96 (1H, m, H-13b), 1.81 (3H, s, H3-4′), 1.79 (1H, m, H-12b), 1.71 (3H, s, H3-25), 1.58 (3H, s, H3-27), 1.55 (1H, m, H-6b), 1.53 (1H, m, H-12a), 1.34 (1H, m, H-5b), 1.21 (1H, m, H-13a), 1.14 (1H, m, H-5a), 1.08 (1H, m, H-6a), 0.88 (3H, d, 6.5, H3-26); 13C NMR (125 MHz, DMSO-d6) δ (assignment): 168.2 (C-1) 166.7 (C-1′), 159.8...
(CONH₂), 154.9 (C-3’), 150.7 (C-3), 134.5 (C-22), 133.4 (C-9), 133.3 (C-14), 132.8 (C-7), 132.2 (C-21), 131.0 (C-10), 129.8 (C-16), 128.0 (C-15), 122.7 (C-24), 122.3 (C-2), 119.6 (C-23), 118.9 (C-2’), 81.6 (C-11), 77.4 (C-7), 76.2 (C-19), 72.1 (C-8), 45.2 (C-18), 38.7 (C-20), 36.6 (C-12), 34.0 (C-4), 31.8 (C-6), 30.9 (C-13), 27.7 (C-4’), 26.1 (C-5), 20.4 (C-5’), 17.7 (C-26), 16.6 (C-27), 13.2 (C-25); LR ESIMS (-) m/z 663.3[M - H]⁺, LRESIMS (+) m/z 567.3 [M + H - H₂SO₄]⁺, HR ESIMS (-) m/z 663.29417 (C₃₃H₄₇N₂O₁₀S requires. 663.29569), HR ESIMS (+) m/z 567.3430 (C₃₃H₄₆N₂O₆ requires 567.3429)

4.2.7 Palmerolide H (63)

Colorless solid; [α]²⁵ₒₒ D -27 (c 0.1, MeOH); IR (thin film) cm⁻¹: 3515, 3400 (br), 2925, 2856, 1653, 1633, 1517, 1208, 1040; UV (MeOH) λmax (ε): 217 (1232), 248 (712); H NMR (500 MHz, DMSO-d₆) δ (multiplicity, J (Hz), assignment): 6.87 (1H, d, 14.5, H-24), 6.75 (1H, ddd, 4, 11.5, 15.7, H-3), 6.02 (1H, dd, 10, 14.5, H-15), 5.87 (1H, d, 14.6, H-24), 5.76 (1H, s, H-2’), 5.74 (1H, m, H-9) 5.70 (1H, d, 14, H-2), 5.63 (1H, m, H-10), 5.57 (1H, d, 11.5, H-16), 5.38 (1H, m, H-14), 5.09 (1H, d, 10, H-21), 4.84 (1H, m, H-19), 4.69 (2H, s, H-6’), 4.66(1H, m, H-11), 4.58 (1H, m, H-7), 4.20 (1H, m, H-8), 3.44 (2H, s, H-4’), 2.68 (1H, m, H-20), 2.15 (1H, m, H-18b),  2.14 (1H, m, H-4b), 2.11 (1H, m, H-4a), 2.00 (1H, m, H-18a), 1.96 (1H, m, H-13b), 1.80 (1H, m, H-12b), 1.76 (3H, s, H₃-8’),  1.72 (3H, s, H₃-25) 1.61 (3H, s, H₃-7’) 1.59 (3H, S, H₃-27), 1.56 (1H, m, H-b), 1.55 (1H, m, H-12a), 1.32 (1H, m, H-5a), 1.21 (1H, m, H-13a), 1.18 (1H, m, H-5a), 1.10 (1H, m, H-6a) 0.89 (3H, d, 6.7, H₃-26); C NMR (125 MHz, DMSO-d₆) δ (assignment): 168.2 (C-1), 166.0 (C-1’), 159.9 (CONH₂), 155.7 (C-3’), 150.7 (C-3), 144.6 (C-5’), 134.5 (C-22),
133.4 (C-14), 132.3 (C-21), 132.3 (C-9), 131.1 (C-10), 129.7 (C-16), 128.1 (C-15), 122.8 (C-24), 122.3 (C-2), 120.5 (C-2′), 119.7 (C-23), 112.6 (C-6′), 81.7 (C-11), 77.5 (C-7), 76.2 (C-19), 72.2 (C-8), 45.2 (C-18), 42.1 (C-4′), 38.7 (C-19), 36.6 (C-12), 33.9 (C-4), 31.8 (C-6), 30.8 (C-13), 26.1 (C-5), 24.8 (C-8′), 22.4 (C-7′), 17.7 (C-26), 16.6 (C-27); LRESIMS (-) m/z 703.3 [M - H]^+, LRESIMS (+) m/z 607.3 [M + H -H_2SO_4]^+, HRESIMS 703.3258 (C_{36}H_{52}N_2O_{10}S requires 703.3270)

4.3 Isolation of Secondary metabolites from *Austrodoris kerguelenensis*

*Austrodoris kerguelenensis* nudibranches were collected by SCUBA around Palmer Station Antarctica. They were frozen immediately after the collection. Upon freeze drying, the nudibranchs were subsequently extracted with CHCl₃ three times for 24 hours, sequentially. After evaporating the solvent under reduced pressure, a gummy, reddish brown solid 2.5 g was obtained. The CHCl₃ extract was further fractionated into 12 fractions by elution of increasing polarity gradient of EtOAc in *n*-hexane on silica gel.

Investigation of each fraction by ^1^H NMR spectroscopy indicated that fractions 1-4 contained a mixture of sterols and fatty acids. Fractions 5 and 6 displayed peaks characteristic of steroids. Fractions 7-11 showed signals indicative of terpenoid glycerides. Further purification of fractions 7, 9 and 10 by HPLC, first on silica gel (EtOAc/*n*-hexane, 2 mL/min) and then on C-18 (MeCN / H₂O, 2 mL, min) afforded palmandorin A (**110**) (24 mg), palmandorin B (**111**) (7 mg) and palamadorin C (**112**) (7 mg).
4.3.1 Palmadorin A (110)

Colorless oil; \([\alpha]^{25}_D +18\) (c 0.05, MeOH); IR (thin film) cm\(^{-1}\): 3400 (br), 2969, 2864, 1712, 1640, 1488, 1382, 1281, 1147, 1100, 1040 and 975. UV (MeOH) \(\lambda_{\text{max}}\) (\(\varepsilon\)): 215 (1001), 248 (499), 266 (484); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) (multiplicity, \(J\) (Hz), assignment): 5.68 (br s, H-14), 4.91 (1H, m, H-2\(^{\prime}\)), 4.48 (2H, d, 1.5, H-18), 3.83 (4H, d, 4.8, H-1\(^{\prime}\) and H-3\(^{\prime}\)), 2.27 (1H, m, H-3b), 2.14 (3H, S), 2.09 (1H, m, H-3a), 1.96 (1H, m, H-12b), 1.87 (1H, m, H-2), 1.85 (1H, m), 1.58 (1H, m, H-6b), 1.50 (1H, m, H-6a), 1.48 (1H, m), 1.47 (1H, m, H-7b), 1.44 (1H, m, H-7a), 1.44 (1H, m, H-11b), 1.43 (1H, m, H-1) 1.39 (1H, m, C-8), 1.33 (1H, m, H-11a), 1.23 (1H, m, H-2), 1.03 (1H, m, H-10), 1.02 (3H, s, H\(_3\)-19), 0.79 (3H, d, 6, H\(_3\)-17), 0.71 (3H, s, H\(_3\)-20), \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) (assignment): 166.7 (C-15), 163.7 (C-13), 160.4 (C-4), 114.3 (C-14) 102.5 (C-18), 74.4 (C-2\(^{\prime}\)), 62.7 (C-1\(^{\prime}\)), 62.7 (C-3\(^{\prime}\)), 48.7 (C-10), 40.0 (C-5), 39.3 (C-9), 37.2 (C-6), 36.7 (C-8), 36.1 (C-11), 34.6 (C-12), 33.0 (C-3), 28.6 (C-2), 27.4 (C-7), 21.6 (C-1), 20.8 (C-19), 19.5 (C-16), 16.00 (C-17), 18.04 (C-20), LRFABMS m/z (%) 379.3 (52 [M\(^{\prime}\)]), 361.2 (10); HRFABMS m/z 379.284200 (C\(_{23}\)H\(_{39}\)O\(_4\) requires 379.2844835).

4.3.2 Ozonolysis of Palmadorin A (110)

Compound 110 (5mg, 0.0132 mmol) was dissolved in 2mL of CH\(_2\)Cl\(_2\) and allowed to react with O\(_3\) for 25 mins. The temperature of the reaction vessel was controlled at -80°C. After 25 minutes the O\(_3\) supply was disconnected and DMS (5 drops) was added and allowed to react for 1.5 hours while allowing the reaction mixture to warm to room temperature. The solvent was evaporated under reduced pressure to obtain a crude product 8 mg as a colorless solid. This crude product was purified by chromatography.
over silica using 30% EtOAc/hexane yeilding the ozonolysis product 113 as a white amorphous solid 1mg (0.038 mmol, 28.65%).

4.3.3 Ozonolyzed product of Palmadorin A (113)

Colorless solid; $\alpha_2^{25}D +0.6$ (c 0.05, MeOH); $^1H$ NMR (500 MHz, CDCl$_3$) $\delta$ (multiplicity, J (Hz), assignment): 2.58 (1H, m, H-3b), 2.34 (1H, m, H-12b), 2.23 (1H, m, H-3a), 2.16 (3H, s, H$_3$-14), 2.13 (1H, m, H-12a), 1.72 (1H, m, H-11b), 1.60(1H, m, H-6b), 1.56(1H, m, H-6a), 1.53 (1H, m, H-7b), 1.46 (1H, m, H-2b), 1.44 (1H, m, H-11a), 1.28 (1H, m, H-7a), 1.24 (1H, m, H-2a), 1.20 (1H, m, H-10), 1.15 (3H, s, H$_3$-17), 0.82 (3H, d, H$_3$-17), 0.73 (3H, s, H$_3$-18); $^{13}C$ NMR (125 MHz, CDCl$_3$) (assignment): 216.3 (C-4), 209.0 (C-13), 50.0 (C-10), 40.6 (C-9) 39.4 (C-5), 37.7 (C-12), 37.6 (C-3), 36.6 (C-8), 33.1 (C-6), 30.8 (C-11), 30. 6 (C-14), 26.5 (C-2), 26.5 (C-7), 18.8 (C-17), 17.00 (C-18), 15.5 (C-15).

EIMS $m/z$ (%): 175 (22), 122 (10), 95 (20), 43 (100), 41 (45), 28 (30).

4.3.4 Palmadorin B (111)

Colorless oil; $\alpha_2^{25}D +24$(c 0.05, MeOH); IR (thin film) cm$^{-1}$ 3360 (br), 2933, 2359, 1718, 1642, 1448, 1383, 1219, 1145, 110, 909. UV (MeOH) $\lambda_{max}$ (E): 214 (1063), 248 (537), 264 (524); $^1H$ NMR (500MHz, CDCl$_3$) $\delta$ (multiplicity, J (Hz), assignment): 5.68 (br s, H-14), 4.91 (1H, m, H-2’) 4.48 (2H, d, 1, H-18) 4.26 (2H, m, H-1’) 3.72 (2H, d, H-3’) 2.27 (1H, m, H-3b), 2.14 (3H, s, H$_3$-16), 2.09 (1H, m, H-3a), 2.05 (3H, S, COOCH$_3$), 1.86 (1H, m, H-2b), 1.85 (1H, m, H-12a), 1.57 (1H, m, H-6b), 1.47 (1H, m, H-6a), 1.47 (1H, m, H-7b), 1.46 (1H, m, H-1b), 1.44 (1H, m, H-7a), 1.42 (1H, m, H-1a), 1.39 (1H, m, H-8), 1.33 (1H, m, H-11a), 1.21 (1H, m, H-2a), 1.03 (1H, m, H-10), 1.02 (3H, s, H$_3$-19), 0.78 (3H, d, 6.5, H$_3$-17), 0.72 (3H, s, H-20) $^{13}C$ NMR (125 MHz, CDCl$_3$) (assignment);
171.2 (\text{COOCH_3}), 166.8 (C-15), 163.9 (C-13), 160.7 (C-4), 114.7 (C-14), 102.9 (C-18), 72.0 (C-2'), 62.9 (C-1'), 62.2 (C-3'), 49.1 (C-10), 40.4 (C-5), 39.7 (C-9), 37.7 (C-6), 37.0 (C-8), 36.4 (C-11), 34.9 (C-12), 34.4 (C-3), 29.0 (C-2), 27.8 (C-7), 22.3 (C-19), 21.8 (C-1), 21.2 (\text{COOCH_3}), 19.8 (C-16), 18.5 (C-20), 16.4 (C-17); LRFABMS m/z (%) 421.4 (36 [M+1]⁺), 379.4(8), 321.3(8), 287.3 (90); HRFABMS m/z 421.2964 (C_{25}H_{41}O_{5} requires 421.2954).

4.3.5. Palmadorin C (112)

Colorless oil; [\alpha]^{25}_D +8 (c 0.05, MeOH); IR (thin film) cm\(^{-1}\): 3389 (br), 2840, 1648, 1228, 1219, 1109, 1015, 894. UV (MeOH) \(\lambda_{\text{max}}(\varepsilon): 216 (1002), 248 (635); \) \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) (multiplicity, \(J\) (Hz), assignment): 5.70 (1H, s, H-14), 5.13 (1H, m, H-3), 4.91 (1H, m, H-2'), 4.02 (1H, m, H-7), 3.82 (4H, d, 4.5, H-1' and H-3') 2.16 (3H, s, H_3-16) 2.10 (1H, m, H-6b), 2.08 (1H, m, H-2b) 1.98 (1H, m, H-2a), 1.98 (1H, m, H-12b), 1.93 (1H, m, H-12a), 1.60 (3H, s, H-18), 1.58 (1H, m, H-1b), 1.54 (1H, m, H-1a), 1.51 (1H, m, H-11b), 1.51 (1H, m, H-8a), 1.39 (1H, m, H-11a), 1.39 (1H, m, H-6a), 1.37 (1H, m, H-10), 1.27 (3H, s, H_3-19), 1.01 (3H, d, 7.5, H-17), 0.99 (1H, s, H_3-20); \(^{13}\)C NMR(125 MHz, CDCl\(_3\)) (assignment): 166.9 (\text{COOCH_3}), 163.4 (C-13), 145.2 (C-4), 120.0 (C-3), 114.8 (C-14), 74.8 (C-2'), 73.9 (C-7), 63.1 (C-1' and C-3'), 46.86 (C-10), 43.1 (C-6), 39.5 (C-8), 38.5 (C-9), 37.8 (C-5), 37.57 (C-11), 35.1 (C-12), 27.0 (C-2), 22.0 (C-19), 20.3 (C-20), 19.7 (C-16), 18.3 (C-18), 18.2 (C-1), 12.7 (C-17): FABMS (+) m/z (%) 377.4 (15, [M + H - H_2O]⁺), 309.1(14), 195.1 (15), 153.1 (45), 135 (45). ESIMS (+) m/z (%) 377.4 (15 [M + H- H_2O]⁺), HRFABMS m/z 378.1503 (C_{23}H_{36}O_{4} requires 378.1467).
4.3.6 Acetylation of Palmadorin C (112)

Palmadorin C (5mg, 0.0127 mmol) was dissolved in CH₂Cl₂ (1 mL) and treated with Ac₂O (30 µL, 0.025 mmol) in the presence of DMAP (3 mg), Et₃N (30 µL) for 24 hrs at 25°C. The reaction was quenched by the addition of few drops of MeOH. Upon evaporation of the solvent by reduced pressure the crude product was separated by chromatography over silica gel (EtOAc /hexane gradient elution) to obtain palmadorin C diacetate (114) (6mg, 0.0125 mmol, 98%).

4.3.7 Palmadorin C diacetate (114)

Colorless solid; [α]₂⁵°D +12 (c 0.05, MeOH); IR (thin film) cm⁻¹: 3389 (br), 2834, 1648, 1408, 1228, 1210, 1040, 1109, 1015, 894. ¹H NMR(500 MHz, CDCl₃) δ(multiplicity, J (Hz), assignment): 5.65 (1H, s, H-14), 5.26 (1H, m, H-2’), 4.23 (4H, m, H-1’ and 3’), 4.03 (1H, m, H-7), 2.15 (3H, s, H₃-16), 2.08 (1H, m, H-6b), 2.06 (3H, s, COOCH₃), 2.05(1H, m, H-2b), 1.98 (1H, m, H-2b), 1.93 (1H, m, H-12b), 1.90 (1H, m, H-12a), 1.60 (3H, s, H₃-18), 1.52 (1H, m, H-8), 1.48 (1H, m, H-11a), 1.38 (1H, m, H-6a), 1.38 (1H, m, H-10), 1.37 (1H, m, H-11), 1.27 (3H, s, H₃-19), 1.02 (3H, d, 7, H₃-17), 1.00 (3H, s, H₃-20).¹³C NMR (125 MHz, CDCl₃) (assignment): 170.8 (COOCH₃), 165.7 (C-15), 163.3 (C-13), 145.2 (C-4), 120.0 (C-3), 114.7 (C-14), 73.9 (C-7), 68.2 (C-2’), 62.7 (C-1’& C-3’), 54.8 (OCH₃), 46.9 (C-10), 43.2 (C-6), 39.5 (C-8), 38.0 (C-9), 37.8 (C-5), 37.5 (C-11), 35.1 (C-12), 26.9 (C-2), 21.8 (C-19), 20.2 (C-20), 19.7 (C-16), 12.7 (C-17); LRESIMS (-) m/z (%) 477.2 (78, [M - 1]⁺), 478.2 (20, [M]⁺).
4.3.8 Preparation of R-MTPA esters of Palmadorin C diacetate (114).

Palmadorin A diacetate (3 mg, 0.0063 mmol) was dissolved in CH₂Cl₂ (500 µL) and allowed to react with R-MTPACl (50 mg, 0.07 mmol) in the presence of Hunig’s base (100 µL) and DMAP (1 mg) for 48 hrs. Conversion of the starting material to the products was monitored by TLC. Evaporation of the solvents gave a crude product of 20 mg. Further purification of this mixture by chromatography on silica gel (EtOAc/hexane gradient elution) gave compound (115) (1.5 mg, 0.0022 mmol, 34%).

4.3.9 Palmadorin C diacetate R-MTPA (115)

Colorless solid; [α]_{25}^{25} D+10 (c 0.05, MeOH); IR (thin film) cm⁻¹: 3401 (br), 2825, 1662, 1510, 1340, 1235, 1032, 1006, 860. ¹H NMR (500 MHz, CDCl₃) δ(multiplicity, J (Hz), assignment): 7.53 (2H, m, Ph), 7.35 (3H, m, Ph), 5.62 (1H, s, H-14), 5.25 (1H, m, H-2'), 4.22 (4H, m, H-1' & 3''), 5.35 (1H, m, H-7), 3.59 (3H, s, OCH₃) 2.13 (3H, s, H₃-16), 2.05 (3H, s, COOCH₃), 2.05 (1H, m, H-2b), 1.98 (1H, m, H-2b), 1.97 (1H, m, H-6b), 1.90 (1H, m, H-12b), 1.88 (1H, m, H-12a), 1.43 (3H, s, H₃-18), 1.52 (1H, m, H-8), 1.48 (1H, m, H-11a), 1.37 (1H, m, H-6a), 1.37 (1H, m, H-11), 1.31 (1H, m, H-10), 0.92 (3H, d, H₃-17), 0.80 (3H, s, H₃-20) 0.53 (3H, s, H₃-19). ¹³C NMR (125 MHz, CDCl₃) (assignment); 170.79 (COOCH₃), 166.9 (COOMTP), 165.7 (C-15), 162.7 (C-13), 145.2 (C-4), 129.7 (Ph) 128.5 (Ph), 127.36 (Ph), 120.6 (C-3), 114.9 (C-14), 78.0 (C-7), 68.3 (C-2'), 62.7 (C-1' & C-3''), 55.8 (OCH₃) 46.4 (C-10), 40.4 (C-6), 39.1 (C-8), 38.2 (C-9), 37.3 (C-5), 37.1 (C-11), 34.9 (C-12), 26.8 (C-2), 21.8 (C-19), 20.2 (C-20), 19.6 (C-16), 12.00 (C-17). HRESIMS m/z 717.3220 (C₃₇H₄₉O₉F₃ requires 717.3219).
4.3.10 Preparation of S-MTPA esters of Palmadorin C diacetate (114)

Palamadorin A diacetate (114) (1mg, 0.0021 mmol) was dissolved in CH₂Cl₂ (300 µL) and allowed to react with S-MTPACl (50 mg, 0.07 mmol) in the presence of Hunig’s base (100 µL) and DMAP (1 mg) for 48 hrs. Conversion of the starting material to the products was monitored by TLC. Evaporation of the solvents gave a crude product of 20 mg. Further purification of this mixture by chromatography on silica gel (EtOAc/hexane gradient elution) gave compound (116) (1 mg, 0.0014 mmol, 69%).

4.3.11 Palmadorin C diacetate S-MTPA (116)

Colorless solid; [α]²⁵_D -5 (c 0.02, MeOH); IR (thin film) cm⁻¹ 3342 (br), 2905, 1658, 1408, 1340, 1235, 1048, 1109, 1020, 870. ¹H NMR (500 MHz, CDCl₃) δ (multiplicity, J (Hz), assignment); 7.59 (1H, m, Ph), 7.53 (2H, m, Ph), 7.37 (2H, m, Ph) 5.61 (1H, s, H-14), 5.24(1H, m, H-2'), 4.22 (4H, m, H-1' & 3'), 5.44 (1H. m, H-7), 3.59 (3H, s, OCH₃) 2.13 (3H, s, H₃-16), 2.07 (1H, m, H-2b), 2.05 (3H, s, COOCH₃), 2.05 (1H, m, H-2b), 1.98 (1H, m, H-2b), 2.06 (1H, m, H-6b), 1.90 (1H, m, H-2a), 1.90 (1H, m, H-12b), 1.86 (1H, m, H-12a), 1.65 (1H, m, H-8), 1.54 (3H, s, H₃-18), 1.48 (1H, m, H-6a), 1.46 (1H, m, H-11b), 1.35 (1H, m, H-10), 1.26 (1H, m, H-11a), 0.75 (3H, d, H₃-17), 0.63 (3H, s, H₃-20) 0.94 (3H, s, H₃-19). ¹³C NMR (125 MHz, CDCl₃) (assignment): 170.8 (C_OOCH₃), 167.0 (C_OOMTP), 165.7 (C-15), 163.3 (C-13), 144.1 (C-4), 129.7 (Ph), 128.6 (Ph), 128.0 (Ph), 127.6 (Ph), 127.6 (Ph), 120.0 (C-3), 114.7 (C-14), 73.9 (C-7), 68.2 (C-2'), 62.7 (C-1' & C-3' ), 46.6 (C-10), 43.2 (C-6), 39.5 (C-8), 38.1 (C-9), 37.5 (C-11), 37.4 (C-5), 35.1 (C-12), 26.9 (C-2), 20.8 (C-19), 20.2 (C-20), 19.7 (C-16), 12.7 (C-17). HRESIMS m/z 717.3220 (C₃₇H₄₉O₉ F₃ requires 717.3219).
REFERENCES


APPENDICES

Appendix A Cytotoxicity profile of palmerolide A

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Note: The table above shows the mean IC50 values for palmerolide A across various cell lines. The values are given in micrograms per milliliter.
About the Author

Thushara Diyabalanage graduated with a BSc degree in Biology from the University of Peradeniya Sri Lanka in 1992. In 1996, he completed a Masters Degree in Organic chemistry at the same university after some extensive research on Sri Lankan medicinal plants. Having spent next three years in industry, where he was employed as a research and development chemist, he came to USA in 2000 to pursue doctoral studies.

His research with Dr Bill J Baker in marine natural products chemistry at the University of South Florida has been very successful, as it led to the discovery of a series of new anticancer agents that show great promise as drug leads. In recognition of this, he has been awarded the ASP student research award 2005, annually given to the most outstanding student research performed by a graduate student, by the American Society of Pharmacognosy. He has presented his research in several national and international conferences.