Drug Discovery from Cold-Adapted Bacteria from Malpeque Bay, Prince Edward Island

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of Master of Science

Department of Chemistry

Faculty of Science

University of Prince Edward Island

Simon Berger

Charlottetown, Prince Edward Island

July 2011

© 2011. Simon Berger

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-82239-5
Our file Notre référence
ISBN: 978-0-494-82239-5

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

CONDITIONS FOR THE USE OF THE THESIS

The author has agreed that the Library, University of Prince Edward Island, may make this thesis freely available for inspection. Moreover, the author has agreed that permission for extensive copying of this thesis for scholarly purposes may be granted by the professor or professors who supervised the thesis work recorded herein or, in their absence, by the Chair of the Department or the Dean of the Faculty in which the thesis work was done. It is understood that due recognition will be given to the author of this thesis and to the University of Prince Edward Island in any use of the material in this thesis. Copying or publication or any other use of the thesis for financial gain without approval by the University of Prince Edward Island and the authors' written permission is prohibited.

Requests for permission to copy or to make any other use of material in this thesis in whole or in part should be addressed to:

Chair of the Department of Chemistry
Faculty of Science
University of Prince Edward Island
550 University Avenue, Charlottetown, PE
Canada C1A 4P3

SIGNATURE PAGE

(iii) + (iv)

REMOVED

ABSTRACT

Marine bacteria represent an excellent renewable source of novel, bioactive natural products. Cold-adapted bacteria have been under-studied and thus temperate environments represent a largely untapped resource for the discovery of microbial natural products.

This thesis examined the bacterial diversity of cold-adapted bacteria from seawater and sediment collected from Malpeque Bay, Prince Edward Island, a unique marine environment, which resulted in the discovery of 16 different groups of bacteria. One isolate was fermented from each group in 11 different media, in a high-throughput method using 96-well plates to maximize the number of media conditions used for each isolate. LC-MS analysis was performed on crude extracts and follow-up fermentations were prioritized by LC-MS data analysis. Fermentations of 6 isolates in 2 media were scaled up in 24-well plates and in 250 mL Erlenmeyer flasks for production confirmation and antimicrobial analysis. A psychrotolerant bacterium, *Figoribacterium* sp., was found to produce a wide variety of cell-associated glycoglycerolipids (GGL) in ACC media. Four glycoglycerolipids, 1-O-acyl-3-[α-D-mannopyranosyl-(1–3)-(6-O-acyl-α-D-mannopyranosyl)]-sn-glycerol (GGL 915, 887, 885a and 885b), including two new analogues which contain *trans*-12-methyl-10-tetradecenoic acid and 12-methyl-tetradecanoic acid were isolated by reverse phase chromatography.

Structure elucidation was performed by LC-ESI-MS/MS, HR-MS and NMR. NMR characterization involved the use of 1D NMR (^{1}H and ^{13}C), 2D NMR (COSY, HSQC, HMBC, NOESY and ROESY) as well as coupling constant analysis. Fermentation optimization by supplementing ACC media with 20 g/L glucose or glycerol

greatly increased production of different GGL analogues. One of the new compounds, GGL 915, was assessed for surface tension, antimicrobial and cytotoxic activities and was found to be inactive. However, GGL 885, 887 and 915 were tested for activity against the *Vaccinia* and Influenza A viruses, where GGL 887 showed preliminary indications of antiviral activity against Influenza A.

ACKNOWLEDGMENTS

I would like to thank Dr. Russell G. Kerr, my supervisor for the opportunity to work in his lab for over 3 years. It has been an interesting experience and I have learned a great deal from him. He has provided me with great insight into what it is like working in a major research group. I appreciate the wide range of equipment that he has provided for our use.

I would like to thank my supervisory committee, Dr. Michael Shaver and Dr. Bob Chapman, for the feedback they have provided. I believe that I have learned a great deal from them and I appreciate their help.

I would like to thank the chemistry department for providing the opportunity of doing research at UPEI as well as the opportunity to teach undergraduate labs. It has been an interesting experience and I believe that I have learned a lot.

I would like to thank Dr. Jim Johnston for running my compounds in his assays. I would also like to thank him for answering my many questions and taking the time to explain how they worked. I learned a lot in a very short time about the difficulties of assays and the difficulties of determining antiviral activity.

I would like to thank Brad Haltli for supervising me throughout the project. I appreciate the time he took out of his busy schedule to answer questions and to try to stimulate learning. I appreciate the patience he has shown in guiding me in my project. I would also like to thank him for telling me not to poke my EtBr gels with my finger.

I would like to thank Dr. Fabrice Berrue for his guidance and help with chromatography, LC-MS and NMR. I have learned a great deal about the use of many different types of instruments. He always made time available to answer questions and to look at MS chromatograms or NMR spectra. I would also like to thank him for providing greatly needed comic relief that helps keep us from taking the many things that do not turn out as desired, too seriously.

I would like to thank Dr. Malcolm McCulloch for helping me with the many random things that need to be learned in a research lab. I would also like to thank him allowing me to rant and rave occasionally (this applies to most other people as well). I would also like to thank him for being totally random and for being an Aussie.

I would like to thank Dr. Chris Kirby and Lloyd Kerry for running NMR samples. Without their service and help, very little could be accomplished here at NRC-INH. I would like to thank Martin Lanteigne for running antimicrobial assays and having patience for answering my many questions. I would like to thank Dr. Gen Wang, Haili Wang and Dominique Schmidt for running the antiviral and cytotoxicity assays. I would like to thank Dr. Bob Chapman again for letting me use his Tensiometer and IR. I would also like to thank him and Lisa Penney for assistance they provided in regards to the Tensiometer, IR; other equipment and supplies. I would like to thank Dr. Junzeng Zhang for letting me borrow the RHM-monosaccharide column and to Dr. Jane Ji for assistance in regards to the column. I would like to thank Sutaporn Bunyajetpong for running the antifouling assay. I would like to thank Steven Locke for running some samples for me and for his aid regarding GC-MS.

I would like to thank the Kerr research group and the personnel at NRC for the help that they have provided and the questions they have answered. I would also like to thank fellow graduate students who are or have been through similar experiences. I would like to thank them for their support and advice they have shared. I would also like to thank the people who have visited our lab for providing a new perspective.

I would like to thank my family and friends for their support. It has been an interesting three years and a great deal has happened.

I would like to thank Canada Foundation for Innovation, Canada Research Chairs, Atlantic Innovation Fund, Natural Sciences and Engineering Research Council of Canada and University of Prince Edward Island for funding and making this all a possibility.

TABLE OF CONTENTS

Title Page..i

Conditions for the Use of the Thesis..ii

Permission to use Postgraduate Thesis..iii

Certification of Thesis Work..iv

Abstract..v

Acknowledgements..vii

Table of contents..x

List of Figures..xvii

List of Tables..xxi

List of Abbreviations..xxiv

Chapter 1-Introduction

1.1. Natural products..1

1.1.1. "What Are Natural Products?"..1

1.1.2. Natural Products as Drugs..1

1.1.3. Historical Use of Natural Products..5

1.1.4. Successful Natural Products..7

1.2. Marine Natural Products..10

1.2.1. "What are Marine Natural Products?"..10

1.2.2. Advantages of Marine Sources Over Terrestrial Sources for Natural Products..10

1.2.3. Technological Hurdles of Marine Natural Products Research....................12

1.2.4. Examples of Marine Natural Products..14

1.2.5. Proven Compounds as Drugs..19

1.3. Marine Bacteria...22

1.3.1. Difficulties in Defining Marine Bacteria..22

1.3.2. Marine Bacteria as Sources of Natural Products...22

1.3.3. Advantages of Marine Bacteria as Sources of Natural Products.................24

1.3.4. Natural Products Discovered from Marine Bacteria..................................26

1.4. Microbial Extremophiles...30

1.4.1. The Different Types of Extremophiles...30

1.4.2. Cold-Adapted Bacteria...31

1.4.3. Advantages of Cold-Adapted Bacteria...32

1.4.4. Relative Abundance of Cold-Adapted Bacteria..34

1.4.5. Natural Products Discovered from Cold-Adapted Bacteria........................35

1.5. Lipids..38

1.5.1. A New Definition for Lipids...38

1.5.2. Glycerolipids...40

1.5.3. Glycosylmonoradylglycerols and Glycosyldiradylglyercols.......................42

1.5.4. Glycoglycerolipids...43

1.5.5. Dimannosylglycerolipids...45

1.6. Research Goals...50

Chapter 2-Materials and Methods

2.1. Preparation of Media....52

2.1.1. Preparation of Bacterial Isolation Media....52

2.1.2. Preparation of Fermentation Media....52

2.1.3. *Actinobacteria* Isolation Media....56

2.1.4. Assay Media....58

2.2. Composition of Buffers and Mixes....58

2.3. Bacterial Isolation from Seawater and Sediment Samples....59

2.3.1. Collection of Sediment Samples from Malpeque Bay, PEI....59

2.3.2 Plating of Sediment Samples onto MA, DMA, NA and DNA....59

2.3.3. Plating of Sediment Samples onto *Actinobacteria* Specific Media....59

2.3.4. Preparation of Isolates from Glycerol Stocks....60

2.3.5. Isolate Purification....61

2.4. Bacterial Molecular Dereplication and Identification....61

2.4.1. DNA Isolation and its Use as Template for Polymerase Chain Reaction (PCR)....61

2.4.2. Agarose Gel Electrophoresis of PCR Products....63

2.4.3. Restriction Fragment Length Polymorphism (RFLP) Analysis of PCR Products....63

2.4.4. 16S rDNA Cleaning and Concentration Measurement....64

2.4.5. Sequencing Data Analysis....64

2.5. Archiving Cultures....65

2.6. Growth Monitoring of Isolates Incubated at Different Temperatures....65

2.7. Small Scale Fermentations for Natural Product Production....................................65

2.7.1. 96-Well Plate Fermentations...65

2.7.2. Extraction of 96-well Plate Fermentations...66

2.7.3. LC-MS analysis of 96-well Plate Fermentations..66

2.7.4. LC-MS Data Analysis...67

2.8. Scale-up of Small Scale Fermentation...68

2.8.1. 24-Well Plate and 250 mL Erlenmeyer Flask Fermentations......................68

2.8.2. Extraction of 24-Well Plate and 250 mL Erlenmeyer Flask Fermentations68

2.8.3. LC-MS analysis of Crude Extracts...69

2.8.4. Preparation of Crude Extracts for Initial Antimicrobial Screening.............70

2.8.5. Preparation of MPY and FF Media Fractions for Antimicrobial Analysis..70

2.8.6. Automated Low Pressure Liquid Chromatography (ALPLC) of Crude Extracts...71

2.9. RKSB-11A Scale-up and Fermentation Optimization...71

2.9.1. Scale up of RKSB-11A Fermentation..71

2.9.2. ALPLC of Extracts from Large Scale Fermentation..................................71

2.9.3. Optimization of Fermentation Extractions..72

2.9.4. Relative Quantitation of GGLs in Three Different Media..........................72

2.9.5. Cell Mass, OD_{600} and Ratio of Different GGLs from Days 1 - 7 for Fermentations at 15 °C and RT...73

2.10. HPLC of GGLs..74

2.11. Sugar Analysis..74

2.11.1. GGL Hydrolysis for Sugar Identification..74

2.11.2. HPLC Sugar Analysis..75

2.12. GGL Characterization..75

2.12.1. NMR..75

2.12.2. HR-MS..75

2.12.3. IR Analysis..75

2.12.4. Polarimetry of Sugars and GGLs..76

2.13. Surface Tension Measurements..76

2.14. Bioactivity Assessment..77

2.14.1. Antimicrobial Assays..77

2.14.2. Cytotoxicity..78

2.14.3. Antiviral Assays..79

2.14.4. Anti-fouling Assay..80

Chapter 3-Results and Discussion

3.1. Bacterial Isolation from Seawater and Sediment Samples..82

3.1.1. Review of Work Performed in Honors Project..82

3.1.2. Bacterial Isolation from Sediment Samples Collected in Winter..83

3.1.3. Rational for Incubation Temperature and Media Selection..85

3.2. Plating of Glycerol Stocks of Previously Isolated Bacteria..86

3.3. Bacterial Molecular Dereplication and Identification..86

3.3.1. DNA Isolation..86

3.3.2. Use of RFLP for Molecular Dereplication..87

3.3.3. Identification from 16S rDNA..88

3.4. Temperature Growth Range Study of 40 Isolates....93

3.5. Small Scale Fermentations for Natural Product Production....96

3.5.1. Selection of Strains and Media Conditions....96

3.5.2. Results from Small Scale Fermentation....97

3.6. Scale-up of Small Scale Fermentation....101

3.6.1. Selection of Isolates and Media Conditions....101

3.6.2. Results from Scale-up of Small Scale Fermentation....102

3.7. RKSB-11A Scale-up and Extraction....104

3.7.1. Harvesting Optimization....104

3.7.2. Effect of Media on GGL Production....105

3.7.3. Timescale Analysis of Cell Pellet Weight, Cell Density and GGL Production by LC-MS....107

3.8. Chromatographic Purification of GGLs 873, 885, 887 and 915....110

3.9. Spectroscopic Characterization of GGL 915....117

3.9.1. Analysis of NMR Data....117

3.9.2. Analysis of MS, IR and Sugar Structure Elucidation....119

3.10. Spectroscopic Characterization of GGL 887....122

3.10.1. Analysis of NMR Data....122

3.10.2. Analysis of MS, IR and Sugar Structure Elucidation....124

3.11. Spectroscopic Characterization of GGLs 885....126

3.11.1. Analysis of NMR Data....126

3.11.2. Analysis of MS, IR and Sugar Structure Elucidation....129

3.12. Spectroscopic Characterization of GGL 873....132

3.12.1. Analysis of NMR Data....132

3.12.2. Analysis of MS, IR and Sugar Structure Elucidation....134

3.13 Discussion of Structure Elucidation Methods for GGLs....135

3.14. Surface Tension Analysis of GGL 915....137

3.15. Antimicrobial Assays....139

3.15.1. Antimicrobial Assay of Crude extracts from 50 mL Fermentations....139

3.15.2. Antimicrobial assay of GGL 915....145

3.16. Cytotoxic Assay....145

3.17. Antiviral Assays....147

3.18. Antifouling Assay....149

Chapter 4-Conclusion....151

Reference....153

Appendix....173

LIST OF FIGURES

Figure 1.1. Captopril and Teprotide....2

Figure 1.2. Chemical Structures of Antibiotics....3

Figure 1.3. Morphine and Enkephalins, Met-enkaphalin and Leu-enkaphalin....4

Figure 1.4. Timeline of Approximate Date of Clinic Use of Antibiotics....7

Figure 1.5. Ivermectin Mixture, B1a and B1b....13

Figure 1.6. Marine Natural Products in Clinical Trials....16

Figure 1.7. Bioactive Bacterial Natural Products....27

Figure 1.8. Omuralide and Salinosporamide A....29

Figure 1.9. Seawater Temperature Profiles at Different Depths in Low, Medium and High Latitudes....32

Figure 1.10. Structures of Natural Products from Cold-Adapted Bacteria....37

Figure 1.11. Eight Classes of Lipids....39

Figure 1.12. Six Classes of Glycerolipids....41

Figure 1.13. Classes of Glycosylmonoradylglycerols and Glycosyldiradylglycerols....43

Figure 1.14. 1-O-acyl-3-[α-D-mannopyranosyl-(1–3)-(6-O-acyl-α-D-mannopyranosyl)]-sn-glycerol....47

Figure 1.15. Water Temperatures for Water less than 12 m in Depth from Malpeque Bay, P.E.I....51

Figure 3.1. Phylogenetic Tree of 28 Bacteria with Closest Match and Reference Strains....90

Figure 3.2. ELSD and MS Chromatograms ACC RKSB-11A Crude Extracts....103

Figure 3.3. ELSD and MS Chromatograms of ACC Media Blank....103

Figure 3.4. ELSD Chromatogram of Crude Extract from Cell Pellet............................105

Figure 3.5. ELSD Chromatogram of Crude Extract from Supernatant..........................105

Figure 3.6. Peak Areas of Different Ions Corresponding to GGLs in Three Fermentation Media..107

Figure 3.7. Dry Cell Pellet Weights and OD_{600} of Fermentations Incubated at 15 °C and RT in Triplicates..108

Figure 3.8. Peak Areas of 885, 887, 915 and 917 m/z Ions at Different Days from 15 °C Fermentations..109

Figure 3.9. Peak Areas of 885, 887, 915 and 917 m/z Ions at Different Days from 22 °C Fermentations..109

Figure 3.10. ALPLC Chromatogram of Crude Extract from 6 L Fermentation of RKSB-11A in ACC+Glucose Media..110

Figure 3.11. Fraction Weights and HPLC Fraction of 6 L Fermentation Fraction 5......112

Figure 3.12. Fraction Weights and HPLC Fraction of 6 L Fermentation Fraction P.3...114

Figure 3.13. Fraction Weights and HPLC Fraction for 12 L Fermentation Fraction 4...115

Figure 3.14. Fraction Weights and HPLC Fraction of 12 L Fermentation Fraction P885M..116

Figure 3.15. Structures of GGLs Isolated from RKSB-11A in ACC Media..................117

Figure 3.16. Major MS^2 Fragmentation Patterns of 915.79 Ion $[M+Na]^+$......................119

Figure 3.17. Major MS^2 Fragmentation Patterns of 887.88 Ion $[M+Na]^+$......................124

Figure 3.18. Major MS^2 Fragmentation Patterns of 885.80 Ion $[M+Na]^+$......................130

Figure 3.19. Major MS^2 Fragmentation Patterns of 873.72 Ion $[M+Na]^+$......................135

Figure 3.20. Surface Tension of SDS at Different Concentrations................................138

Figure 3.21. Surface Tension of GGL 915 at Ten Different Dilutions....138

Figure 3.22. Cytotoxic Activity of GGL 915 Results....146

Figure 3.23. GGL 887 with Huh7 Cells and *Vaccinia* Virus....148

Figure 3.24. Trial 1 and 2 of RKDP Cells Infected with H1N1 Virus with Controls of Uninfected Cells....149

Figure S1. HPLC Chromatogram of Sugars from GGL 887 Hydrolysis....173

Figure S2. HPLC Chromatogram of D-Mannose Standard....173

Figure S3. IR Spectrum of GGL 885....174

Figure S4. IR Spectrum of GGL 915....174

Figure S5. ^{1}H NMR Spectrum of GGL 885 (600 MHz) in CD_3OD....175

Figure S6. ^{13}C NMR Spectrum of GGL 885 (150 MHz) in CD_3OD....175

Figure S7. COSY NMR Spectrum of GGL 885 (600 MHz) in CD_3OD....176

Figure S8. HSQC NMR Spectrum of GGL 885 (600 MHz) in CD_3OD....176

Figure S9. HMBC NMR Spectrum of GGL 885 (600 MHz) in CD_3OD....177

Figure S10. NOESY NMR Spectrum of GGL 885 (600 MHz) in CD_3OD....177

Figure S11. ^{1}H NMR Spectrum of GGL 915 (600 MHz) in CD_3OD....178

Figure S12. ^{13}C NMR Spectrum of GGL 915 (150 MHz) in CD_3OD....178

Figure S13. ^{1}H NMR Spectrum of GGL 887 (600 MHz) in CD_3OD....179

Figure S14. ^{13}C NMR Spectrum of GGL 887 (150 MHz) in CD_3OD....179

Figure S15. HSQC NMR Spectrum of Hydrolyzed GGL 873 (600 MHz) in CD_3OD...180

Figure S16. HSQC NMR Spectrum of GGL 873 Mixture (600 MHz) in DMSO - d_6....180

Figure S17. HR-MS Spectrum of GGL 885....181

Figure S18. ^{1}H NMR Spectrum of GGL 887 Sugar Digest and D-Mannose Standard (600 MHz) in Phosphate Buffered D_2O at pH 7.65 181

LIST OF TABLES

Table 1.1. NP-derived drugs in NDA or Phase III Stage of Development (March 2008)...8

Table 1.2. Drug Development Pipeline of Marine Natural Products (March 2011).........15

Table 1.3. Selected Bioactive NP or NP-derived Compounds from Marine Bacteria......26

Table 1.4. Classification of Selected Extremophiles...30

Table 1.5. Natural Products from Cold-Adapted Bacteria..36

Table 1.6. 1-O-acyl-3-[α-D-mannopyranosyl-(1–3)-(6-O-acyl-α-D-mannopyranosyl)]-sn-glycerol analogues...47

Table 2.1. PCR Master Mix...61

Table 2.2. Thermal Cycler Program..62

Table 2.3. RFLP Master Mix...63

Table 2.4. UPLC Solvent Gradient..67

Table 2.5. HPLC Solvent Gradient..73

Table 3.1. Identity of Sequenced Bacteria...82

Table 3.2. Number of Isolates from the Winter Sediment Sample using *Actinobacteria*-Specific Media with Different Pre-treatments..83

Table 3.3. Number of Isolates from Cold-Adapted Bacteria Isolation from Winter Sediment Sample..84

Table 3.4. Grouping Based on RFLP Patterns of 16S rDNA..88

Table 3.5. Basic Local Alignment Search Tool (BLAST) Search Results of Isolates Sharing 97 % Identity of 16S rDNA...89

Table 3.6. Growth Temperature Range of Selected Isolates..95

Table 3.7. Media Designations...97

Table 3.8. LC-MS Data Analysis of 96-Well Plate Fermentations....................................98

Table 3.9. 1D NMR Data (600 MHz, CD_3OD) of GGL 915 and Literature Reference for Sugars and Literature Reference for Fatty Acids....................................118

Table 3.10. Coupling Constants of GGL 915 and Mannose Disaccharide....................................122

Table 3.11. 1D and 2D NMR Data (600 MHz, CD_3OD) of GGL 887....................................123

Table 3.12. Coupling Constants of GGL 887 and Mannose Disaccharide....................................125

Table 3.13. 1D and 2D NMR Data (600 MHz, CD_3OD) of GGL 885....................................128

Table 3.14. Coupling Constants of GGL 885 and Mannose Disaccharide....................................131

Table 3.15. NMR Data (600 MHz, DMSO-d_6) of GGL 873 Mixture and GGL 915 Standard....................................133

Table 3.16. Antimicrobial Assay of 5 μL Extracts against *S. aureus* (Averaged Duplicates)....................................140

Table 3.17. Antimicrobial Assay of 5 μL Extracts against *E. faecalis* (Averaged Duplicates)....................................141

Table 3.18. Antimicrobial Assay of 5 μL Extracts against *C. albicans* (Averaged Duplicates)....................................141

Table 3.19. Antimicrobial Assay of 5 μL Extracts against *P. aeruginosa* (Averaged Duplicates)....................................142

Table 3.20. Antimicrobial Assay of 50 μL Extracts against *S. aureus* (Averaged Duplicates)....................................142

Table 3.21. Antimicrobial Assay of 50 μL Extracts against *E. faecalis* (Averaged Duplicates)....................................143

Table 3.22. Antimicrobial Assay of 50 μL Extracts against *C. albicans* (Averaged Duplicates)....143

Table 3.23. Antimicrobial Assay of 50 μL Extracts against *P. aeruginosa* (Averaged Duplicates)....144

Table 3.24. Antifouling Assay of GGL 915....150

LIST OF ABBREVIATIONS

10-DAB: 10-Deactylbaccatin III

16S rDNA: Ribosomal RNA Gene which has a Sedimentation Coefficient of 16

ACC: Ampicillin Cycloheximide Chloramphenicol

ACN: Acetonitrile

AIA: *Actinomyces* Isolation Agar

ALPLC: Automated Low Pressure Liquid Chromatography

APCI: Atmospheric Pressure Chemical Ionization

Ara-A: Arabinofuranosyladenine

Ara-C: Arabinofuranosylcytosine

ATR: Attenuated Total Reflectance

BLAST: Basic Local Alignment Search Tool

BMS: Bristol-Myers Squibb

BSA: Bovine Serum Albumin

CAMH: Cation-Adjusted Mueller-Hinton

CFU/mL: Colony Forming Units per mL

CLSI: Clinical and Laboratory Standards Institute

CMC: Critical Micelle Concentration

COSY: Correlation Spectroscopy

DMA: 1/10 Dilute Marine Agar

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DNA: 1/10 Dilute Nutrient Agar

DNB: Dilute Nutrient Broth

EC_{50}: Settlement Inhibition Concentration of 50%

EDTA: Ethylenediaminetetraacetic Acid

ELSD: Evaporative Light Scattering Detector

ESI: Electrospray Ionization

ETBr: Ethidium Bromide

EtOH: Denatured Alcohol

FA: Formic Acid

FAB-MS/MS: Fast Atom Bombardment-Tandem Mass Spectrometry

FAME: Fatty Acid Methyl Ester

FBS: Fetal Bovine Serum

FDA: US Food and Drug Administration

G+C: Guanosine + Cytosine

GC-MS: Gas Liquid Chromatography-Mass Spectrometry

GGLs: Glycoglycerolipids

GI_{50}: Growth Inhibition of 50%

HAVA: Humic Acid-Vitamin Agar

HMBC: Heteronuclear Multiple bond Coherence

HPI: Hours Post Infection

HPLC: High Pressure Liquid Chromatography

HRFAB-MS: High Resolution Fast Atom Bombardment-Mass Spectrometry

HR-MS: High Resolution-Mass Spectrometry

HSQC: Heteronuclear Single Quantum Coherence

IC_{50}: Inhibition Concentration of 50%

IO: Instant Ocean

ISP2: International Streptomyces Project Medium 2

ISP3: International Streptomyces Project Medium 3

LC_{50}: Lethal Concentration of 50%

LC-MS: High Pressure Liquid Chromatography-Mass Spectrometry

LD_{50}: Lethal Dose Concentration of 50%

LDH: Lactate Dehydrogenase

LSU: Light Scattering Units

m/z: Mass to Charge Ratio

MA: Marine Agar

Man*p:* Mannopyranosyl

MB: Marine Broth

MBI: Malpeque Bay Isolate

MDCK: Madin-Darby Canine Kidney

MEGA: Molecular Evolutionary Genetics Analysis

MEM: Minimal Essential Medium Eagle

MeOH: Methanol

MMAE: Monomethyl Auristatin E

MMAF: Monomethyl Auristatin F

MMM: Marine Minimum Media

MOI: Multiplicity of Infection

MPY: Malt Peptone Yeast Extract

MRSA: Methicillin-Resistant *Staphylococcus aureus*

MS^2: Tandem Mass Spectrometry

MTT: 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide

mV: Millivolts

NA: Nutrient Agar

NB: Nutrient Broth

NCI: National Cancer Institute

NDA: New Drug Application

NEB: New England BioLabs

NOESY: Nuclear Overhauser Effect Spectroscopy

NPs: Natural Products

NR: Non-Redundancy

OD_{600}: Optical Density at 600 nm

P/S: Pencillin/Streptomycin

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDA: Photodiode Array

PEI: Prince Edward Island

RFLP: Restriction Fragment Length Polymorphism

ROESY: Rotating-frame Overhauser Effect Spectroscopy

RT: Room Temperature

spp.: Species

TAE: Tris Acetate

TLC: Thin Layer Chromatography

TOF: Time of Flight

TPCK: Tosyl Phenylalanyl Chloromethyl Ketone

UPLC: Ultra Performance Liquid Chromatography

VRE: Vancomycin-Resistant *Enterococcus*

WYE: Water-Yeast Extract

YE/SDS: Yeast Extract/Sodium Dodecyl Sulfate

YpSs: Yeast Protein Soluble Starch

CHAPTER 1-INTRODUCTION

1.1. Natural Products

1.1.1. "What are Natural Products?"

Primary metabolites are compounds produced by an organism which are necessary for its basic survival. Natural products (NPs) are secondary metabolites which are compounds produced by an organism that are not necessary for its basic survival but are believed to give it an adaptive advantage. They are believed to play important roles in defence, predation and communication.[1,2] NPs have been isolated from many different organisms such as plants like yew trees or periwinkles, animals like sharks or sponges and microorganisms like bacteria or fungi. They are found in many places like in your back yard or in hard to reach places like in hydrothermal deep-sea vents. NPs can be small and simple like aspirin or large and complex like taxol. They can have many different possible functions in organisms, many of which are not well understood.[1]

1.1.2. Natural Products as Drugs

NPs are considered "privileged structures" which means that they have "intrinsic biological activity"[3] and are viewed as structures selected by evolutionary pressures to bind to select biological targets.[4] Over billions of years, organisms have evolved ways to interacted with coexisting organisms. NPs are compounds that organisms developed to interact with other organisms in order to achieve a specific biological response. Since there are such a large number of species and an almost infinite number of different ways they can interact with each other, it's not surprising that there are an astounding number of NPs. They are both structurally and stereochemically complex, designed to interact

with biological targets in order to help preserve the survival of the producing organism.[5] This means that species diversity or biodiversity has a direct correlation with chemical diversity where increased biodiversity would lead to an increase in chemical diversity.[6]

NPs and NP-derived drugs have therapeutic activity in many areas. These areas include, but are not limited to, antiviral, antibacterial, antifungal, anti-inflammatory, anticancer, antiparasitic, or specific diseases or malignant conditions like osteoporosis and Alzheimer's disease. Over 75% of anticancer, anti-infectives and anticholesterolaemic compounds have some part of their development linked with a NP. NP-derived could mean a synthetic derivative which shows improved activity or a cytotoxic compound coupled to a delivery agent like antibody-drug conjugates. Alterantively, it can also mean a synthesized compound mimicking a NP, like the drug captopril mimicking the peptide teprotide (**Figure 1.1**).[7-9]

Figure 1.1. Captopril and Teprotide

Some NPs have been shown to have specific targets which can be exploited for human benefit like the compound penicillin (**Figure 1.2**) which is an antibiotic produced by the fungus *Penicillium notatum*. It kills bacteria by inhibiting the enzyme that cross-links bacterial cell walls which results in their destabilization.[8]

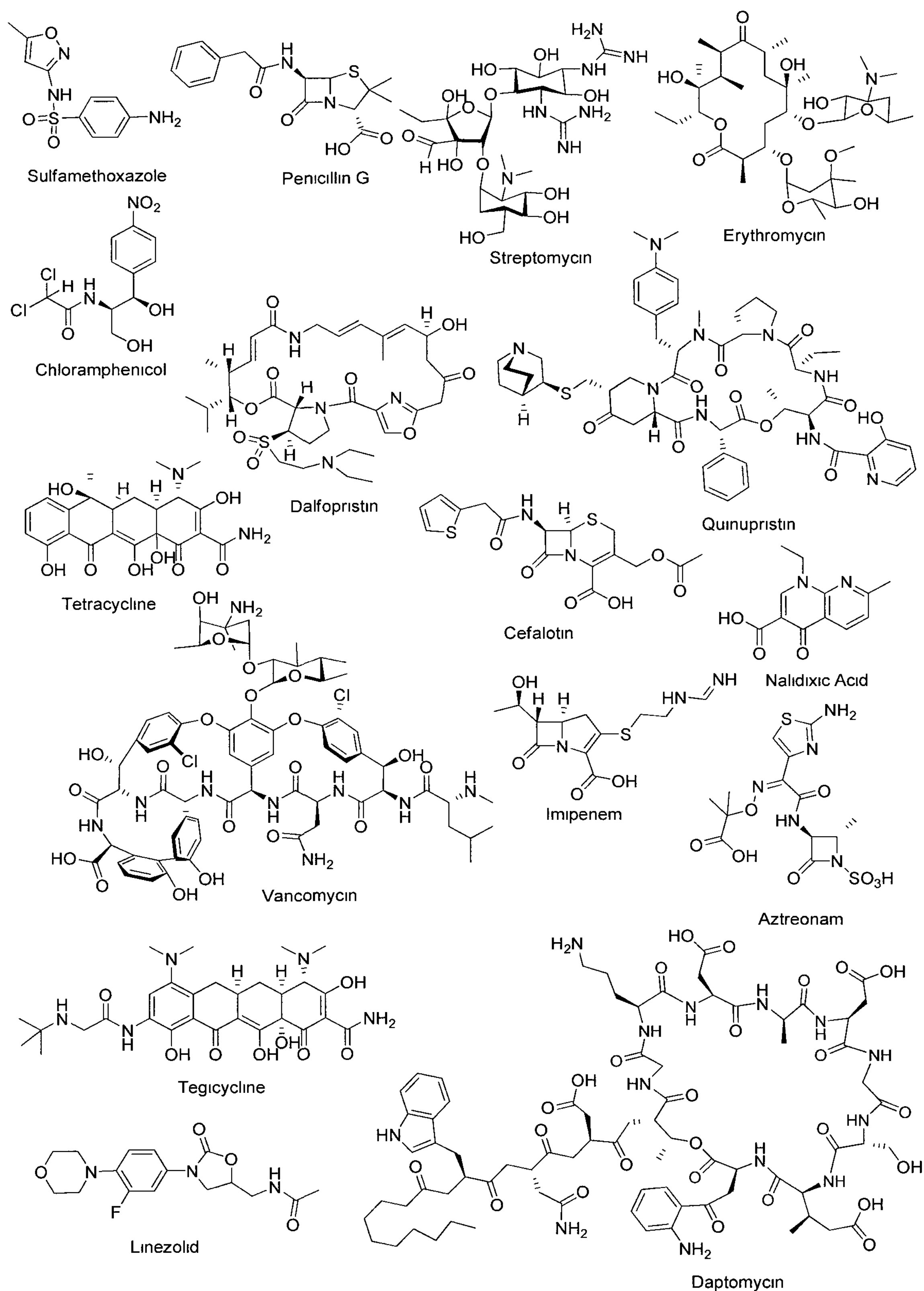


Figure 1.2. Chemical Structures of Antibiotics [9]

Some NPs mimic other naturally occurring compounds but are completely different. They may have good drug properties unlike the mimicked compound. For example, peptides are known to inhibit many different functions as hormones and neurotransmitters. However, peptides are rarely good oral drugs since they get rapidly degraded in the stomach and have poor bioavailability. This creates the challenge of finding non-peptide compounds that will fit the same active site. This has been achieved in nature with the production of morphine which fits the active site of enkephalins, the brains analgesic (**Figure 1.3**). Morphine also has many properties that make it a good drug.[8]

Morphine

Met-enkaphalin, R= CH_2SCH_3
Leu-enkaphalin, R=$CH(CH_3)_2$

Figure 1.3. Morphine and Enkephalins, Met-enkaphalin and Leu-enkaphalin

A historical problem with drug discovery in combinatorial chemistry is the solubility of compounds.[10] Combinatorial chemistry is the rapid synthesis of a large number of compounds or "libraries" using a small number of smaller compounds.[11] To increase the chance of producing compounds which are more orally bioavailable, chemists followed Lipinski's rule of five. Lipinski's rule of five states that poor absorption or permeation of compounds are likely if there are more than five hydrogen bond donors or ten hydrogen bond acceptors, the molecular weight is greater than 500 daltons or the log P is greater than five.[12] NPs, however, are exempt from these rules due

to the ability of nature to create very large compounds with low hydrophobicity that mimic biosynthetic intermediates or other metabolites in order to take advantage of active transport mechanisms.[10] NPs generally have a larger number of chiral centers and oxygen atoms, more steric complexity and contain fewer aromatic rings than compounds from combinatorial libraries. These attributes have greatly contributed to the success of NPs in drug development.[4, 13]

1.1.3. Historical Use of Natural Products

NPs have been used for thousands of years all over the world. People have always been dependent on nature to provide for them, be it food, shelter, clothing or medicine. Ancient documents written on clay tablets from Mesopotamia from as far back as 2600 B.C describe about 1000 different substances used to treat different illnesses that were extracted from plants. An important Egyptian document *Ebers Papyrus*, dating back to 1500 B.C, contains about 700 drugs. It contains formulas for infusions, poultices, ointments and various others, which are made from plants and sometimes animals. The *Materia Medica* from China is an ancient document that has changed extensively over the centuries, starting with 52 prescriptions in 1100 B.C, 365 prescriptions in 100 B.C and 850 prescriptions in 659 A.D.[14]

An important Greek physician in 100 A.D was Dioscorides who studied the medicinal application of herbs and had written several important books. He had compiled more than 800 drugs with sources like plants, animals and minerals as well as their uses and locations to obtain them.[15] Galen was another important Greek physician who contributed greatly to medicine between 130 and 200 A.D. He wrote over 30 books on

pharmacy and medicine including *Methodo medendi*. One of his most important contributions was his classification system of grouping medicines, while incorrect by modern view, provided a rational for selecting medicines.[14-16]

It wasn't until after 500 A.D that Arabians had set up privately owned pharmacies and started to test the efficacy of their drugs that medicine moved from guesswork and philosophy to more of a science.[15] Avicenna compiled most of the, then current, medicinal data in the *Canon medicinae*. It contained descriptions of 800 drugs, mostly from plants, and 650 compound prescriptions. It was one of the main texts used in teaching medicine and pharmacy until the 19th century.[15, 17] However, complex mixtures were still being used and it would not be until the 1800's that pure compounds would be considered as drugs. The commercial production of morphine **(Figure 1.3)** in 1826 by E. Merck is considered to be the first pure NP.[14]

NPs have continued to contribute greatly to the development of products for human and animal health. NPs have provided a lot of new chemical scaffolds for various applications such as anticancer, antifungal, anti-inflammatory and antibiotic as well as many others. Between 1940 and 2010, 13 new classes of antibiotics were discovered from NPs or produced using NPs previously isolated. What constitutes a new class is debatable with the number of new antibiotic classes ranging from twelve[18] and thirteen[19] to as high as twenty-seven.[20] Dates of clinical introduction of antibiotics can also vary considerably. **Figure 1.4** is a timeline of the different classes of antibiotics and their dates of discovery. In italics are synthetic antibiotics while the rest are NPs or NP-derived. Chemical structures are shown in **Figure 1.2**.[18-20]

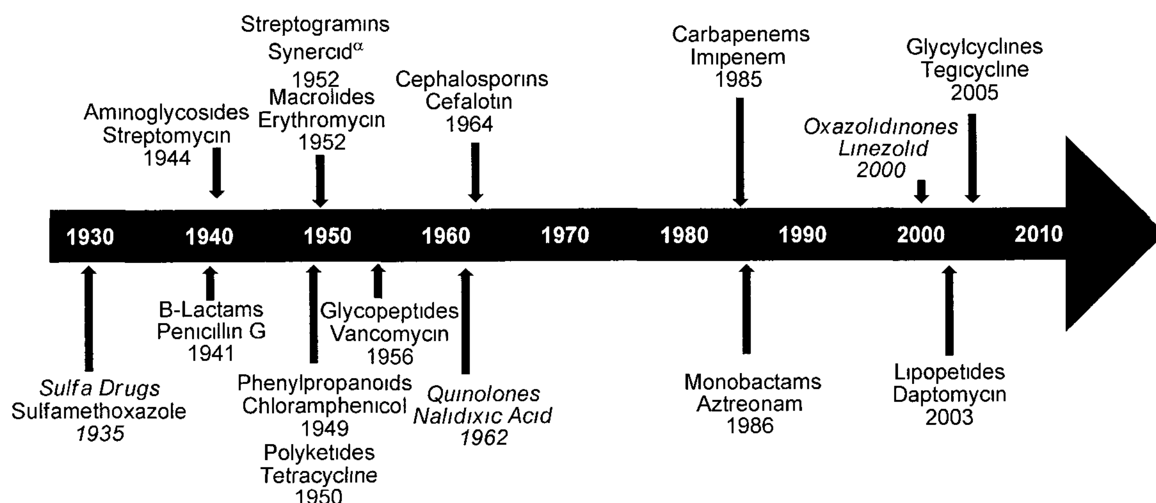


Figure 1.4. Timeline of Approximate Date of Clinic Use of Antibiotics[20]
$^{\alpha}$Synercid is a drug that is composed of Quinupristin and Dalfoprisitin

1.1.4. Successful Natural Products

NPs have had a very large impact on successful drug production with up to 60% of commercial drugs being NPs or having a NP scaffold.[21, 22] Of the top 25 best selling drugs in 1997, 42% were NPs or NP-derived.[22] NPs, NP-derived or inspired compounds in Phase III clinical trials or in the New Drug Application (NDA) stage of drug development are outlined in **Table 1.1**. The names of the compounds, how they relate to NPs, the inspiring NP and their treatment area are listed. There are six compounds at the NDA stage and 31 compounds in Phase III clinical trials which could potentially become live saving and enhancing drugs.

Table 1.1. NP-derived drugs in NDA or Phase III Stage of Development (March 2008)[9]

Compound	Classification	Parent Compound	Disease Area
NDA or equivalent			
Ceftobiprole medocaril	NP-derived	Cephalosporin	Antibacterial
Dalbavancin	Semi-synthetic NP	Vancomycin	Antibacterial
Telavancin	Semi-synthetic NP	Vancomycin	Antibacterial
Oritavancin	Semi-synthetic NP	Vancomycin	Antibacterial
Methylnaltrexone	NP-derived	Morphine	Opiod induced constipation and pain
Rubuxistaurin	NP-derived	Staurosporine	Diabetic retinopathy
Phase III			
Tebipenem pivoxil	NP-derived	Carbapenem	Antibacterial
Faropenem daloxate	NP-derived	Faropenem	Antibacterial
Omignan	NP-derived	Indolicidin	Antibacterial
Cethromycin	Semi-synthetic NP	Erythromycin	Antibacterial
Tiacumicin	NP	N/A	Antibacterial
Eritoran	NP-derived	Lipopolysaccharide Rs-DPLA	Antibacterial
Morphine-6-glucuronide	Semi-synthetic NP	Morphine	Pain
Dapagliflozin	NP-derived	Phlorizin	Type 2 Diabetes
Ilepatril	NP-derived	Captopril	Hypertension and Kidney Disease
SCH 530348	NP-derived	Himbacine	Cardiovascular diseases
Voclosporin	Semi-synthetic NP	Cyclosporin A	Multiple sclerosis and transplantation
Fingolimod	NP-derived	Myriocin	Multiple sclerosis
BNP-1350	Semi-synthetic NP	Camptothecin	Oncology
Combretastatin A-4 Phosphate	Semi-synthetic NP	Combretastatin A-4	Oncology
AVE-8062	NP-derived	Combretastatin A-4	Oncology
Vinflunine	Semi-synthetic NP	Vinblastine	Oncology
Cabazitaxel	Semi-synthetic NP	Paclitaxel	Oncology
Larotaxel	Semi-synthetic NP	Paclitaxel	Oncology
DHA-paclitaxel	Semi-synthetic NP	Paclitaxel	Oncology
Homoharringtonine	NP	N/A	Oncology
Phenoxodiol	NP-derived	Daidzein	Oncology
Alvocidib	NP-derived	Rohitukine	Oncology
Tanespimycin	Semi-synthetic NP	Geldanamycin	Oncology
Everolimus	Semi-synthetic NP	Rapamycin	Oncology
Deforolimus	Semi-synthetic NP	Sirolimus	Oncology

Continued next Page

Table 1.1. Continuation of NP-derived drugs in NDA or Phase III Stage of Development (March 2008)[9]

Compound	Classification	Parent Compound	Disease Area
Enzastaurin	NP-derived	Staurosporine	Oncology
Lestaurtinib	NP-derived	Staurosporine	Oncology
Epothilone B	NP	N/A	Oncology
Irofulven	Semi-synthetic NP	Illudin	Oncology
Eribulin	NP-derived	Halichondrin B	Oncology
Panobinostat	NP-derived	Psammaplin	Oncology

1.2. Marine Natural Products

1.2.1. What are Marine Natural Products?

Marine NPs are NPs isolated from marine organisms. Many different organisms have been studied for NPs such as sponges, corals, ascidians, algae, and a large number of different microorganisms. Marine organisms can be found in different habitats such as high or low temperatures and in environments ranging from mangroves to deep sea hydrothermal vents. Marine organisms have populated virtually every area in the seas and oceans.[23, 24]

1.2.2. Advantages of Marine Sources over Terrestrial Sources for Natural Products

Marine Organisms as Sources of Natural Products

Over 70% of the earth is covered with water and the oceans represent 95% of the earth's biosphere.[25] It was over 3500 million years ago that organisms first appeared in the sea while the first metazoans appeared over 800 million years ago. Over time, evolution of marine organisms has equipped them with unique adaptations for survival. Adaptations could be physical or chemical, resulting in the organisms being able to withstand a hostile environment. Organisms with no physical defence are believed to have evolved chemical defences that protect from predation. Many benthic organisms like sponges are sessile with no apparent physical defences, yet they have survived. It's believed that they, or microorganisms that they host, have evolved chemical defences to prevent attack by parasitic microorganisms, fouling by other organisms, or overgrowth by neighbouring organisms. To prevent these activities, they would need to have or produce antimicrobial, antifouling or cytotoxic compounds.[7, 25] Some organisms, such as cone

snails, use compounds for predation. They use a complex mixture of peptides to paralyze prey, like fish, via a hollow harpoon; which is also used to defend itself against predators.[1] Such compounds would have to be extremely potent because of dilution in water and as such, are view as being analogues to pheromones from insects but with a different purpose.[26] They would also have to be extremely selective and be able to easily enter cells to be effective. These are the same properties that make good drugs and are highly sought for in the pharmaceutical industry.[7]

Biological Diversity of Marine Organisms

Marine organisms are much more phylogenetically diverse than terrestrial organisms. Of the 33 recognized different animal phyla, 32 are observed in a marine environment and 18 in a terrestrial environment. Fifteen animal phyla are only found in marine environments with some examples being *Ctenophore*, *Echinodermata* and *Porifera*.[25, 27] Functionally, marine phyla display more diversity with sessil organisms like sponges and corals that are filter feeders which have no terrestrial counterparts.[6, 27] This also suggests that marine phyla likely diverged long ago and have been on separate evolutionary paths for much longer than terrestrial phyla.[28] The consensus of Marine Life Project in 2010 predicted that there are 1 to 1.4 million different marine species on earth. With the number of known species estimated at 230,000, it leaves many more species left to be characterized.[1, 29, 30]

1.2.3. Technological Hurdles of Marine Natural Products Research

Accessing Marine Environments

Marine NPs research has been stymied by the lack of technology for deep-water sample collection. Shore wading can be used to collect the more easily accessible sample however samples in deeper water need alternative methods.[31] Dredging can be used to collect samples however, it's very invasive and destroys much of the natural habitat. For non-invasive sample collecting, there are several different methods. For example, scuba diving can be used to collect samples but was only introduced in the 1940s.[26] It wasn't widely used for NPs research until it became popular in the 1970s. The disadvantages are that scuba diving only allows for a limited amount of dive time and depth. If prolonged dives are necessary, an underwater laboratory may be used of which there is only one in the world. It's called *Aquarius* and was deployed in 1986.[32] If scuba diving is not capable of reaching the desired depth, submersibles with robotic arms may be used to collect samples from much greater depths.[33] However, this is an extremely expensive endeavour to undertake which can cost between $10,000 and $45,000 a day for the use of the submersible and support ship; of which there are very few available.[32, 34]

Natural Products Chemistry Technological Limitations

NPs chemistry has had difficulties in several parts of the drug discovery path which include compound purification, dereplication, and structure elucidation. High pressure liquid chromatography (HPLC), which is a necessary technique for NPs isolation, was not generally available until the 1970s.[26] Some compounds like ivermectins

(Figure 1.5) reported in 1980, were developed and marketed as mixtures since separation technology had not developed sufficiently. Without adequate preparative separation,

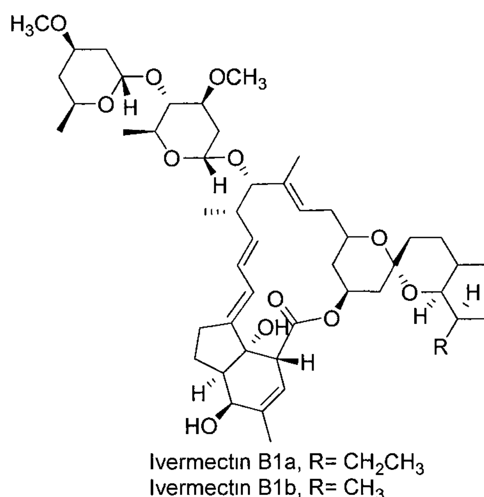


Figure 1.5. Ivermectin Mixture, B1a and B1b

sufficient compound cannot be purified to be produced at a commercial scale.

Dereplication is the rapid identification of known compounds. Without dereplication, there would be a large amount of time and money wasted on rediscovering already known NPs. High pressure liquid chromatography-mass spectrometry (LC-MS) and NP databases, which are critical for dereplication, have only been available for a little over 30 years[35] and less than 20 years respectively.[36] Since NPs can be very complex, like the peptides from the venom of cone snails, and are usually present in minute quantities, such as 1 mg for every 3 kilograms of sample,[26] very sensitive techniques are required to obtain the structure. NMR has improved with the introduction of 2D NMR methods for structural determination. Sensitivity has increased with the development of stronger magnetic fields by superconducting magnets, cryogenic electronics and micro-probe technology. The introduction of high-resolution-mass spectrometry (HR-MS) is a

technique that greatly facilitates structure elucidation. With the molecular mass, the molecular formula can be calculated which greatly limits the possible structure of a compound. This tool also greatly facilitates dereplication since with the exact mass, databases can be analyzed for natural compounds with the same molecular formula.[4]

1.2.4. Examples of Marine Natural products

There are 17 marine NPs or derived marine NPs in clinical trials (**Table 1.2, Figure 1.6**).[37] Five are approved by the FDA or European commission for clinical use as human therapeutics of which eribulin mesylate was only approved November 2010.[39] Eribulin mesylate is a derivative of halichondrin B and was approved by the FDA for metastatic breast cancer.[38] Arabinofuranosyladenine (Ara-A) is an antimetabolite, which is a derivative of adenosine, that competes with the natural nucleoside and results in the inhibition of DNA polymerase and DNA synthesis. Ara-A was approved in 1976 by the FDA for treatment against the herpes virus and is currently obtained from *Streptomyces antibioticus.*[38] Ara-C is an antimetabolite which was approved in 1969 against leukemia. It was the first marine NP approved by the FDA. Ziconatide is a very potent analgesic approved by the FDA in 2004. It's currently approved for chronic pain in patients who have cancer or AIDS. Trabectedin is an alkaloid with activity against soft tissue sarcoma and relapsed platinum resistant ovarian cancer. Currently, it is being investigated for activity against breast, lung, prostate and pediatric cancer. Arabinofuranosylcytosine (Ara-C), ziconatide and trabectedin will be discussed more in detail in **Section 1.2.5.**[38]

Table 1.2. Drug Development Pipeline of Marine Natural Products (March 2011)[37, 38]

Compound Name	Marine Source	Chemical Class	Company	Disease Target
Approved				
Cytarabine, Ara-C	Sponge	Nucleoside	Bedford, Enzon	Cancer
Vidarabine, Ara-A	Sponge	Nucleoside	King Pharmaceuticals	Antiviral
Ziconatide	Cone Snail	Peptide	Elan Corporation	Pain
Trabectedin	Tunicate	Alkaloid	Pharmamar	Cancer
Eribulin Mesylate	Sponge	Macrolide	Eisai Inc.	Cancer
Phase III				
Brentuximab vedotin (SGN-35)	Mollusk	Monoclonal Antibody and Peptide	Seattle Genetics	Cancer
Phase II				
DMXBA (GTS-21)	Worm	Alkaloid	Comentis	Cognition Schizophrenia
Plinabulin (NPI-2358)	Fungus	Diketopiperazine	Nereus Pharmaceuticals	Cancer
Plitidepsin	Tunicate	Depsipeptide	Pharmamar	Cancer
Elisidepsin	Mollusc	Depsipeptide	Pharmamar	Cancer
PM00104	Nudibranch	Alkaloid	Pharmamar	Cancer
CDX-011 (CR011-vcMMAE)	Mollusk	Monoclonal Antibody and Peptide	Celldex Therapeutics	Cancer
Pseudopterosins	Soft Coral	Diterpene glycoside	Terosin Group	Wound Healing
Phase I				
Bryostatin 1	Bryozoa	Polyketide	National Cancer Institute	Cancer
E7974	Sponge	Tripeptide	Eisai Inc.	Cancer
SGN-75	Mollusk	Monoclonal Antibody and Peptide	Seattle Genetics	Cancer
Marizomib (Salinosporamide A)	Bacterium	Beta-lactone-gamma lactam	Nereus Pharmaceuticals	Cancer

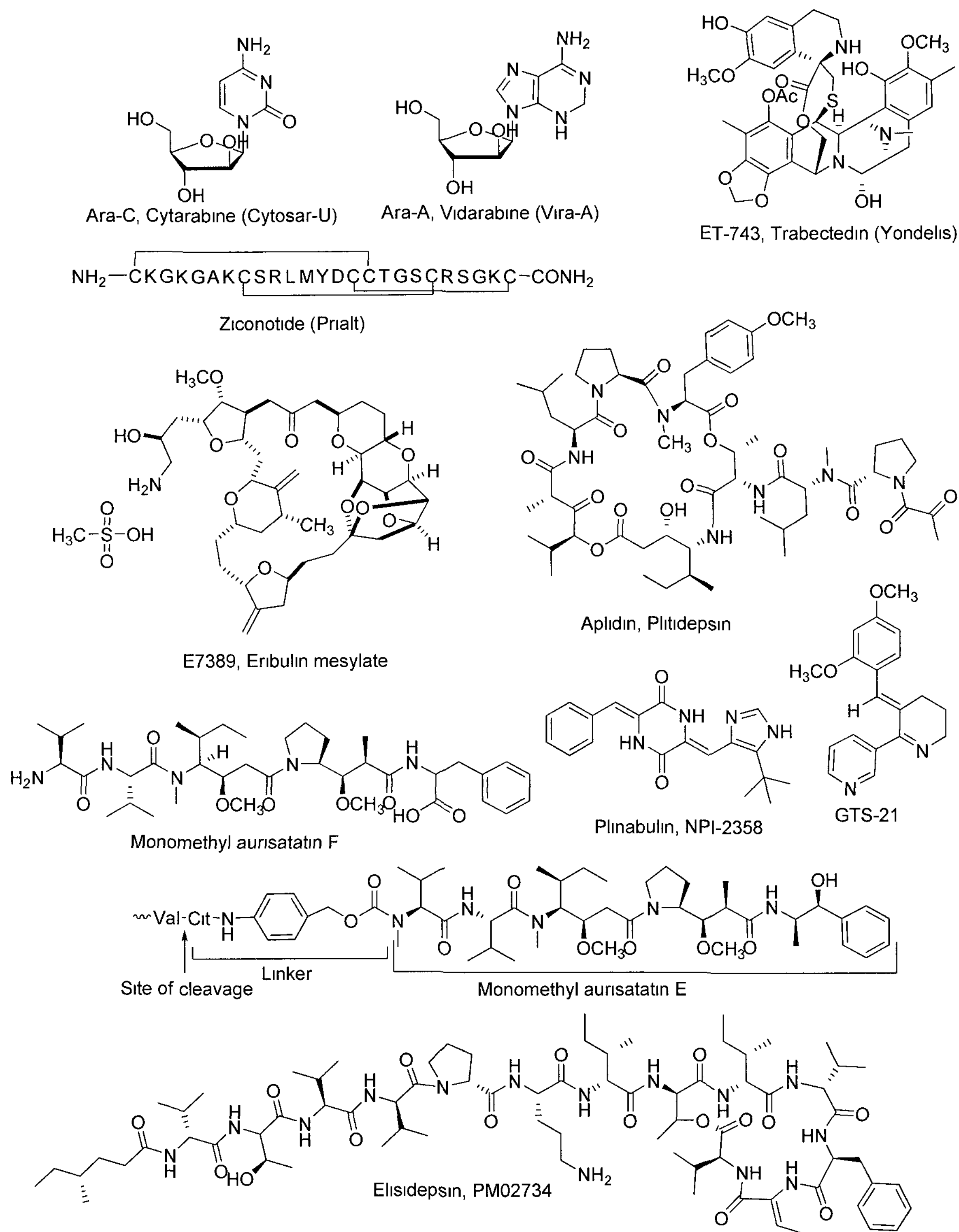


Figure 1.6. Marine Natural Products in Clinical Trials. In brackets are the drug names and is continued on next page

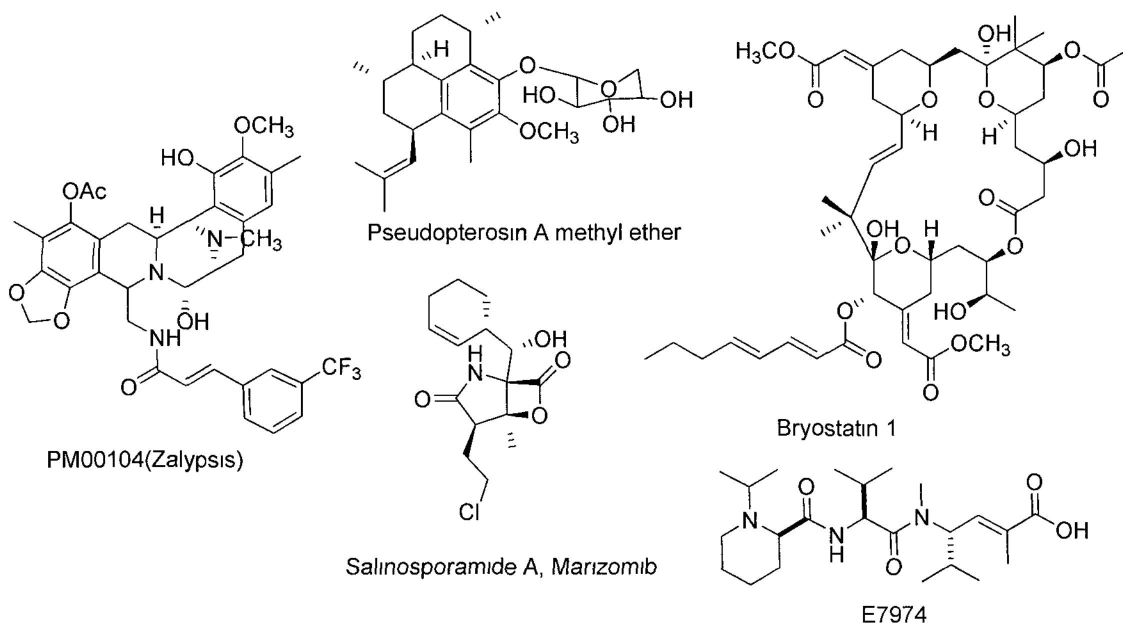


Figure 1.6. Continuation of Marine Natural Products in Clinical Trials. In brackets are the drug names.

Brentuximab vedotin is an antibody-drug conjugate that is composed of a warhead, monomethyl auristatin E, attached to a monoclonal antibody, anti-CD30, which is the drug's targeting system.[40] It is being investigated for activity against Hodgkin lymphoma.[41] Two other antibody-drug conjugates are CDX-011 and SGN-75. CDX-011 is composed of a glycoprotein NMB targeting antibody, CR-011, linked to monomethyl auristatin E (MMAE). It is currently being investigated for activity against metastatic breast cancer and melanoma.[42] SGN-75 is composed of an anti-CD70 monoclonal antibody attached to monomethyl auristatin F (MMAF). It is is currently being investigated for activity against renal cell carcinoma and non-Hodgkin lymphoma.[41]

DMXBA is a derivative of anabaseine and has shown improvement for schizophrenics. It is currently being developed by Comentis Inc., a company dedicated to

finding treatments for Alzheimer's disease.[43] Plitidepsin is a depsipeptide that shows very potent activity with a half maximal inhibitory concentration (IC_{50}) in the low nanomolar range. It is currently being investigated for activity against multiple myeloma and T cell lymphoma. Elisidepsin is a relative of the kahalide family of compounds. Testing for activity against lung cancer started in 2008.[38, 44] PM00104 is a relative of jorumycin and shows very potent multiple myeloma activity with IC_{50} values in the low nanomolar to the picomolar range. PM00104 showed good *in vivo* activity against breast, prostate, liver, bladder and gastric cancers. It is currently being investigated for activity against solid tumours.[45] Pseudopterisin A methyl ether is a terpenoid that has very potent anti-inflammatory and wound healing properties. Pseudoptersin A methyl ether is currently being examined in phase 2 clinical trials for its wound healing ability.[38]

Bryostatin 1 is a NP isolated from *Bugula neritina* but subsequent research indicates its biosynthetic source is from a symbiotic bacterium *Candidatus endobugula sertula*. Bryostatin 1 has been in over 80 clinical trials against cancer and is now also being tested against Alzheimer's.[38] E7974 is a hemiasterlin derivative with very potent activity with an IC_{50} in the low nanomolar to picomolar range. It is currently being evaluated for activity against solid tumors.[46] Marizomib is a very promising compound isolated from *Salinispora tropica.* It displays very potent anticancer activity in the low nanomolar range and is currently being evaluated in combination with vorinostat against several different cancers such lung, pancreatic, melanoma or lymphoma.[47] Marizomib will be discussed in more detail in **Section 1.3.4**.

1.2.5. Proven Compounds as Drugs

Ara-C, Cytarabine (Cytosar-U)

Ara-C, also called cytarabine, is marketed under many different names including Cytosar-U. The synthesis of Ara-C was inspired by the discovery of unusual nucleosides, spongothymidine and spongouridine, in the early1950s from *Cryptotethia crypta.*[14, 48] This challenged the accepted paradigm in the 1950s that a nucleoside had to have a ribose or deoxyribose in order to have activity, but that the base could be extensively modified. These discoveries lead to an explosion in derivatives that spawned a series of new important drugs like Ara-A, acycloguanosine, azidothymidine and didanosine.[14, 26] Ara-C was reported in 1966 to show activity for acute leukemia in children which prompted its rapid development and subsequent FDA approval in 1969.[38, 49] It has been approved for the prevention and treatment of meningeal leukemia by the FDA. It has been approved for the treatment of acute myeloid leukemia, acute lymphoblastic leukemia and chronic myelogenous leukemia in combination with other drugs by the FDA.[50]

Ecteinascidin 743, Trabectedin (Yondelis)

Trabectedin, previously named Ecteinascidin 743, is currently sold under the drug name Yondelis (**Figure 1.6**). Trabectedin was first reported from the tunicate *Ecteinascida turbinata* in 1990[51, 52] but was isolated in a very low yield of 0.0001%. Trabectedin was discovered to have a unique mechanism of action when it was tested against the National Cancer Institute (NCI) 60 cell line panel.[53, 54] Later research revealed that the mechanism of action was by binding to the minor groove of DNA and also by inhibiting different proteins involved in the nucleotide excision repair system which leads

to cell apoptosis.[38] It was licensed by the University of Illinois to PharmaMar in 1994. Trabectedin is currently being supplied by semi-synthesis from cyanosafracin B which is obtained from the fermentation of *Pseudomonas fluorescens.*[53] Trabectedin was approved for advanced and metastatic soft tissue sarcoma by the European commission in 2007. It was also approved in combination with doxil for relapsed platinum-sensitive ovarian cancer by the European commission in 2009. It has been granted orphan drug status for ovarian cancer and soft tissue sarcoma. It is currently in phase 2 trials for breast cancer, lung cancer, prostate cancer and paediatrics tumours.[44]

Ziconatide (Prialt)

Cone snails are marine snails found mostly in the western and southern pacific. They are known for having a harpoon-like projectile that contains a mixture of potent peptide toxins which they use to stun, paralyze or kill prey like small fish.[14] Ziconatide (**Figure 1.6**) is a 25 amino acid peptide, one of many different analogues, which was first isolated from the cone snail *Conus magus.*[14, 38, 55] It is a very potent analgesic, up to 1000 times more potent than morphine,[55] and does not induce dependence which is a problem with other analgesics like opiates. It also does not produce tolerance which would require a gradual increase of the administered compound over long term treatment and is also a major problem with opiates. Ziconatide was discovered to have a new mechanism of action that gave it great selectivity and potency. Since ziconatide is a peptide, it is readily supplied by synthesis and does not have a supply issue which is a common with other NPs. It was approved in 2004 by the FDA for chronic pain in patients with cancer or

AIDS and by the European commission for severe chronic pain. It's currently licensed to Elan Corporation under the drug name Prialt.[56]

1.3. Marine Bacteria

1.3.1. Difficulties in Defining Marine Bacteria

There is some debate on what constitutes marine bacteria. Marine bacteria that require salt are considered marine bacteria or salt obligate bacteria. However, near shores, bacteria are adapted to marine environments to different degrees so it makes it difficult to distinguish between marine bacteria and salt tolerant terrestrial bacteria. Bacteria isolated from near the shore could be marine bacteria that show the capability of growing without salt or could, and is more commonly believed, be terrestrial bacteria that were washed into the sea. It makes sense to study all the bacteria isolated from marine samples since they have been shown to produce new compounds with good biological activity which have not previously been observed in their terrestrial counterparts.[57]

1.3.2. Marine Bacteria as Sources of Natural Products

Diversity and Abundance of Marine Bacteria

It was once believed that the oceans were biological deserts[58] and in the 1950s, the very existence of marine bacteria was questioned. However, in 1977, it was shown that the oceans harbour a large diversity of organisms with the discovery that there were about 10^6 bacteria per mL of seawater[59] and subsequent discovery that marine sediments may contain 10^9 bacteria per mL. Bacteria from new genera such *Oceanospirillum*, *Marinomonas* and *Salinispora* have been isolated only from marine sources which indicates a vast untapped biodiversity of which have been shown to produce new NPs. One genus, *Salinispora,* shows great potential with the production of a potent compound

named Salinosporamide A (**Table 1.2, Figure 1.6**) which is in Phase I clinical trials and will be discussed later.[60]

It's been recently predicted that there are tens of millions to hundreds of millions of new species of marine microorganisms present on earth with less than 1% having been cultured.[1, 29] It is believed that marine bacteria account for 80% of life on earth with a population of 3.67×10^{30} organisms.[61] Due to evolutionary pressures spanning about 4 billion years, they have evolved and colonized virtually every area of surface of the earth. They have developed diverse mechanisms to survive in the competitive environment that is highly variable with different temperatures, salinity, pressure, pH and nutrients.[58, 61] Marine microbes have shown activity for many therapeutic applications such as antimicrobial, anticancer, antiparasitic, anti-inflammatory and antiprotozoan activity.[62, 63] This indicates that there is vast potential for the discovery of new unique organisms producing novel NPs.

Trends in Microbial Research

By 2002, microbes were the source of 22500 biologically active NPs,[22] 62% of which have been obtained from bacteria.[64] Bacteria also produce over 70% of naturally occurring antibiotics,[65] a drug class that had a market value of 32 billion dollars in 2001.[22] Terrestrial bacteria have proven to be a rich source of antibiotics,[66] however novel antibiotics discovery from terrestrial microorganisms is decreasing as more previously isolated NPs are being discovered. With the growing emergence of antibiotic resistant infectious diseases, new sources of antimicrobials need to be found. Since new biodiversity directly correlates with new chemical diversity, it is logical that exploration

of microbial NPs will move to the oceans.[6, 61] NPs discovery from marine bacteria is a budding research area that has only started recently with 3000 compounds characterized by the end of 2008. Despite this, many are already showing great potential with some compounds showing high levels of activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococcus* (VRE).[66, 67]

1.3.3. Advantages of Marine Bacteria as Sources of Natural Products

Determining the True Producers of Natural Products

It is believed that microorganisms are the true producers of many NPs isolated from marine invertebrates however, obtaining firm proof of the biosynthetic source has been difficult.[68] With over 50% of the mass of sponges coming from microorganisms, it seems quite likely that they are the true producers of many NPs that have previously been isolated.[69] Current supporting evidence is the localization of the NPs in microbial cells as well as their close structural similarity to NPs isolated from microorganisms.[68] It's not surprising that symbiotic bacteria produce many biologically active compounds given that they face large competition from surrounding seawater which typically contain 10^6 bacteria per mL.[28] There has been a lot of recent research conducted on the microbial communities of sponges[69] as well as coral communities.[59] It has been shown in corals of different states of health that there are shifts in the bacterial composition[59] and that there is little overlap with bacterial communities in the surrounding seawater.[62] They have been shown to be essential for the normal development of the host organism.[62] The red alga *Delisea pulchra* has been shown to be able to influence the composition of its epiphytic bacteria by the use of quorum-sensing inhibitory compounds called furanones.[67]

There have been a number of compounds that were originally isolated from macroorganisms but have been shown to be produced by microorganisms. An example is hypericin, which was isolated from an endophytic fungus isolated from *Hypericum perforatum.*[1] Manzamine A (**Figure 1.7**) and 8-hydroxymanzamine A were produced by a *Micromonospora sp.* isolated from a sponge that was shown to contain the same compounds.[26] Swinholide A, which can be extracted from the sponge *Theonella swinhoeei,* was found to be localized in unicellular bacteria within the sponge but not located in the filamentous cyanobacteria. Swinholide A was also recently isolated from marine cyanobacteria. This raises the question of whether both types of bacteria can produce the compound or whether the compound may diffuse from the cyanobacteria into the unicellular bacteria.[68, 70]

Marine Bacteria as Renewable Sources of Natural Products

Marine NPs have traditionally had a problem with a lack of supply of the desired compound for clinical trials and commercialization. Marine samples were typically invertebrates that needed to be collected by scuba diving in large quantities since they contained the bioactive compounds in such small quantities. Mass harvests were generally not economically and environmentally sustainable. The bioactive compounds were also structurally complex with many chiral carbons which can make total synthesis difficult and impractical on a large scale.[1, 4, 62] An example is halichrondrin B, which is very complex and is found in very low abundance in the sponge *Lissodendoryx sp.* From 1 metric tonne of sponge, only 300 mg of product was obtained. It's estimated that 1 to 5 Kg of halichrondrin B per year would be required if it successfully became a drug. This

would require the unsustainable harvest of 3,000 to 16,000 metric tons of sponge per year.[55] NPs from bacteria however, do not have this limitation since they can be cultured and manipulated in bioreactors to provide a sustainable supply of NPs.[62] This makes microbial NPs much more attractive as a renewable source of therapeutic compounds.

1.3.4. Natural Products Discovered from Marine Bacteria

There have been many NPs from bacterial sources discovered. Listed in **Table 1.3** are some of the many different compounds produced by bacteria, or believed to have bacterial origins, that have biological activity. Listed are their names, class of compounds, biological activity, and microbial source. Structures are located below in **Figure 1.7**.

Table 1.3. Selected Bioactive NP or NP-derived Compounds from Marine Bacteria

Name and Reference	Class	Activity	Bacteria
Tasidotin[71]	Peptide	Anticancer	*Symploca hydnoides*
Soblidotin[71]	Peptide	Anticancer	*Symploca hydnoides*
Bryostatin 1[71]	Polyketide	Anticancer	*Candidatus endobugula sertula*
Manzamine A[71]	Alkaloid	Antimalarial	*Micromonospora sp.*
Thiocoralin[60]	Peptide	Anticancer	*Micromonospora sp.*
Resistoflavine[63]	Polyketide	Anticancer	*Streptomyces chibaensis*
Marinomycin A[63]	Polyketide	Anticancer	*Marinispora sp.*
MC21-B[63]	Brominated Aromatic	Antibacterial	*Pseudoalteromonas phenolica*
Curacin A[63]	Polyketide	Antifungal	*Lyngbya majuscula*
BE-43472B[63]	Polyketide	Antibacterial	*Streptomyces sp.*
Abyssomycin C[60]	Polyketide	Antibacterial	*Verrucosispora sp.*
Platensimycin[66]	Terpene	Antibacterial	*Streptomyces platensis*
Lynamicin A[66]	Alkaloid	Antibacterial	*Marinispora sp.*

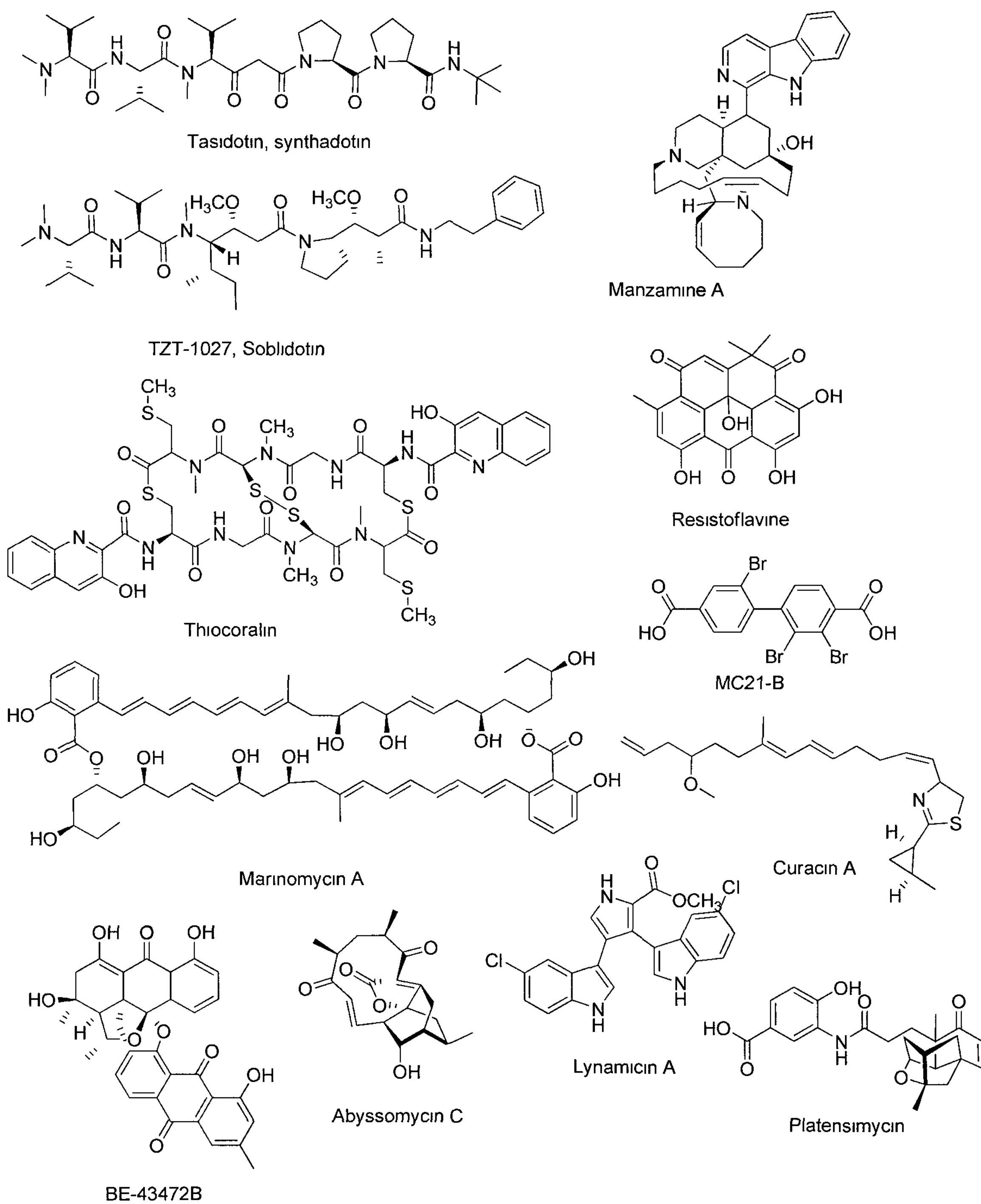


Figure 1.7. Bioactive Bacterial Natural Products

Discussed below is a very promising compound in clinical trials that was isolated from marine bacteria. Salinosporamide A displays potent activity which has generated excitement in the scientific community. It continues to show good prospects for becoming a drug.

Salinosporamide A (Marizomib)

Salinosporamide A (**Figure 1.8**) was first reported in 2003 by Fenical *et al.*[72] It was isolated from *Salinispora tropica* from Chub Cay, Bahamas. It showed potent activity against the HCT-116 cell line with an IC_{50} of 11 ng/mL. The NCI 60 cell line panel indicated that it was a highly selective tumour cell growth inhibitor with 4 log difference for the median lethal concentration (LC_{50}) between resistant and susceptible cell lines. The average median growth inhibition (GI_{50}) for the 60 cell line panel was <10 nM with NCI-H226 non-small cell lung cancer, MDA-MB-435 breast cancer, SF-539 CNS cancer and SK-MEL-28 melanoma all having LC_{50} lower than 10 nM. Salinosporamide A has a structure very similar to omuralide (**Figure 1.8**) which is a well known selective proteolytic activity inhibitor of the 20S proteasome. The 20S proteasome is responsible for the degradation of most non-lysosomal proteins which is necessary for cell survival. Testing salinosporamide A against purified 20S proteasome resulted in an IC_{50} of 1.3 nM, which was 35 times more potent than that of omuralide which is considered the gold standard for 20S proteasome inhibition.[72, 73]

These impressive results resulted in the rapid licensing of Salinosporamide A to Nereus Pharmaceuticals in 2001 and accelerated preclinical development in 2003. It was only four years from its discovery that it entered clinical trials which is a very rare feat.[7]

The first phase 1 clinical trials started against advanced solid tumors or lymphomas in 2006 followed by multiple myeloma in 2007.[73] Phase 1b clinical trials in combination with vorinostat (Zolinza, Merck & Co.) against non-small cell lung cancer, pancreatic cancer, melanoma or lymphoma started in 2008.[47]

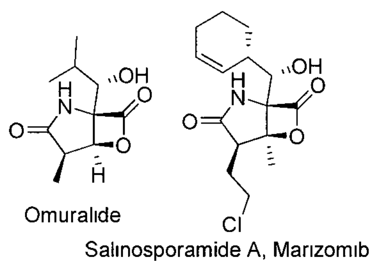


Figure 1.8. Omuralide and Salinosporamide A

1.4. Microbial Extremophiles

1.4.1. The Different Types of Extremophiles

The name extremophile originates from the Greek words *extremus* (extreme) and *philus* (lover). Examples are thermophile (heat-lover) and psychrophile (cold-lover). Bacterial extremophiles are characterized as bacteria that display optimum growth outside of traditional life supporting conditions.[74] There are many different types of extremophiles due to the many different possible conditions that bacteria can grow in as observed in **Table 1.4.**

Table 1.4. Classification of Selected Extremophiles[74, 75]

Environmental Factor	Classification	Growth Range or Definition
Temperature	Hyperthermophile	>80 °C
	Thermophile	60-80 °C
	Psychrophile	<20 °C
Pressure	Piezophile (Barophile)	>35 MPa
pH	Alkaliphile	pH > 9
	Acidophile	pH < 4
Salinity	Halophile	> 3% NaCl

Some bacteria are extremophiles in more than one category resulting in polyextremopiles such as *Shewanella violacea*, a deep sea piezopsychrophile which was isolated from a depth of 5,110 m in Ryukyu Trench.[76] Another example is *Aquifex pyrophilus* which is a thermohalophile that grows optimally at 85 °C and 3% NaCl. It was isolated from a hydrothermal vent system north of Iceland. Not all bacteria that grow in harsh conditions will flourish in them. Those organisms that grow optimally at conditions more moderate but can grow in extreme conditions are named extreme-tolerant.[74] The next sections will be focusing on cold-adapted bacteria that are classified as psychrophilic and psychrotolerant.

1.4.2. Cold-Adapted Bacteria

Psychrophilic bacteria are characterized as having a maximum growth temperature at or below 20 °C with an optimal growth temperature at or below 15 °C and a minimal growth temperature of 0 °C or lower. There is an ongoing debate about the temperature ranges that differentiates psychrophiles from psythrotolerant (psychrotrophic) bacteria however, the definition most accepted is the one above proposed by Morita, R.Y in 1975. Psychrotolerant bacteria are characterized as having a maximum growth temperature above 20 °C with an optimum growth temperature above 15 °C and a minimal temperature at or near 0 °C.[77] In comparison, mesophiles have an optimum growth temperature of 30-40 °C with a minimal growth temperature from 5-10 °C and a maximum at about 47 °C.[78]

Not all bacteria fit nicely into the definitions assigned since some bacteria have an optimum growth temperature above 15 °C but a maximum growth temperature below 20 °C. Also, bacteria may have an optimum growth temperature below 15 °C but an maximum temperature above 20 °C.[79] Bacteria with a very limited growth range may also fall outside the definitions. Bacteria with a growth range from 10 °C to 20 °C have been found and do not fit into any existing group.[80] The maximum temperature of many bacteria isolated from the Arctic had maximum temperatures under 25 °C but above 20 °C. It has been suggested that another group be defined which have a maximum temperature at 25 °C as "moderately psychrophilic".[81]

1.4.3. Advantages of Cold-Adapted Bacteria

Most of the Biosphere is Cold

The earth's biosphere is predominantly cold with about 70% of the earth's surface covered by oceans with more than 90% of the volume of the oceans being 5 °C or lower.[77] About 95% of the surface area of the oceans are deeper than 1000 m[76] and after a depth of 1000 m, the water temperature is about 4 °C.[82] At a depth of 2000 m or greater, temperatures drop to about 2 °C.[76] See **Figure 1.9** for the general seawater temperature-depth trend at low, mid and high latitudes.[83]

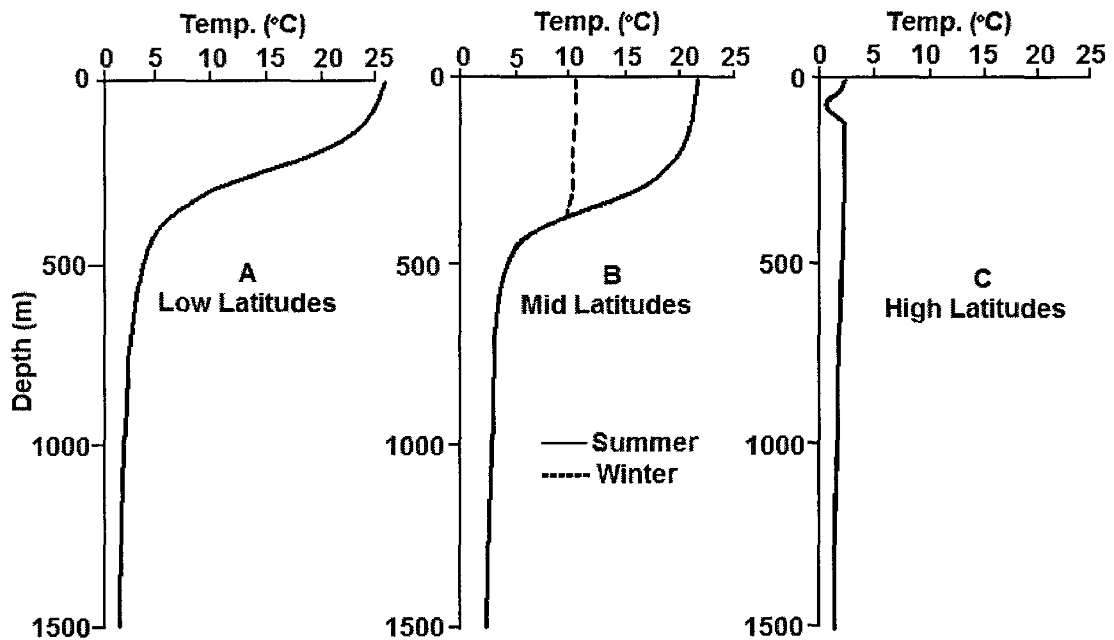


Figure 1.9. Seawater Temperature Profiles at Different Depths in Low, Medium and High Latitudes[83]

Sea ice is another major potential source of psychrophilic bacteria. It covers up to 13% of the earth's surface with the largest expanse being 20 million km^2, and has a thickness of about 1 m. Sea ice can get as cold as -20 °C, necessitating good cold

adaptation by bacteria in order to survive.[84] The ocean bottoms are also covered with a layer of sediment which may be up to 1 km deep.[85] Bacteria have been isolated from sediment samples deeper than 500 m at five Pacific Ocean sites and the rate of decline suggests that viable bacteria may be found much deeper. This significantly extends the perceived marine biosphere.[86]

Cold-Adapted Enzymes

Most research on cold-adapted bacteria have centered on their use as sources of cold-adapted enzymes, which can be used for many purposes.[85] They have possible applications for every process which uses enzymes or microbes. Cold-adapted enzymes have several advantages over mesophilic enzymes. They are more active at lower temperatures which allows for the reduction in energy consumption used for heating. Examples of applications are laundry, synthesis, and leather production. Cold-adapted enzymes can be used for the transformation of heat labile products such as milk, juice, synthetic products and molecular biological products. They can also be heat inactivated to stop their activity. Examples are in baking, molecular biology and food preservation. Cold-adapted enzymes may also be used for processes where increased activity at low temperatures is desired, such as for bioremediation, baking and waste treatment. They have also found applications for their use in controlling ice crystal formation which has subsequently been applied to the manufacturing of ice cream.[87]

1.4.4. Relative Abundance of Cold-Adapted Bacteria

There has not been a complete consensus on the relative abundance of psychrophilic bacteria to psychrotolerant bacteria in consistently cold habitats or even in areas with large seasonal temperature variations. It's been reported that psychrophilic bacteria are very abundant and outnumber psychrotolerant bacteria in consistently cold habitats[77, 88-90] and it has also been reported that they are not abundant in nature.[79, 91, 92] Explanations for low isolation numbers of psychrophilic bacteria have been focused on source location, such as sampling from areas where there is significant seasonal temporal variation. Also, poor sample handling resulting in sample and isolate exposure to elevated temperatures have also been cited as causes of poor yield in psychrophilic bacteria isolation.[77, 89] It has been argued that psychrotolerant bacteria are "ecophysiologically resilient and nutritionally versatile"[88] and that rigorous temperature-controlled isolations have supported the predominance of psychrotolerant bacteria.[79]

Psychrotolerant bacteria are believed to be predominant in areas with seasonal or diurnal thermal fluctuations[93] but psychrophilic bacteria may be the dominant population present.[81] It has been shown that in sea ice, psychrophiles become more dominant the older the ice is whereas in new ice and underlying seawater, psychrotolerant bacteria dominate. This suggests that given time, psychrophilic bacteria will outcompete psychrotolerant bacteria when there is consistent low temperatures and stable nutrient sources.[94] It has been shown that psychrophilic bacteria can grow faster than their psychrotolerant counterparts below 10 °C[77, 95] however, it is said that some psychrotolerant bacteria grow as well or better at low temperatures than their psychrophilic counterparts.[79, 88]

1.4.5. Natural Products Discovered from Cold-Adapted Bacteria

There has been little NPs research completed on cold-adapted bacteria.[96] Even with a continent and large areas of coastline in polar regions, less than 3% of reported marine NPs have been reported from organisms collected from these regions.[97] Also, NP isolation can be difficult from cold-adapted bacteria. It has been observed that for some microorganisms, antimicrobial production was only achieved at 8 °C and not at 20 °C, indicating that low temperature incubation was required for antimicrobial production.[98] This would not be an obvious temperature to conduct fermentations for psychrotolerant bacteria since the optimum growth temperatures are above 15 °C.

Some examples of NPs from cold-adapted bacteria are listed in **Table 1.5** with their chemical structures shown in **Figure 1.10**. Determining which NPs are from cold-adapted bacteria can be difficult due to the lack of bacterial characterization performed by NP chemists. Many bacteria that produce NPs are not classified according to their growth temperature and some are not even identified. The following examples are of NP production from marine bacteria isolated from cold waters and/or deep locations. If the source did not explicitly state they were cold-adapted bacteria, the culture conditions were used to rule them out as psychrophilic bacteria.

Table 1.5. Natural Products from Cold-Adapted Bacteria

Selected NP and Reference	Class	Bacteria	Bacterial Type
Cyclo-(D-pipecolinyl-L-Isoleucine)[99]	Alkaloid	*Pseudoalteromonas haloplanktis*	Psychrophilic
Macrolactin A[100]	Polyketide	Unknown	Psychrotolerant
γ-indomycinone[101]	Polyketide	*Streptomyces sp.*	Psychrotolerant
Cyclo-(L-proline-L-methionine)[74]	Alkaloid	*Pseudomonas aeruginosa*	Psychrotolerant
Mixirin A[74]	Peptide	*Bacillus sp.*	Psychrotolerant
Helquinoline[74]	Alkaloid	*Jannibacter limosus*	Psychrotolerant
Glyciapyrrole A[74]	Alkaloid	*Streptomyces sp.*	Psychrotolerant
Caprolactin A[102]	Alkaloid	Unknown	Psychrotolerant
Bisdehydro-B-carotene-2-carboxylic acid[103]	Terpene	*Micrococcus roseus*	Psychrophilic
Lanost-8(9)-en-3β-ol[104]	Terpene	*Methylosphaera hansonii*	Psychrophilic
9Z,9'Z-bacterioruberin[105]	Terpene	*Arthrobacter agilis*	Psychrotolerant
Violacein[106]	Alkaloid	*Janthinobacterium sp.*	Psychrotolerant
2-phenylethylamine[107]	Alkaloid	*Psychroflexus torquis*	Psychrophilic

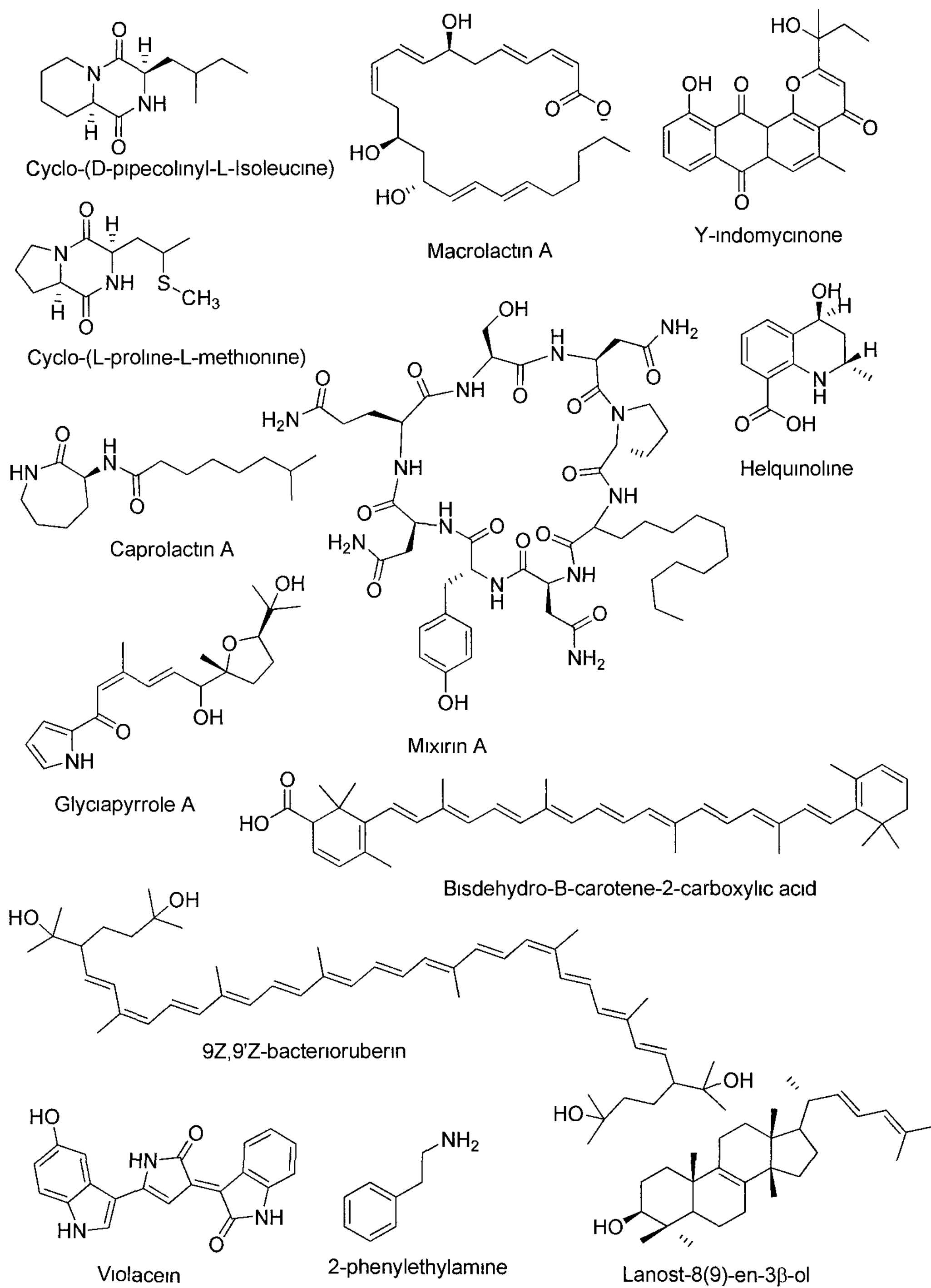


Figure 1.10. Structures of Natural Products from Cold-Adapted Bacteria

1.5. Lipids

1.5.1. A New Definition for Lipids

Lipids have been generally defined as "natural products which may be isolated from biological materials by extraction with organic solvents and which are usually insoluble in water".[108] However this has been considered a very broad and loose definition and does not accurately describe the compounds. In 2005, a new classification system was proposed that considers their structural and biosynthetic features, is lipidomics compatible and has a precise definition of lipids.[109] The new definition of lipids is "hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units".[109, 110] This new classification system was originally targeted toward mammalian lipids but international demand has broadened it to include all lipids and has now been internationally accepted as the classification system of lipids.[110] The classification system is currently being used on LIPID MAPS[111] and will be used as a guide for the introduction of lipids in this section. Due to the recent development of this classification system, new classes and subclasses are continuously being developed resulting in the evolution of the system over time.

There are eight main classes of lipids. These classes are fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides.[109, 110] An example of a compound in each class is given in **Figure 1.11**. Fatty acyls are the simplest lipids and are composed of different types of long alkyl chain molecules. They are separated into 14 different subclasses. Glycerolipids are all glycerol

containing lipids except for phosphate containing lipids which are a separate class due to their abundance.

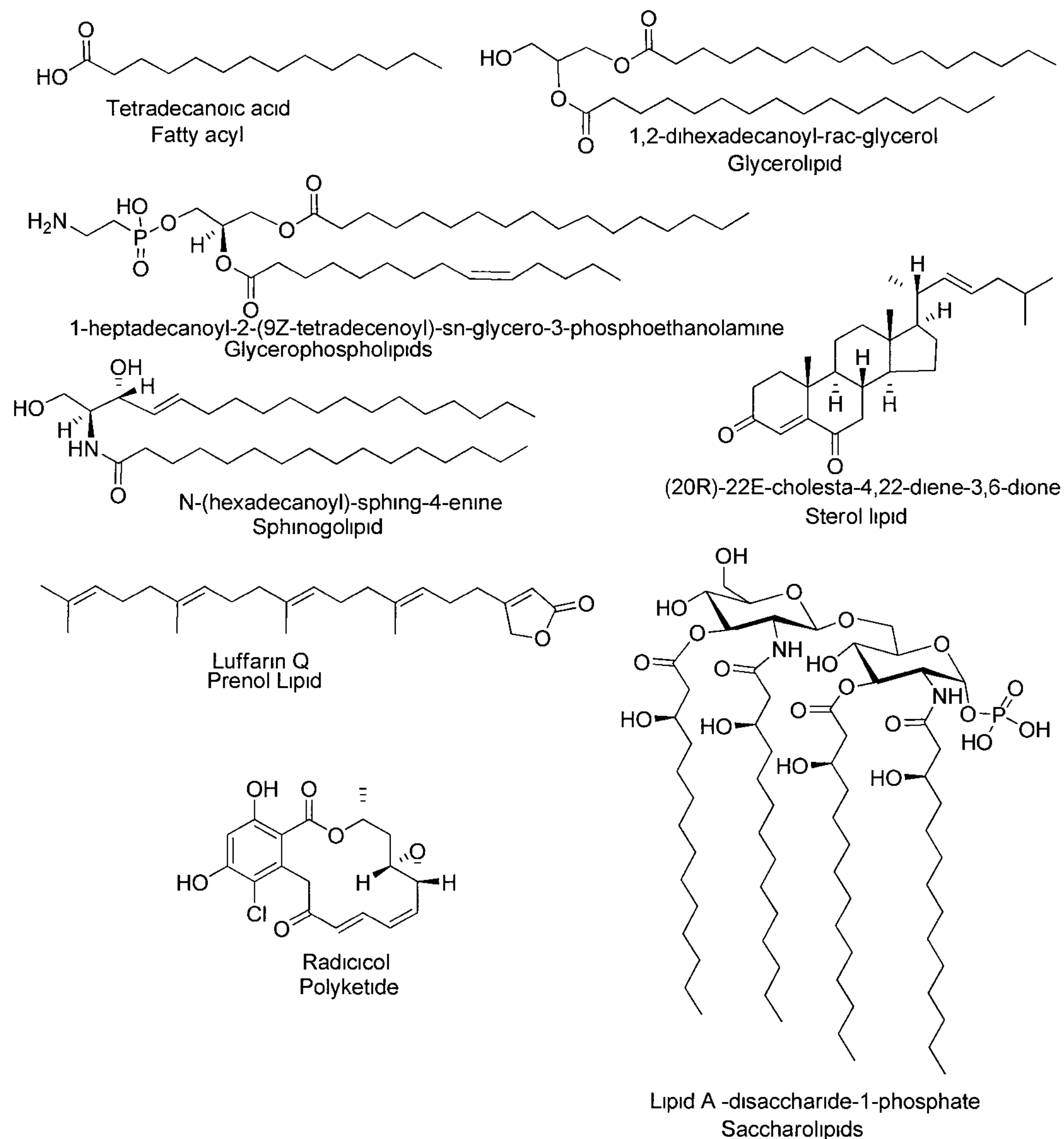


Figure 1.11. Eight Classes of Lipids

Glycerophospholipids are glycerol and phosphate containing lipids which are separated into 21 different subclasses. They are primarily separated by the different head

groups attached to the phosphate. Sphingolipids are lipids that contain a sphingoid backbone which is a serine molecule fused to a long alkyl chain. Spingolipids are separated into ten different subclasses. Sterol lipids are cholesterol-like compounds. They are separated into six different subclasses. Prenol lipids are terpenoid compounds that are composed of isopentenyl diphosphate and dimethylallyl diphosphate. They are separated into five different subclasses. Saccharolipids are sugars connected directly with a fatty acid containing compounds. They are separated into six different subclasses. The last major class is polyketides which are synthesized by polyketide synthases. They are separated into 15 different subclasses.[109] We will focus further on glycerolipids and describe the different subclasses.

1.5.2. Glycerolipids

Glycerolipids are all lipids which contain glycerol except for phosphate containing lipids. Glycerolipids are separated into six different subclasses of which are monoradylglycerols, diradylglycerols, triradylglycerols, glycosylmonoradylglycerols, glycosyldiradylglycerols and other glycerolipids (**Figure 1.12**). Monoradylglycerols, diradylglycerols and triradylglycerols are glycerol containing lipids which contain monosubstituted, disubsitituted and trisubstituted glycerols respectively. Glycosylmonoradylglycerols and glycosyldiradylglycerols are glycerol containing lipids which are glycosylated on the glycerol and contain one and two additional substitutions respectively on the glycerol. Other glycerolipids is a subclass which contains glycerolipids which contain different head groups or very unusual alkyl groups which do

not fit into any of the previous subclasses.[109, 110] The following section will focus only on glycosylmonoradylglycerols and glycosyldiradylglycerols.

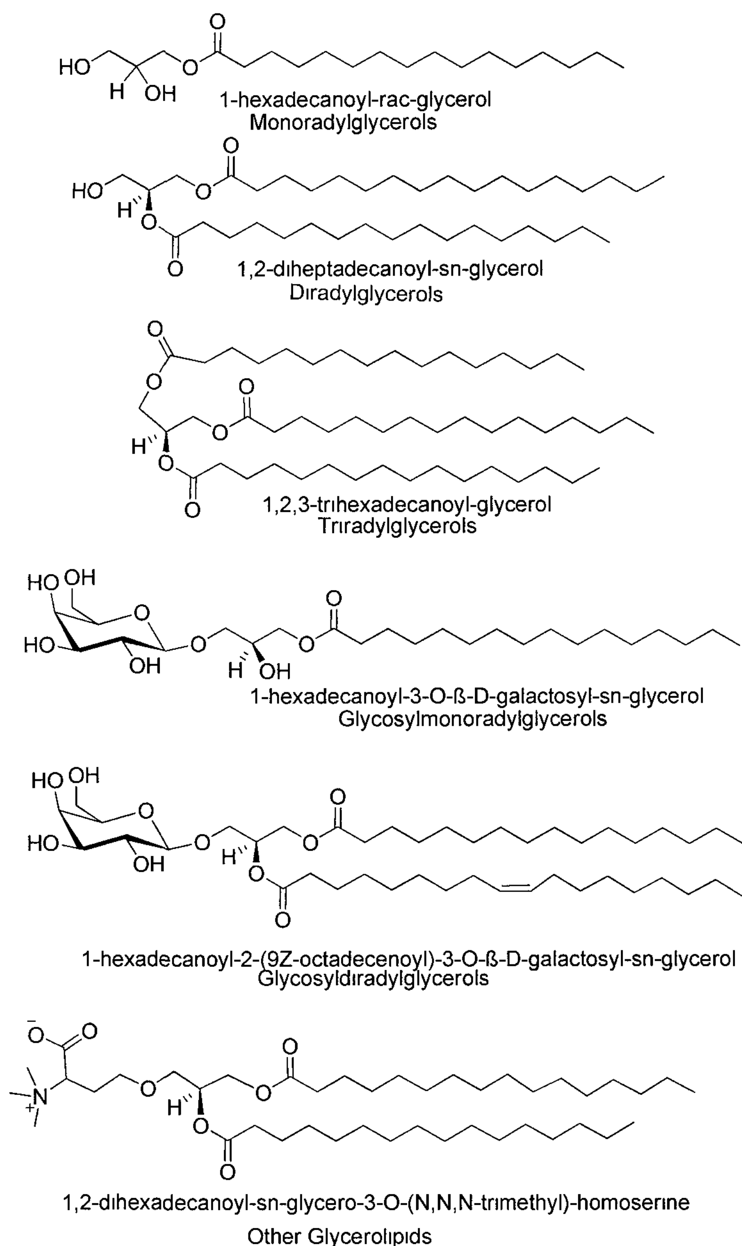


Figure 1.12. Six Classes of Glycerolipids

1.5.3. Glycosylmonoradylglycerols and Glycosyldiradylglycerols

Glycosylmonoradylglycerols are glycosylated glycerolipids with a substitution on only one of the two locations on the glycerol. They are divided into two subclasses, glycosylmonoacylglycerols and glycosylmonoalkylglycerols (**Figure 1.13**). Glycosylmonoacylglycerols have a fatty acid attached to one of the locations on the glycerol and glycosylmonoalkylglycerols have an ether linkage with a hydrocarbon chain.[109, 110]

Glycosyldiradylglycerols are glycosylated glycerolipids which are substituted on both remaining positions on the glycerol. There are three different subclasses which are glycosyldiacylglycerols, glycosylalkylacylglycerols and glycosyldiakylglycerols (**Figure 1.13**). Glycosyldiacylglycerols, glycosylalkylacylglycerols and glycosyldiakylglycerols have two fatty acids, one fatty acid and one ether linked hydrocarbon chain, and two ether linked hydrocarbon chains respectively attached to the glycerol in either of the two remaining positions.[109, 110]

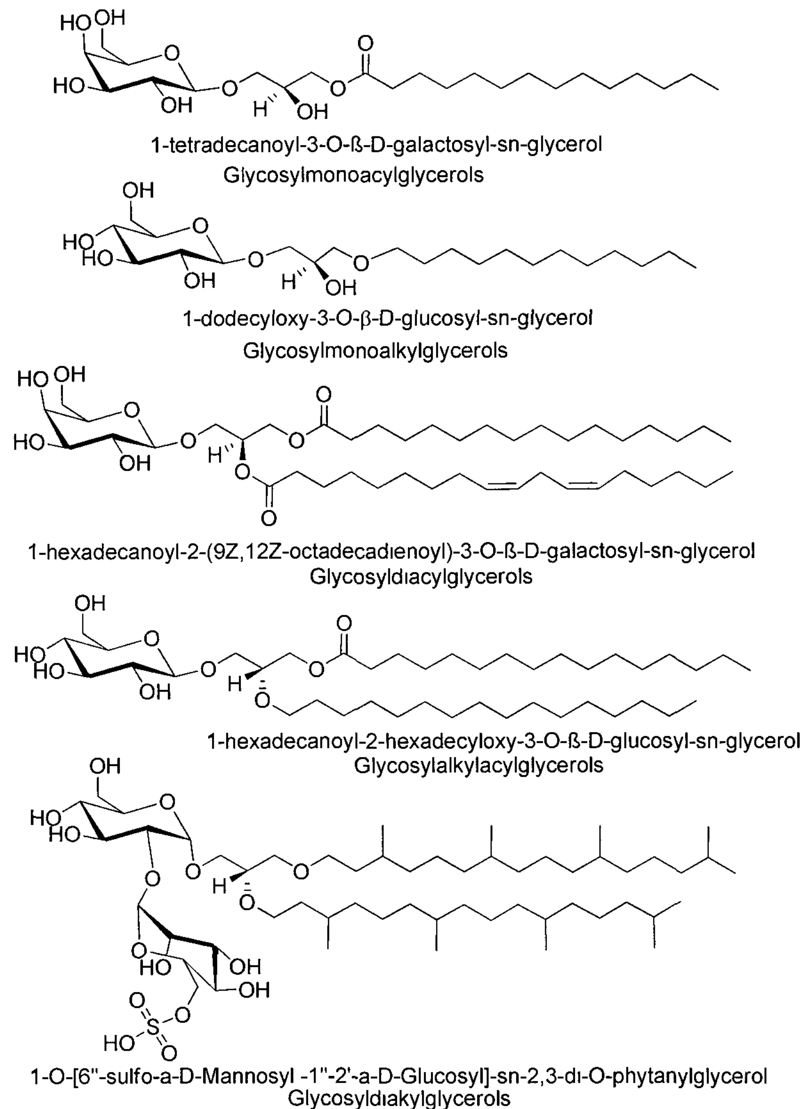


Figure 1.13. Classes of Glycosylmonoradylglycerols and Glycosyldiradylglycerols

1.5.4. Glycoglycerolipids

There are many different sugars which compose glycoglycerolipids (GGLs). Sugars observed in GGLs in plants and bacteria include galactose, glucose, mannose, α-glucuronic acid and sulfoquinovose. The carbohydrate portion of GGLs can be composed of one or more sugars. Disaccharides, trisaccharides or polysaccharides may contain the same sugar repeating or they may contain different sugars[112] and the sugars can have an

alpha anomeric configuration or beta. Sugars may also have different substitution locations. For example, a disaccharide may be linked with a 1-2 glycosidic linkage or by a 1-3, 1-4 or 1-6 glycosidic linkage.[112, 113] The sugars can also be substituted with different acyl functional groups in any of the previously mentioned locations. This results in great structural complexity of GGLs for the carbohydrate region alone.

GGLs have two different locations on the glycerol which may be substituted. The fatty acids may be located at the *sn*1, *sn*2 or *sn*3 position depending on the location of the sugar unit. The *sn*2 location on the glycerol has two different enantiomers. This means that the fatty acid location needs to be determined and the stereochemistry of the *sn*2 position needs be determined in order to obtain the relative and absolute configuration of a GGL.[109, 110]

There are many different fatty acids which can substitute GGLs. There are currently 17 different classes of fatty acids however, the most common fatty acids fall into three classes, straight fatty acids, branched fatty acids and unsaturated fatty acids. Straight fatty acids contain a single unbranched saturated alkyl chain. Unsaturated fatty acids contain double or triple bonds on an unbranched alkyl chain. There can be any number of unsaturations and the double bonds can have two different configurations, cis or trans. Branched fatty acids contain an alkyl chain that is substituted by at least one or more methyl groups. A methyl group on the second last carbon is called an *iso* fatty acid and a methyl group on the third last carbon is called an *ante-iso* fatty acid of which we will be focusing on later.[109, 110]

There are many different forms that GGLs can take. Nature makes a large variety of different analogues and synthesis allows the development of many more novel and

unusual GGLs. The following section will only discuss diacyl-3-[α-D-mannopyranosyl-(1-3)-α-D-mannopyranosyl]-sn-glycerols.

1.5.5. Dimannosylglycerolipids

Biological Sources of Dimannosylglycerolipids

Dimannosylglycerolipids have only been reported from gram-positive bacteria with high G+C (guanosine + cytosine) content.[114] Complete characterization was rarely completed on dimannoglycerolipids in the past because of the difficulty in structural elucidation and also because most work completed was for taxonomic purposes, which aimed for rapid lipid characterization or the rapid characterization of different types of lipids. Characterization was generally not completed for many glycolipids. The fatty acid attachment locations on the carbohydrate core were not always determined. Also, the locations of individual fatty acids were not determined. It seems that it was generally assumed that the fatty acids would be both located on the glycerol. The list of microorganisms from which mannoglycerolipids have been isolated but do not have fatty acid locations determined include *Micrococcus lysodeikticus,*[115] *Arthrobacter globiformis,*[116, 117] *A. pascens and A. crystallopoietes,*[117] *Corynebacterium aquaticum,*[118] *Microbacterium lacticum,*[119] and *M. thermosphactam.*[120]

Dimannosylglycerolipids may also have fatty acids located on the sugars. Dimannosylglycerolipids with a fatty acid attached to the primary carbon of the glycerol and the sixth carbon of the sugar adjacent to the glycerol have been discovered from a number of different microorganisms. From the following microorganisms have 1-O-acyl-3-[α-D-mannopyranosyl-(1–3)-(6-O-acyl-α-D-mannopyranosyl)]-*sn*-glycerol **(Figure**

1.14) been isolated: *Micrococcus luteus*[121] (formerly *Micrococcus lysodeikticus*), *A. globiformis* and *A. scleromae,*[122] *A. atrocyaneus,*[114] *Leifsonia aquatic* (formerly *corynebacterium aquaticum*[123]), *Nocardiopsis dassonvillei*, *Rothia dentocariosa,*[124] *Saccharopolyspora sp, S. hirsuta, S. rectivirgula, S. erythraea.*[125]

Structure Elucidation of GGL Analogues

A lot of GGL research involves the hydrolysis of the fatty acids and their characterization and relative quantitation by gas chromatography-mass spectrometry (GC-MS). A sugar back bone is characterized separately so fatty acid location information is lost. Some perform LC-MS analysis of the mixture of intact GGLs however, information of the locations of individual fatty acids are not always obtained. Sometimes information on fatty acid locations are determined but information regarding the location of different individual fatty acids are not determined. This results in at least two different possible structures for the GGL.[122] Below is a table of GGL analogues where the location of the fatty acids has been determined as well as the type and length of the fatty acids for each location **(Table 1.6**).

By fast atom bombardment-mass spectrometry (FAB-MS/MS), GGL 887, 901, 913, 913, 915, 929 and 943 were characterized after the complete characterization of GGL 915 by NMR and FAB-MS/MS. However, the double bond locations for both 913 GGLs could not be determined unambiguously. Stereochemistry of the glycerol and fatty acids were not determined.[114] GGL 915 was characterized by NMR and high resolution fast atom bombardment mass spectrometry (HRFAB-MS). The fatty acids and sugars were determined by GC-MS. The fatty acid locations on the sugar backbone were

determined by selective enzymatic hydrolysis. The stereochemistry of the glycerol was determined to be *R* by using the coupling constants and chemical shifts of the glycerol protons.[123] The *ante-iso* fatty acids were determined to have the *S*-configuration by HPLC.[126] A GGL 887 which had different fatty acids was reported. It was characterized by NMR and HR-MS. Acetylation was performed to determine the fatty acid locations and selective enzymatic hydrolysis was performed to determine absolute fatty acid locations. GC-MS was performed to determine the fatty acids. The stereochemistry of the glycerol was determined to be R by using the coupling constants and chemical shifts of the glycerol protons.[121] GGL 885 has not previously been reported.

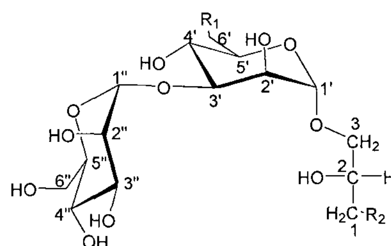


Figure 1.14. 1-O-acyl-3-[α-D-mannopyranosyl-(1–3)-(6-O-acyl-α-D-mannopyranosyl)]-sn-glycerol

Table 1.6. 1-O-acyl-3-[α-D-mannopyranosyl-(1–3)-(6-O-acyl-α-D-mannopyranosyl)]-sn-glycerol analogues

Analogue and Reference	$[M+Na]^+$	[M]	R1COOH	R2COOH
1(Current Thesis)	885	862	C15:1ai	C15:0ai
2(Current Thesis)	885	862	C15:0ai	C15:1ai
3[114]	887	864	C15:0ai	C15:0ai
4[121]	887	864	C15:0	C15:0i
5[114]	901	878	C16:0	C15:0ai
6[114]	913	890	$C17:1^C$	C15:0ai
7[114]	913	890	C17:0ai	$C15:1^C$
8[123]	915	892	C17:0ai	C15:0ai
9[114]	929	906	C17:0ai	C16:0
10[114]	943	920	C17:0ai	C17:0ai

ai: anteiso, i: iso, otherwise straight fatty acid; C: double bond location not determined

Research Applications and Activity of GGLs

There are several different important research applications that 1-O-acyl-3-[α-*D*-mannopyranosyl-(1–3)-(6-O-acyl-α-*D*-mannopyranosyl)]-*sn*-glycerols may provide. One of the most frequently reported is that of its potential use as a taxonomic biomarker. The first attempt for bacterial classification by lipid composition was reported in 1963 by Abel, K. et *al.* and was shown to be an effective tool for bacterial classification and taxonomy.[108] With the use of 16S rDNA for bacterial identification, much less emphasis is on bacterial lipids as a classification tool however, it has been used effectively to differentiate closely related bacteria.[127] Bacterial lipid composition does need to be characterized along with many other bacterial properties when describing a new bacterial species.[128, 129]

Arthrobacter bacteria can be difficult to identify by conventional methods. They have biotechnological potential for bioremediation due to their catabolising activity[114] but they have also been found to be opportunistic pathogens.[122] *Rothia dentocariosa* is an opportunistic bacterium that has the same type of GGLs.[124] It was previously classified as *Actinomyces dentocariosus*, then *Nocardia dentocariosa* and then *Nocardia salivae* before being classified as *R. dentocariosal.* The exclusion of *R. dentocariosal* from *Actinomyces* and *Nocardia* was based on its "L-Lys-L-Ala_3 type cell wall, a fatty acid profile composed of iso-, anteiso-, and normal fatty acids, a major glycolipid characterized previously as the dimannosyl diglyceride and a lipomannan identified recently".[124] *Saccharopolyspora* is a bacterial genus which contains the same mannolipids and is implicated as the cause of Farmer's lung, a respiratory illness. The mannolipids

were shown to have potential as a diagnostic and taxonomic tool for the identification of Farmer's lung.[125]

Mannolipids such as 1-O-Acyl-3-[α-*D*-mannopyranosyl-(1–3)-(6-O-acyl-α-*D*-mannopyranosyl)]-*sn*-glycerols have been screened in many different assays, many of which may not have been reported. Specific mannolipids have been reported to display no antibacterial activity and no anticancer activity.[114] Influenza-virus binding activity has been reported for specific mannolipids due to their ability to mimic sialic acid which is the receptor that hemagglutinin on the influenza virus binds.[130, 131] Specific mannolipids have been shown to be competitive to the protein antigens prepared for the specific diagnosis of Farmer's lung. They have been shown to have immunoregulatory activity in interferon synthesis and tumor necrosis factor, the later which has been shown to be important for hypersensitivity pneumonitis causing *Saccharopolyspora rectivirgula* in mice.[125]

1.6. Research Goals

The research goal of this thesis was to isolated NPs from psychrophilic or psychrotolerant bacteria from Malpeque Bay, Prince Edward Island (P.E.I). There has been very little research into NPs isolation from cold-adapted bacteria and therefore is a vast untapped source of potential novel NPs. As well, there has been no NPs research completed on bacteria from Malpeque Bay. Malpeque Bay was chosen because it is an unusual body of water in that it exhibits a large seasonal temperature swing (**Figure 1.15**). Given this unusual physical environment, it is very likely that there will be new and interesting biological diversity and a potential source of psychrophilic and psychrotolerant bacteria. The research project looked at the biological diversity from Malpeque Bay in regards to cold-adapted bacteria. Fermentation extracts of selected bacteria were screened for production of NPs. Structural characterization was completed by NMR, MS and IR. Biological activity of the compounds was determined using assays targeting antimicrobial, anticancer and antiviral activity.

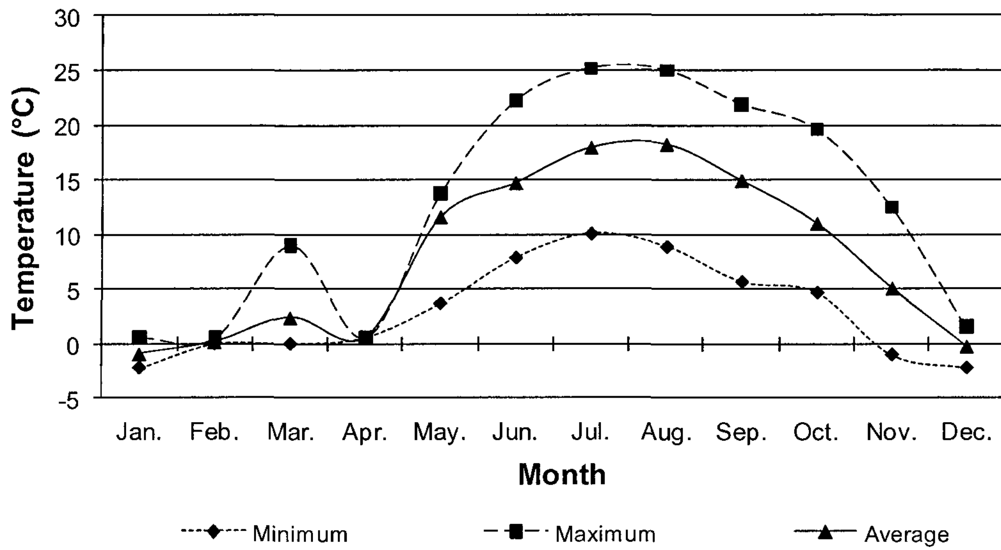


Figure 1.15. Water Temperatures for Water less than 12 m in Depth from Malpeque Bay, P.E.I[132]

CHAPTER 2-MATERIALS AND METHODS

2.1. Preparation of Media

Media reagents and microbiology supplies were purchased from Sigma-Aldrich, Fisher Scientific or VWR unless otherwise stated. Media were prepared using Milli-Q water obtained from a Millipore Biocel System. The pH of the media was adjusted using 5 M NaOH and/or 5 M HCl unless from a premix or otherwise stated. Media were autoclaved at 122 °C for 20 minutes at 16 psi.

2.1.1. Preparation of Bacterial Isolation Media

EMD Nutrient Agar (NA) was prepared from a premix as directed by the manufacturer. EMD Nutrient Broth (NB) was prepared from a premix as directed by the manufacturer. 1/10 Dilute Nutrient Broth (DNB) was prepared by preparing NB at 1/10 concentration. 1/10 Dilute Nutrient Agar (DNA) was prepared by adding 16.0 g/L agar to DNB. Difco Marine Agar 2216 (MA) was prepared from a premix as directed by the manufacturer. Difco Marine Broth 2216 (MB) was prepared from a premix as directed by the manufacturer. 1/10 Dilute Marine Broth (DMA) was produced by adding 16 g/L Agar to 1/10 diluted MB.

2.1.2. Preparation of Fermentation Media

Ampicillin Cycloheximide Chloramphenicol (ACC) media[133, 134] consisted of:

Protease Peptone # 3	20.0 g
Glycerol	1.5 g
Potassium Sulfate	1.5 g
Magnesium Sulfate Heptahydrate	1.5 g
Instant Ocean (IO)	18.0 g

Water	1000 mL
pH	7.2 ± 0.2

The pH was adjusted to 7.5 for the 96-well plate, 24-well plate and 50 mL fermentations. The pH was adjusted to 7.2 for the scaled up fermentations of 1 L or more.

Ancylobacter Spirosoma Medium[135] consisted of:

D-(+)-Glucose	1.0 g
Protease Peptone #3	1.0 g
Yeast Extract	1.0 g
Instant Ocean	18.0 g
Water	1000 mL
pH	7.0 ± 0.2

Benzoate Minimal Salts Medium[133] consisted of:

Magnesium Sulfate Heptahydrate	0.2 g
Dipotassium Phosphate	10.0 g
Sodium Ammonium Phosphate Tetrahydrate	3.5 g
Citric Acid	0.2 g
Instant Ocean	18.0 g
Sodium Benzoate	2.5 g
Water to	950 mL
pH	7.0 ± 0.2

Sodium Benzoate (2.5g/50 mL) was filter sterilized and added aseptically to the sterile media.

Cyclohexanone Medium[133] consisted of:

Magnesium Sulfate Heptahydrate	0.2 g
Dipotassium Phosphate	0.25 g
Ammonium Nitrate	3.0 g
Calcium Chloride Dihydrate	0.01 g
Iron (III) Chloride Hexahydrate	1.0 mg
Instant Ocean	18.0 g
Cyclohexanone	1 mL
Water to	999 mL

Cyclohexanone was filter sterilized and added aseptically.

Sigma-Aldrich Czapek Dox Broth was prepared from a premix as directed by the manufacturer and was supplemented with IO. The media was dissolved by heating until near boiling.

Emerson Yeast Protein Soluble Starch (YpSs) Broth was prepared as Emerson YpSs Agar[133] and consisted of:

Soluble Starch	15.0 g
Yeast Extract	4.0 g
Dipotassium Phosphate	1.0 g
Magnesium Sulfate Heptahydrate	0.5 g
Instant Ocean	18.0 g
Water to	1000 mL
pH	7.0 ± 0.2

FF Media contained:

Fish Meal	25.0 g
Yeast Extract	2.0 g
Casimino Acids	2.0 g
Bacto Peptone	2.0 g
Maltose	2.0 g
Instant Ocean	18.0 g
Water to	1000 mL
pH	7.0 ± 0.2

Marine Minimum Media (MMM)[136] consisted of:

Magnesium Sulfate Heptahydrate	7.0 g
Magnesium Chloride Hexahydrate	5.3 g
Potassium Chloride	0.7 g
Calcium Chloride	1.25 g
Tris Base	6.1 g
Instant Ocean	18.0 g
Iron (II) Sulfate Heptahydrate	0.025 g
Copper (II) Sulfate Pentahydrate	5.0 mg
Dipotassium Phosphate	0.075 g
Monosodium Glutamate	2.0 g

Water to	900 mL
pH	7.4 ± 0.2

Monosodium Glutamate (2.0 g/100 mL) was filter sterilized and added to the sterilized media aseptically.

Malt Peptone Yeast Extract (MPY) Broth was prepared as MPY Agar (ATCC Medium 582)[133] and consisted of:

α-D-Lactose	2.0 g
Bacto Protease Peptone	1.0 g
Yeast Extract	1.0 g
Malt Extract	5.0 g
D-(+)-Xylose	2.0 g
D-Fructose	2.0 g
Instant Ocean	18.0 g
Water to	1000 mL
pH	7.0 ± 0.2

Myxobacteria Medium[133] consisted of:

Yeast Extract	0.5 g
Skim Milk Powder	5.0 g
Instant Ocean	18.0 g
Water to	1000 mL

B-Vitamin Solution[137] consisted of:

Thiamine-HCl	50.0 mg
Riboflavin	50.0 mg
Nicotinic Acid	50.0 mg
Pyridoxine-HCl	50.0 mg
D-pantothenic Acid Calcium Salt	50.0 mg
D-*myo*-Inositol	50.0 mg
p-Amino-Benzoic Acid	50.0 mg
D-Biotin	25.0 mg
Water	100 mL

B-Vitamin solution was filter sterilized.

2.1.3. *Actinobacteria* Isolation Media

All of the following media were supplemented after sterilization, aseptically with filter sterilized 15 mg/L Nalidixic Acid in 0.45M NaOH. Also added aseptically was 50 mg/L Cycloheximide in dimethyl sulfoxide (DMSO) and 50 mg/L Nystatin in absolute ethanol.

Humic Acid-Vitamin Agar (HAVA)[137] consisted of:

Agar	18.0 g
Humic Acid in 10mL 0.2 M NaOH	1.0 g
Dipotassium Phosphate	0.5 g
Potassium Chloride	1.7 g
Magnesium Sulfate Heptahydrate	0.05 g
Iron (II) Sulfate Heptahydrate	0.01 g
Calcium Carbonate	0.02 g
B-Vitamin Solution	1 mL
Instant Ocean	18.0 g
Water to	999 mL
pH	7.2 ± 0.2

B-Vitamin Solution was aseptically added after media was autoclaved.

Actinomyces Isolation Agar (AIA)[133] consisted of:

Agar	15.0 g
Sodium Caseinate	2.0 g
DL-Asparagine	0.1 g
Sodium Propionate	4.0 g
Dipotassium Phosphate	0.5 g
Magnesium Sulfate	0.1 g
Iron (II) Sulfate Heptahydrate	1.0 mg
Glycerol	5.0 g
Instant Ocean	18.0 g
Water	1000 mL
pH	8.2 ± 0.2

International Streptomyces Project Medium 3 (ISP 3)[138] consisted of:

Agar	18.0 g
Oatmeal, Rolled Oats	20.0 g
Iron (II) Sulfate Heptahydrate	1.0 mg
Manganese Chloride Tetrahydrate	1.0 mg
Zinc Sulfate Heptahydrate	1.0 mg
Instant Ocean	18.0 g
Water to	1000 mL
pH	7.0 ± 0.2

The oatmeal was boiled for 20 min after pH was adjusted to 7.2. The liquid was filtered through a cheese cloth. The supernatant was diluted to 1L and the other reagents were added.

Water-Yeast Extract (WYE)[139] consisted of:

Agar	18.0 g
Dipotassium Phosphate	0.5 g
Yeast Extract	0.25 g
Instant Ocean	18.0 g
Water	1000 mL

Chitin Agar[133, 140] consisted of:

Agar	20.0 g
Chitin	4.0 g
Magnesium Sulfate	0.5 g
Dipotassium Phosphate	1.2 g
Potassium Phosphate Monobasic	0.3 g
Iron (II) Sulfate Heptahydrate	0.011 g
Manganese Chloride Tetrahydrate	1.0 mg
Zinc Sulfate Heptahydrate	1.0 mg
B-Vitamin Solution	1 mL
Instant Ocean	18.0 g
Water to	999 mL
pH	8.2 ± 0.2

B-Vitamin Solution was added aseptically after media was autoclaved.

EMD International Streptomyces Project Medium 2 (ISP 2) was prepared as a premix as directed by the manufacturer and supplemented with 18.0 g/L IO.

2.1.4. Assay Media

Sigma-Aldrich Dulbecco's Modified Eagle Medium (DMEM) was prepared from a premix as directed by the manufacturer. The media was adjusted using 1M HCl and/or 1M NaOH. The media was filter sterilized.

Sigma-Aldrich Minimal Essential Medium Eagle (MEM) was prepared from a premix as directed by the manufacturer. The media was adjusted using 1M HCl and/or 1M NaOH. The media was filter sterilized.

Difco Mueller-Hinton II Broth, Cation-adjusted (CAMH) was prepared from a premix as directed by the manufacturer.

Difco Sabouraud Dextrose Broth was prepared as directed by the manufacturer from a premix.

2.2. Composition of Buffers and Mixes

6X Loading Dye[141] was composed of:

Bromophenol Blue	0.25 %
Xylene cyanol FF	0.25 %
Glycerol in Water	30 %

50X Tris Acetate Buffer (TAE)[141] was composed of:

Tris Base	242 g
Glacial Acetic acid	57.1 mL
EDTA*, 0.5 M@ pH 8.0	100 mL
Water to	1000 mL

* Ethylenediaminetetraacetic acid

2.3. Bacterial Isolation from Seawater and Sediment Samples

2.3.1. Collection of Sediment Samples from Malpeque Bay, PEI

A sediment sample was collected from Malpeque Bay, PEI in April 2009. The sample was collected in a sterile 50 mL Falcon tube and glycerol was added to a final concentration of 25 %. The sediment sample was frozen at -80 °C for 5 months.

2.3.2. Plating of Sediment Samples onto MA, DMA, NA and DNA

The sediment sample was thawed at room temperature (RT) for approximately 6 hours. Glycerol was removed from the sediment by washing three times with 20 mL of 50 % IO prepared with Milli-Q water. Washing was completed by centrifuging the sediment at 3000 rpm for 5 min and discarding the liquid. Washing was completed to remove the glycerol and seawater bacteria. IO was used in the wash in order to prevent osmotic shock. Three serial dilutions of 1 mL sediment into 9 mL NB yielded 10^{-1}, 10^{-2} and 10^{-3} dilutions which were spread on six MA, DMA, NA and DNA agar plates. One triplicate was incubated at 4 °C and the other at 8 °C for three weeks. All Petri dishes were wrapped in Parafilm to prevent desiccation and possible contamination.

2.3.3. Plating of Sediment Samples onto *Actinobacteria* Specific Media

Two different pre-treatment methods were used for the select isolation of spore forming *Actinobacteria*. An untreated method was also used as a control and in case the pre-treatments killed all isolates. For the untreated method, washed sediment was serially

diluted as in **Section 2.4**; 100 µL from the 10^{-2} and 10^{-3} dilutions were spread in triplicate on five agar media, AIA, HAVA, ISP3, WYE and Chitin agar.

In order to selectively isolate spore forming *Actinobacteria*, a dry heat/stamp method[140] was used to isolate *Actinobacteria*. For this method, 5-9 mL washed sediment was transferred to an empty Petri dish and dried in a laminar flow hood overnight. The dry sediment was transferred to a sterile glass bottle and heated at 100 °C for 1 h. The sediment was transferred to a sterile Petri dish and polyurethane foam plugs (38 mm x 18 mm) were used to serially dilute and stamp sediment onto agar plates. A new foam plug was used for each plate and stamping was performed in six replicates for each of the five media.

A yeast extract/sodium dodecyl sulfate (YE/SDS) treatment method[142] was also used to selectively isolate spore forming *Actinobacteria*. The method consisted of transferring 1 mL of washed sediment to 9 mL of sterile 0.005 M phosphate buffer at pH 7.0 containing 6 % YE and 0.05 % SDS. The 10^{-1} dilution sample was heated at ~36 °C for 20 min and serially diluted in 9 mL NB twice, to yield 10^{-2} and 10^{-3} dilutions, of which 100 µL was spread on the five media in triplicate. All the plates from the two pre-treatments and the untreated were wrapped in Parafilm and were incubated at 8 °C in a New Brunswick Scientific InnovaR shaker incubator.

2.3.4. Preparation of Isolates from Glycerol Stocks

Glycerol stocks of previously isolated bacteria were cultured on NA and incubated at 4 °C. A 10 µL loop was used to transfer frozen sample onto solid media in Petri dishes.

2.3.5. Isolate Purification

Isolates were purified by serially sub-culturing onto solid media until the desired colony was obtained pure.

2.4. Bacterial Molecular Dereplication and Identification

2.4.1. DNA Isolation and its Use as Template for Polymerase Chain Reaction (PCR)

Non sterile pipet tips were sterilized at 122 °C at 16 psi for 20-60 min. PCR reagents were stored on ice when thawed. Barrier pipet tips were used for all PCR work.

To rapidly obtain DNA from bacteria, a modified DMSO cell lysis method was used.[143] This method consisted of the addition of 50 μL sterile DMSO to sterile shallow 96-well plates or PCR tubes and then the addition of isolates to their respective wells by using sterilized tooth picks. 96-well plates were then covered with a sterile 96-well plate aluminum foil lid and stored at -20 °C prior to use.

The boiling method was also used to obtain DNA from bacteria. Isolates were transferred with tooth picks to 50 μL sterile Milli-Q water in a 96-well plate. The plate was then covered with an aluminum foil lid and silicon mat. Following, the plate was heated in a thermal cycler at 98 °C for 3 hours.

PCR reactions of 30 μL were performed to amplify the ribosomal RNA gene which has a sedimentation coefficient of 16 (16S rDNA) for each isolate (**Table 2.1**).

Table 2.1. PCR Master Mix

Reagent	Volume (μL)
Sterile Water, Nuclease Free	10.5
GoTaq Green Master Mix, 2X	15.0
Forward Primer (10 μM)	1.5
Reverse Primer (10 μM)	1.5

Sterile water was autoclaved Milli-Q water or nuclease-free water supplied with the kit, 2X GoTaq Green Master Mix was a polymerase mix from Promega, the forward primer was 16S F27 with a sequence of 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer was 16S R1525 with a sequence of 5'-AAGGAGGTGATCCAGCCGCA-3'. PCR master mixes were prepared by adding the reagents in the indicated order and the total volume was calculated by adding the total number of isolates to be analyzed, a positive control, a negative control, and additional volumes to compensate for pipetting error. The reagents and the PCR master mix were vortexed and pulse centrifuged prior to use. A 28.5 μL volume of PCR master mix was transferred to each well in a sterile PCR tube strip or sterile PCR 96-well plate. 1.5-2.0 μL template from each isolate was added to their respective wells. DMSO or sterile Milli-Q water which was used for cell lyses was used as a negative control. The DNA of a sample that previously successfully amplified was used as a positive control. PCR 96-well plates were covered with sterile aluminum foil lids. The wells were gently agitated by running a finger nail across the bottom of them. The following thermal cycler program was used for the PCR (**Table 2.2**).

Table 2.2. Thermal Cycler Program

Step	Time (min)	Temperature (°C)	Action
1	1.5	95	Initial Denaturation
2	1	95	Denaturation
3	1.5	54	Annealing
4	2	72	Extension
5	Go to step 2	Repeat steps 2-4	30 times
6	5	72	Final Extension
7	Rest	4	End/Storage

2.4.2. Agarose Gel Electrophoresis of PCR Products

PCR products were analyzed using 1 % agarose gels prepared in 1 % TAE Buffer. In order to visualize the DNA in the agarose gels, Ethidium Bromide (EtBr) from a 1 mg/mL solution was added prior to pouring to obtain a final concentration of 0.5 µg/mL. Analysis was performed on 5 µL of PCR products.

2.4.3. Restriction Fragment Length Polymorphism (RFLP) Analysis of PCR Products

Enzymatic digestion reactions of 20 µL were performed using the New England BioLabs (NEB) enzymes, *Hha*I and *Bfu*CI. NEB buffers used were 10X NEBuffer 4 and 100X Bovine Serum Albumin (BSA). Sterile nuclease free water was autoclaved Milli-Q water or supplied with the kit. Master mixes for RFLP were prepared as in **Table 2.3.**

Table 2.3. RFLP Master Mix

Reagent	Volume (µL)
Sterile Water, Nuclease Free	12.7
10X NEBuffer 4	2.0
100X BSA	0.2
Enzyme (*Hha*I or *Bfu*CI)	0.1

The reagents were added in the indicated order to a sterile 1.7 mL or 2.0 mL microcentrifuge tube. To sterile shallow 96-well plates or PCR tubes, 15 µL master mix and 5 µL PCR products were transferred to their respective wells and incubated $\geq$ 3 hours. Loading dye of 3.3 µL was added to each well prior to analysis by agarose gel electrophoresis using 1 or 2 % agarose gels.

2.4.4. 16S rDNA Cleaning and Concentration Measurement

DNA Clean & Concentrator-5kits were used to purify amplified 16S rDNA as directed by the manufacturer. After DNA cleanup, DNA concentrations were measured using a spectrophotometer and agarose gel electrophoresis. A working volume of 1 μL was used for DNA concentration measurements using a NanoDrop ND-1000 spectrophotometer attached to a Dell computer running ND-1000 V3.3.0 software. Agarose Gel electrophoresis was performed with a working volume of 1 μL and a 1% agarose gel.

2.4.5. Sequencing Data Analysis

Invitrogen NTI ContigExpress[144] was used for sequence chromatogram error correction and sequence alignment with a 97 % sequence identity cut-off for all bacterial isolates. Taxonomic affiliations of the isolates were determined by comparison to sequences contained in the GenBank database[145] using the Basic Local Alignment Search Tool (BLAST)[146] application.

Phylogenetic analysis was completed using Molecular Evolutionary Genetics Analysis (MEGA).[147] A phylogenetic tree was drawn using the Neighbor-Joining method.[148] Evolutionary distances were calculated using the Maximum Composite Likelihood method.[149] A total of 939 positions were in the final dataset. Default settings were used for the alignment and the phylogenetic tree construction. For the bootstrap test, 3000 replicates were selected.

2.5. Archiving Cultures

Isolates were archived as glycerol stocks stored at -80 °C. Isolates were cultured in 5 mL NB at 15 °C shaken at 150 rpm until confluent growth was observed. Glycerol stocks were prepared by adding 1 mL 50 % glycerol to 1 mL culture in 2 mL sterile cryogenic vials.

2.6. Growth Monitoring of Isolates Incubated at Different Temperatures

To sterile 24-well plates was added 1.5 mL molten NA and left to cool. Media was inoculated with 35 μL culture from 1.5 mL samples. The 24-well plates were incubated at 4, 15, 22, 30 and 37 °C. Growth was monitored on days 3, 5, 7, 10 and 12 and growth scored as none, very low, low, medium, high or very high. The 24-well plates were discarded on day 32. Optimum growth temperatures were estimated based on the different rates of growth at different temperatures.

2.7. Small Scale Fermentations for Natural Product Production

2.7.1. 96-Well Plate Fermentations

96-well plates were prepared by autoclaving at 121 °C for 30 min. Media was added aseptically to each well and a replicate of each plate was prepared. All plates were covered with plastic lids and stored at 4 °C.

Seed cultures were prepared by inoculating isolates into 5 mL MB in sterile disposable culture tubes. The seed cultures were incubated at 15 °C, shaken at 200 rpm in a New Brunswick Scientific InnovaR shaker incubator with a 5 cm circular orbit. Seed culture densities were monitored by spectrophotometry using 2 μL for the measurement.

Isolates were inoculated when their Optical Density at 600 nm (OD_{600}) was above 0.1 if possible.

96-well plate fermentations were prepared by inoculating with 75 µL seed cultures. The plates were covered with a breathable membrane and incubated at 15 °C, shaken at 200 rpm for 7 days.

2.7.2. Extraction of 96-well Plate Fermentations

The growth was scored for each fermentation culture by visual inspection. The 96-well plates were covered with aluminum foil and stored at -20 °C until processing. Plates were dried on a EZ-2 or EZ-2^{plus} Genevac centrifugal evaporator overnight at ~30 °C. Water (100 µL) was added to each well and left to sit with occasional shaking. Acetonitrile (ACN) or methanol (MeOH) of 1 mL was added to each well and mixed by repeatedly aspirating. The 96-well plates were centrifuged for 2-5 min at 1000 rpm and the ACN or MeOH solution transferred to another 96-well plate. The 96-well plates were dried on a centrifugal evaporator, covered with aluminum foil and stored at -20 °C until LC-MS analysis.

2.7.3. LC-MS analysis of 96-well Plate Fermentations

To each well in the 96-well plates was added 5 µL water. The water was shaken to the bottom and left to sit for 5-10 min. The extracts were resuspended in 70 µL MeOH and centrifuged for 1 min at 1000 rpm. The samples were analyzed by Ultra High Performance Liquid Chromatography-Mass spectrometry (UPLC-MS) using a Thermo Accela UPLC equipped with a Photodiode Array (PDA), autosampler and pump coupled

to a Sedex 80 Sedere Evaporative Light Scattering Detector (ELSD). With a flow splitter, the UPLC was connected to a Finnigan LXQ Thermo MS, using an Atmospheric Pressure Chemical Ionization (APCI) probe. An Acquity Waters UPLC column (Bridged Ethyl Hybrid, 2.1 x 50 mm, C_{18}, 1.7 μm, 130 Å) was used with a VanGuard Waters guard column to run all samples for UPLC. Mass spectrometry was performed in positive mode and was run using a Dell computer loaded with Xcalibur.[150] Water and MeOH were the mobile phase and were supplemented with 0.1 % formic acid (FA). The UPLC solvent gradient is as observed in **Table 2.4.**

Table 2.4. UPLC Solvent Gradient

Time (min)	Water (%)	MeOH (%)	Flow Rate (μL/min)
0.00	95	5	500.0
0.20	95	5	500.0
2.40	0	100	500.0
3.00	0	100	900.0
4.50	0	100	900.0
4.70	0	100	600.0
4.90	95	5	500.0
5.00	95	5	600.0
6.00	95	5	600.0

2.7.4. LC-MS Data Analysis

LC-MS data was analyzed using an Xcalibur software suite. ELSD, MS and UV chromatograms of media blanks were compared to the fermentation media. Unique MS ions, UV peaks or ELSD peaks observed in fermentation extracts but absent in the media blanks were tabulated. The data processing function in Xcalibur used retention times for UV peaks and ELSD peaks, retention times and mass to charge ratio (m/z) signals for MS ions, to compare relative abundance in each well in the 96-well plates. Using a Microsoft

excel spreadsheet custom template, a 3-D graph was produced where the size of the cones are represented by the intensity of the corresponding peaks or ions. This process was repeated for each plate and for each replicate. The number of unique ions present for each media and isolate were tallied to determine the isolates and media that produced the most new compounds. Results which did not repeat in the replicate were not considered when selecting the media and isolates.

2.8. Scale-up of Small Scale Fermentation

2.8.1. 24-Well Plate and 250 mL Erlenmeyer Flask Fermentations

For each isolate, two media were selected that displayed the most diverse production of unique MS ions and which displayed the largest coverage of new MS ions. To scale up fermentations, parallel fermentations of 5 mL and 50 mL were conducted in triplicate using 24-well plates and 250 mL Erlenmeyer flasks respectively. Media was aseptically added to 24-well plates and 250 mL Erlenmeyer flasks and inoculated with 1 % inoculums from seed cultures in NB. The fermentation cultures were incubated at 15 °C and shaken at 200 rpm for 7 days.

2.8.2. Extraction of 24-Well Plate and 250 mL Erlenmeyer Flask Fermentations

Activated Diaion HP-20 resin (Styrene-Divinylbenzene, 250-850 μm) was added to Milli-Q water in a 500 mL bottle until the bed of settled HP-20 was 1-2 cm from the surface of the water. Fermentation cultures of 5 mL were extracted using 250 μL suspensions of the HP-20. The 24-well plates were incubated at 15 °C for 2.5 hours, shaken at 200 rpm and subsequently centrifuged for 10 min at 5000 rpm. The media was

removed and the HP-20 washed twice with 1 vol. water. The plates were incubated at 15 °C for 5 hours and 1 hour respectively, shaken at 200 rpm and centrifuged at 5000 rpm for 10 min. The 24-well plates were dried under vacuum. Denatured Alcohol (EtOH) composed of 90 % ethanol, 5 % MeOH and 5 % isopropyl alcohol was used to elute the organics from the HP-20. The HP-20 was washed twice with 1 vol. EtOH. The 24-well plates were shaken for 1 hr at 150 rpm, and centrifuged at 5000 rpm for 10 min. The extracts were transferred to pre-weighed scintillation vials which were dried under vacuum. Dried samples were stored at -20 °C.

Fermentation cultures of 50 mL were extracted using 2.5 mL suspensions of HP-20 resin. The flasks were shaken at 200 rpm for 3.5 hours at RT. Fermentation cultures were transferred to 50 mL centrifuge tubes and centrifuged at 4000 rpm for 12 min or 5000 rpm for 10 min. The supernatant was removed and the pellets washed with ~40 mL Milli-Q water. Fermentation cultures that poorly pelleted due to high viscosity of the media were washed by filtering through glass wool in 60 mL syringes. To elute the organics, 20 mL EtOH for pelleted samples or 30 mL EtOH for syringe filtered samples, was used twice to wash the HP-20. The centrifuge tubes were gently shaken lengthwise at 50 rpm for 1 hour and 5-8 hours respectively. The tubes were centrifuged for 12 min at 5000 rpm. The extracts were combined and dried under vacuum.

2.8.3. LC-MS analysis of Crude Extracts

Crude extracts were extracted twice with 0.75 mL MeOH and transferred to a 96-well plate. Suspended particulates were pelleted by centrifugation and the top 1 mL was transferred to another 96-well plate. The plate was covered with a pre-slit plastic lid and

centrifuged to pellet any remaining particulates. The samples were analyzed by using the Electrospray Ionization (ESI) probe. The crude extracts were diluted 5 fold by transferring 40 μL to 160 μL MeOH in another 96 well plate. They were then re-analyzed by LC-MS. Antibase[151] and MarinLit[152] were used to aid in dereplication.

2.8.4. Preparation of Crude Extracts for Initial Antimicrobial Screening

To 1.7 mL tubes was transferred 0.5 mL of 1 mL resuspended crude extracts from the 50 mL fermentations. The extracts were dried under vacuum and submitted for antimicrobial assays.

2.8.5. Preparation of MPY and FF Media Fractions for Antimicrobial Analysis

MPY media blanks, FF media blanks, FF media inoculated with RKSB-14A and FF media inoculated with RKSB-11A were fermented in duplicates at 50 mL volumes for 7 days at 15 °C, shaken at 200 rpm. The fermentation cultures and media were extracted with 5 mL HP-20 suspensions using a similar protocol as described previously. Crude extracts were dissolved in MeOH:water and mixed with C_{18}. The samples were dried and loaded on flash columns. A stepwise gradient of 20 % MeOH/water, 40 % MeOH/water, 60 % MeOH/water, 80 % MeOH/water, 100 % MeOH and 50 % MeOH/DCM using 10 mL solvent was performed. A 96-well plate was prepared and diluted with 500 μL MeOH. Dilutions of 10^{-1} and 10^{-2} were prepared by transferring 5 μL and 50 μL to 96-well plates in duplicates. The plates were dried under vacuum and submitted for antimicrobial assays.

2.8.6. Automated Low Pressure Liquid Chromatography (ALPLC) of Crude Extracts

Crude extracts of RKSB-11A in ACC media were combined, dried under vacuum, dissolved in MeOH and concentrated in Phenomenex Sepra C_{18} resin (C_{18}-E, 50 μm, 65 Å). ALPLC was performed using a Teledyne ISCO Combi*Flash* R_f 200 System with 15.5 g C_{18} Redi*SepR*$_f$ Gold High Performance Columns with a gradient mobile phase of 10 % MeOH:water-100 % MeOH at a flow rate of 20 mL/min. UV detection was set to wavelengths 214 nm and 254 nm. Tubes were combined into fractions and dried under vacuum. Samples of each fraction were submitted for LC-ESIMS analysis and the last three fractions were submitted for NMR analysis. This was repeated with the crude extracts of RKSB-16A in *Ancylobacter Spirosoma* Media. EtOH was used to wash the column after each run.

2.9. RKSB-11A Scale-up and Fermentation Optimization

2.9.1. Scale up of RKSB-11A Fermentation

Fermentation cultures were scaled up to a total of 1 L using 50 mL fermentations. Twenty fermentations were performed in 250 mL Erlenmeyer flasks using ACC media. Fermentations were incubated at 15 °C, shaken at 200 rpm for 7 days. Extractions of fermentation cultures were performed using 5 mL HP-20 suspensions.

2.9.2. ALPLC of Extracts from Large Scale Fermentation

Crude extracts were handled as described previously. ALPLC was performed using 43 g C_{18} Redi*Sep* R_f Columns or 50 g C_{18} Redi*Sep* R_f Gold High Performance

Columns with a flow rate of 40 mL/min. Tubes were combined into 7 fractions and the last three fractions before the EtOH wash fraction were analyzed by LC-ESIMS.

2.9.3. Optimization of Fermentation Extractions

From a 50 mL fermentation culture, 20 mL was removed and pelleted by centrifugation. The supernatant was transferred to another centrifuge tube and extracted using a 2 mL HP-20 suspension. The tube was shaken at 200 rpm for 20 min. The HP-20 was pelleted by centrifugation and the supernatant removed. The HP-20 and the cell pellet were separately washed with 40 mL water and extracted twice with 10 mL EtOH. The cell pellet was vortexed and sonicated for 3 min for each extraction. The extracts were transferred to scintillation vials, dried under vacuum, diluted in 1 mL MeOH and filtered through cotton plugs. The samples were analyzed by LC-ESIMS.

2.9.4. Relative Quantitation of GGLs in Three Different Media

Twenty-five fermentations of 50 mL were inoculated with 500 μL RKSB-11A seed cultures and were incubated at 15 °C, shaken at 200 rpm for 7 days. Eleven fermentations contained ACC media, seven contained ACC media supplemented with 20 g/L glycerol, and seven contained ACC media supplemented with 20 g/L glucose, where each media contained a media blank. Cells were harvested by centrifugation at 4000 rpm for 12 min, the supernatant decanted and cell pellets washed with 40 mL Milli-Q water by vortexing and centrifugation. Pelleted cells were extracted twice using 20 mL EtOH with vortexing and sonication. Extracts were transferred to scintillation vials which were dried under vacuum and extracted with 1 mL MeOH. Dilutions of 1/6 were analyzed by a Finnagin

Surveyor Plus Thermo HPLC which consisted of a autosampler connected to a HPLC Pump coupled to a PDA Detector and a Sedex 60 LT Sedere ELSD all run by a Dell computer using ChromQuest 4.2.34 software. All quantitation was performed using an Analytical C_{18} Prodigy Column. Dilutions of 1/24 were required for ACC + glycerol fermentations in order to stay within the dynamic range of the ELSD. The gradient mobile phase conditions are shown in **Table 2.5.**

Table 2.5. HPLC Solvent Gradient

Time (min)	Water (%)	MeOH (%)	Flow Rate (μL/min)
0.01	3	97	1000
38	3	97	1000
39	0	100	1000
48	0	100	1000
49	3	97	1000
55	3	97	1000

2.9.5. Cell Mass, OD_{600} and Ratio of Different GGLs from Days 1-7 for Fermentations at 15 °C and RT

RKSB-11A was fermented in 1 L fermentation cultures of ACC + glucose media in 2.8 L fernbach flasks shaken at 200 rpm for 7 days. Fermentations of 3 L were conducted at 15 °C and 3 L were conducted at RT for a total of 6 L. The RT fermentations were conducted on an Innova 5000 New Brunswick Scientific Digital Tier Shaker with a 5 cm circular orbit. Aliquots of 10.5 mL fermentation culture were removed daily with 0.5 mL being used for OD_{600} measurement by spectrophotometry with a working volume of 2 μL. The 10 mL aliquot was pelleted, washed twice with water and freeze dried to obtain the mass of the cell pellet. The cell pellets were extracted twice with 5 mL EtOH by vortexing, sonicating for 30 min and centrifugation. The

samples were extracted with MeOH and analyzed by LC-ESIMS using the analytical C_{18} prodigy column. Peak areas were selected manually because of some retention time shifting. Peaks were generated by selecting for the desired ion ± 0.5 m/z as the base peak in the MS chromatogram.

2.10. HPLC of GGLs

Fractionation was performed on a Waters HPLC which was composed of a 1525 Binary HPLC Pump coupled to a 2489 UV/Visible Detector and a 2424 ELSD all run by a Lenovo IBM computer running MassLynx V4.1 software. A HPLC Semiprep Luna Phenomenex column (C_{18}, 250 x 10.00 mm, 5 μ, 100 Å) was used interchangeably with HPLC Semiprep Gemini Phenomenex column (C_{18}, 250 x 10.00 mm, 5 μ, 110 Å,). They were used with an isocratic mobile phase of 90 % MeOH:water up to 97 % MeOH:water at a flow rate of 3 mL/min. Another column used was a HPLC analytical Prodigy Phenomenex column (C_{18}, 250 x 10.00 mm, 5 μ, 100 Å) for the final fractionation of low yield compounds.

2.11. Sugar Analysis

2.11.1. GGL Hydrolysis for Sugar Identification

Acid hydrolysis was performed by adding 5 mL of 2 M trifluoroacetic acid to about 1-3 mg of sample followed by refluxing for 4 hours.[153] The solution was extracted three times with 3 mL hexane and the hexane fraction was extracted three times with 3 mL water. The aqueous layers were combined, dried under vacuum and then dried using the freeze drier.

2.11.2. HPLC Sugar Analysis

Sugar analysis was performed on the Waters HPLC using a Rezex Phenomenex Analytical Ion Exclusion Monosaccharide column (300 mm x 7.80 mm, 8 μ, 8 % H^+ cross-linked sulfonated styrene-divinylbenzene) protected by a Phenomenex Security Guard guard column. The isocratic mobile phase consisted of 100 % water at 0.5-0.6 mL/min with the column heated to 80-85 °C in a water bath.

2.12. GGL Characterization

2.12.1. NMR

NMR was performed using a Bruker 600 MHz NMR equipped with a microprobe. NMR was performed in methanol-d_4 for most experiments. Other solvents used were pyridine-d_5, dimethyl sulfoxide-d_6, chloroform-d, and deuterium oxide. Samples were dissolved in 50 μL solvent and transferred to a capillary NMR tube. NMR analysis was performed using MestRec.[154] Scifinder[155] and Combined Chemical Dictionary[36] were used to identify known compounds.

2.12.2. HR-MS

HR-MS data was obtained from Dalhousie University and performed on a Bruker micro Time of Flight (TOF) Focus MS run in ESI mode.

2.12.3. IR Analysis

IR spectroscopy was performed by Attenuated Total Reflectance (ATR) using a Thermo Scientific Nicolet 6700 Fourier Transform-Infrared equipped with a Smart iTR

with a diamond plate coupled to a Photoelastic Modulation module. It was run using a Dell Computer using OMNIC software. Samples in MeOH were repeatedly added to the crystal and left to dry to obtain a concentrated sample.

2.12.4. Polarimetry of Sugars and GGLs

Polarimetry was performed using a Rudolph Research Analytical Autopol III Automatic Polarimeter. The Polarimeter lamps were left to stabilize for 30 min before any measurements were preformed. A quartz control plate calibration standard with an optical rotation of 11.500° at 589 nm was used to calibrate the instrument. A 50 x 5 mm stainless steel cell with a volume of 1 mL was used. For sugar identification, a sugar standard that had undergone the same pre-treatment as the sample was measured alongside the sample.

2.13. Surface Tension Measurements

Surface tension measurements were performed using a Kyowa Interface Science Full Automatic Surface Tensiometer Model CBVP-Z with a Kyowa Interface Science platinum plate using the Wilhelmy plate method. The fully automatic measurement, standard data out method for low viscosity samples was used. A 1 mL Kyowa Interface Science Teflon dish designed for low volume measurements and a Petri dish (60 x 15 mm) were used.

A solution of 10 g/L SDS sonicated solution was used as a standard for method validation. SDS dilution standards were prepared in 30 mL volumes at 10 different concentrations (w/v) of, 0, 0.001, 0.025, 0.050, 0.100, 0.200, 0.400, 0.600, 0.800, and 1.000. Forceps were used to handle the platinum plate at any time. The platinum plate

was cleaned using Milli-Q water, MeOH and was then heated using an alcohol burner until glowing red. The plate was left to cool to RT for 30 s-2 min. Multiple measurements were performed per sample and cleaning was performed between each sample. GGL 915 was prepared in serial dilutions from 10^4 μg/mL-10^{-2} μg/mL. Analysis was performed the same as for SDS.

2.14. Bioactivity Assessment

2.14.1. Antimicrobial Assays

Antimicrobial assays were performed by Martin Lanteigne (Kerr Group) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines M7-A6.[156] Assays were performed using the test organisms *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Candida albicans*, MRSA and VRE. The standard concentration used of the test organisms were 5 x 10^4 Colony Forming Units per mL (CFU/mL). The standard media used was CAMH broth for *S. aureus*, *E. faecalis*, *P. aeruginosa*, MRSA and VRE. Sabouraud dextrose broth was used for *C. albicans*. Samples were run in triplicate or more in the 96-well plate and alongside the test samples were, blank controls which contained 2 % DMSO and CAMH broth or other media used, positive controls which contained 2 % DMSO and the diluted organism, and a negative control which contained a serial dilution of an antibiotic known to kill the organism. The antibiotics used as positive controls were: penicillin G for *S. aureus*, vancomycin or amoxicillin for *E. faecalis* and MRSA, rifampicin for VRE, nystatin for *C. albicans* and gentamicin for *P. aeruginosa.* The final volume in the 96-well plate wells was 100 μL.

Plates were incubated static at 37 °C for 22 hours. The OD_{600} was measured at T = 0 and T = 22 hours using a 96-well plate reader.

2.14.2. Cytotoxicity

Cytotoxicity assays were performed by Haili Wang in Jim Johnston's research group.

A Roche 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed with GGL 915 to test for cell toxicity using the cell lines HeLa, Huh7 and A549. The MTT assay was performed according to the manufacturer with the exception of replacing the media when adding the MTT labelling reagent. The growth medium used was DMEM. The concentration of cells used was about 1 x 10^5 cells/well and they were analyzed at 24 and 48 hours. The samples were tested in quadruplets with controls of 0, 0.2, 0.3 and 10 % DMSO in media to act as three negative and one positive control respectively. The eight sample concentrations tested were 5, 10, 15, 20, 25, 30, 40, and 50 μM. The plate was analyzed at 570 nm using a plate reader and diluted 1:2 with phosphate buffered saline (PBS) if required.

Data was processed by obtaining the averages and standard deviations of the absorbances. The averages and standard deviations of 5 μM-30 μM concentrations were normalized by dividing by the media control averages. Average and SDs of 40 μM and 50 μM were normalized by dividing by the combined average of the vehicle controls, 0.2 and 0.3 % DMSO, in order to account for experimental variation in the vehicle controls.

2.14.3. Antiviral Assays

Antiviral assays were performed by Gen Wang, Haili Wang and Dominique Schmidt in Jim Johnston's research group. Three assays were performed.

An anti-viral assay was performed using the *Vaccinia* virus strain Western Reserve in Hela, Huh7 and A549 cell lines. The growth media, DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 % Penicillin/Streptomycin (P/S) in 1X PBS, was warmed to 37 °C. GGL at concentrations of 0, 20, 30, 40, and 50 μM were prepared with a positive control containing the vehicle used to dissolve the GGL. The seed media was removed from the cells which were subsequently washed with PBS. Treated media of 200 μL was added to the cells in each of the wells in the 24-well plate. The 24-well plate was incubated at 37 °C for 2 hours. The cells were than washed with PBS and infected with the *Vaccinia* virus at a multiplicity of infection (MOI) of 1, 0.1 and 0.01 for 1 hour. The media was removed and the cells washed with PBS. Treated media was prepared using 5 % FBS and 1 % P/S of which 0.5 mL was added to the cells. The cells were harvested 24 and 68 hours post infection (hpi). Images were obtained using fluorescent microscopy and digital photography.

Anti-viral assays were performed using the laboratory-adapted Influenza A virus H1N1, strain A/PR/8/34, using Madin-Darby Canine Kidney (MDCK) cells. The growth medium was MEM supplemented with 10 % FBS and 1 % P/S. The concentration of cells used were 1.5 x 10^4 cells per well in a 96-well plate. The next day, the cells were treated with the antiviral compounds for 24 hours at concentrations of 5, 10, 20 μM for GGL 885; 10, 20, 30 μM for GGL 887 and 915. The media was removed and the cells were infected with the virus at an MOI of 0.1 for 4 hours in infection media MEM

supplemented with 10 U/mL Tosyl Phenylalanyl Chloromethyl Ketone (TPCK)-Trypsin, 0.125 % BSA, 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid and 1 % P/S. After, the infection media was removed and replaced with the original growth medium with half the concentration of the antiviral compound. Images were obtained using digital photography. For trial 1, regular media was used which resulted in a more cell friendly environment, and for trial 2, infection media was used which resulted in an environment favouring the propagation of the virus due to the presence of TPCK-Trypsin.[157]

Wells were scored using a cell death rating based on monolayer health. The scores ranged from 0-5 based on the following system where, 0 = even monolayer and no dead cells, 1 = some gaps in the monolayer around the edges, 2 = gaps in the monolayer everywhere with the presence of some dead cells, 3 = significant gaps in the monolayer with the presence of many dead cells, 4 = very large gaps everywhere in the monolayer with few living cells and 5 = all cells are dead. The results were processed by dividing the average scores and standard deviations by the average scores of DMSO controls.

2.14. Anti-fouling Assay

An antifouling assay was performed by Sutaporn Bunyajetpong (Kerr Group) using a modification of a similar method to Cirino, *et al.*[158] The fouling organism used was the tunicate *Ciona intestinalis*. *C. intestinalis* was collected 24-72 hours prior to use. Sperm and eggs were harvested from *C. intestinalis* using a needle and a Pasteur pipet respectively. The sperm and the eggs were mixed and left to sit for 1-3 hours to ensure fertilization. The solution was separated, diluted 5 fold and left to sit for ~16 hours for larval formation. Ten free swimming larvae were transferred to each well in a pre-treated

12-well plate. The bottoms of the wells were coated with 100 μg samples in triplicates. Negative controls in triplicate containing the vehicle were included. The 24-well plates were dried before use so any residual solvent (e.g MeOH) was expected to have evaporated. After one day, tunicates were examined and categorized as settled, swimming, morphologically changed, or dead.

CHAPTER 3-RESULTS AND DISCUSSION

3.1. Bacterial Isolation from Seawater and Sediment Samples

3.1.1. Review of Work Performed in Honors Project

Sediment and seawater samples were collected August, 2007 from Malpeque Bay, P.E.I. Samples were serially diluted and plated on MA, DMA, NA and DNA. They were incubated at 4 and 10 °C for up to five weeks. Unique isolates were sub-cultured until pure, resulting in 124 isolates from MA, 106 from DMA, 58 from NA and 67 from DNA. In total 355 isolates were isolated. Following morphological dereplication, 190 isolates were carried forward and fermented in 24-well plates for 5 days. Growth was assessed after 24 and 48 hours. Glycerol stocks were prepared and the remaining sample transferred to 96-well plates for pelleting by centrifugation. Agar well diffusion antimicrobial assays were performed against *S. aureus*, *P. aeruginosa* and *C. albicans.* DNA was isolated from positive hits using the freeze-thaw method and the 16S rDNA amplified by PCR. The PCR products were run on agarose gel electrophoresis and the bands cut out that corresponded to the 16S rDNA. The excised DNA was purified using a QIAquick gel extraction kit and submitted for sequencing. The results from Blastn searches resulted in *Pseudomonas anguilliseptica*, *Rhizobium sp. 4_C16_39*, *Shewanella sp. TP4* and *Marinomonas sp. JL-55* as having the highest percent identity corresponding to submitted sequences (**Table 3.1**).

Table 3.1. Identity of Sequenced Bacteria

Sample	Species (% Identity)
SWA 8°C-33	*Pseudomonas anguilliseptica* (98)
SWA 8°C-38	*Rhizobium* sp. 4_C16_39 (98)
SWA 8°C-27	*Shewanella* sp. TP4 (98)
SWA 8°C-25	*Marinomonas* sp. JL-55 (96)

3.1.2. Bacterial Isolation from Sediment Samples Collected in Winter

A replicate of the previous work was completed with a sediment sample obtained in April 2009 to compare and contrast bacteria communities in winter and summer. Also performed was a separate study targeting cold-adapted *Actinobacteria* using *Actinobacteria*-specific media. Two pretreatments and an untreated method were performed on the sediment sample. The two pretreatments, YE/SDS and dry heat/stamp, were obtained from different literature sources and have been shown to select for *Actinobacteria*. An untreated method was chosen in case the pre-treated samples showed very poor yield. The YE/SDS method is based on using yeast extract at 40 °C as a spore activating agent and SDS as a germicide.[142] The dry heat/stamp method was chosen as a pretreatment because it was simple and shown to be effective.[140] Isolates were incubated at 8 °C and sub-cultured about every 3 weeks until pure isolates were obtained.

A total of 70 isolates were obtained from the *Actinobacteria*-specific media (**Table 3.2**). The dry heat/stamp method was shown to be the most oppressive method with 9 isolates obtained. YE/SDS and untreated methods yielded 28 and 33 isolates indicating that the YE/SDS method may not provide enough selective pressure. For media, WYE was by far the best with a yield more than tripling that of any other media

Table 3.2. Number of Isolates from the Winter Sediment Sample using *Actinobacteria*-Specific Media with Different Pre-treatments*

Pre-Treatments	HAVA	ISP3	WYE	AIA	Total
Dry Heat/ Stamp	0	4	5	0	9
YE/SDS	2	3	16	7	28
Untreated	3	7	21	2	33
Total	5	14	42	9	70

*Chitin Agar did not yield any isolates

used. Chitin agar was the worst with no isolates obtained.

A total of 165 isolates were obtained from the cold-adapted bacteria isolation experiment from the winter sample (**Table 3.3**). This is about half of that obtained from the summer samples. There were some very large differences in the treatment and collection of the sediment sample. The winter sample consisted of one sediment sample while the summer samples consisted of two water samples and two sediment samples. The winter sediment sample was washed while the summer samples were not. There were also differences between the time of collection and plating where the summer samples were plated about 1 month after collection and the winter sediment sample was plated about 5 months after collection. For the winter sample, NA media yielded about the same number of isolates as MA which is quite different compared to the summer sample where MA and DMA yielded about twice as many as that of the NA and DNA. This could be due to the fact that no isolation work was performed on seawater samples from the winter. Of interest is that the 4 and 8 °C incubations both yield about the same number of isolates. However, the difference of 4 °C in temperature is quite small. A larger difference would likely be observed if the first incubation temperature was at 0 °C.

Table 3.3. Number of Isolates from Cold-Adapted Bacteria Isolation from Winter Sediment Sample

Incubation Temperature	MA	DMA	NA	DNA	Total
4 °C	20	19	22	17	78
8 °C	33	5	33	16	87
Total	53	24	55	33	165

3.1.3. Rational for Incubation Temperature and Media Selection

Incubation temperatures of 4 and ~8 °C were selected to obtain psychrophilic and psychrotolerant bacteria and to reduce the number of mesophilic bacteria obtained. MA and NA were chosen in order to have rich media with salt and rich media without salt in order to obtain bacteria with varying salt requirements. DMA and DNA were chosen to provide a low nutrient source for bacteria that can only be cultured on lean media. Generally found was that the rich media provided the most culturable bacteria or were simply the fastest growing and outcompeted the slower growing bacteria. The *Actinobacteria*-specific media were chosen from widely different literature sources and contain very different carbon sources and reagents. Selected were two lean media and three rich media. HAVA is a media designed for *Actinobacteria* isolation from soil and has been widely investigated with different antibiotics to target unique *Actinobacteria.*[159] Humic acid is known as a spore activating agent[142] and is a unique carbon source. AIA is a media designed to isolate *Actinobacteria* and contains several carbon sources. ISP3 is a standard *Actinobacteria*-specific media using oatmeal as the carbon source. WYE is a nutrient poor media using yeast extract as a carbon source. WYE was reported to inhibit the growth of eubacteria and to be a good selective media.[139] Chitin agar has been used as far back as 1961 to isolate *Actinobacteria* using chitin as a unique carbon source.[43, 160] Media were supplemented with antibiotics in order to inhibit the growth of non-*Actinobacteria* isolates. Nalidixic acid is used to inhibit the growth of gram-positive and gram-negative bacteria; Cycloheximide and nystatin are used to inhibit the growth of fungi. This is a common technique which is widely used for the isolation of *Actinobacteria.*[140, 159, 161, 162]

3.2. Plating of Glycerol Stocks of Previously Isolated Bacteria

Glycerol stocks containing isolates from the summer samples were plated on NA. NA was chosen due to the ability of isolates to be maintained on NA when previous work was completed. NA plates were divided into quarters and approximately 50 μL of frozen glycerol stock was streaked on the NA. The cultures were left to grow for ~2 weeks at 4 °C.

Many of the isolates were impure so they were sub-cultured onto individual plates. Pure isolates were sub-cultured on plates divided into quarters. The colonies were incubated at 4 °C. The revival of isolates from glycerol stocks was quite low with 102 of 186 isolates recovered.

The isolates that did not grow on NA were later cultured on MA. It was believed that although NA could support the growth of all isolates, growth on MA was required to obtain a necessary critical cell mass before they could be transferred to NA. Of the 84 that did not grow on NA, 78 more isolates were recovered on MA. Some glycerol stocks yielded more than one isolate. They were sub-cultured until pure and carried forward for molecular dereplication with the isolates from the winter sample including those from *Actinobacteria*-specific media.

3.3. Bacterial Molecular Dereplication and Identification

3.3.1. DNA Isolation

DNA isolation was carried out using a modified DMSO DNA extraction method. Bacteria lysis by DMSO is a very inexpensive, easy and fast method and is therefore often the first method used for DNA isolation from bacteria. The success rate of PCR

amplification was very high for the initial 102 bacteria. The success rate of PCR amplification decreased dramatically when performed on the isolates from the winter sediment sample and the 78 recovered isolates. A boiling method was used with 90 more isolates that had failed to previously amplify. Increased success was observed however, a phenol-chloroform DNA isolation method[141] would be required for many of the winter sediment samples and the 78 recovered isolates. Due to time restrictions, the isolates from the winter sediment sample and the 78 recovered isolates were not processed further.

3.3.2. Use of RFLP for Molecular Dereplication

RFLP analysis was performed on all 102 isolates using the restriction endonucleases *Hha*I and *Bfu*CI. RFLP analysis is an easy dereplication method and is one of many such tools available.[163] Dereplication was performed by grouping isolates with the same RFLP pattern which resulted in 20 different groups (**Table 3.4**). A 2% agarose gel was found to give much better separation of the DNA bands than the 1% agarose gel. Isolates showing similar RFLP patterns were run side by side and then three isolates or less from each group were preserved in glycerol stocks. Two isolates of each group were then prepared for sequencing by cleaning the DNA using a QIAquick clean and concentrator kit and measuring the concentration of DNA by agarose gel electrophoresis and spectrophotometry. DNA was sequenced by Genome Quebec using Sanger sequencing.

Table 3.4. Grouping Based on RFLP Patterns of 16S rDNA

Group	Total Isolates grouped Together	Selected Isolates for Growth Study	Sequenced Isolates
1	22	B10, G9, B12	B10, G9
2	10	F11, "6", E2	F11, "6"
3	4	A4, A12, A11	A4, A12
4	19	B8, A1, E5	A1, B8
5	2	A5, D6	A5, A6
6	1	B6	B6
7	10	D9, C11, E8	D9, C11
8	2	E10, E11	E10, E11
9	1	B7	B7
10	1	E3	E3
11	1	G12	G12
12	3	F3, F9, H7	F3, F9
13	15	G10, C6, C8	G10, C6
14	1	C4	C4
15	1	C12	C12
16	1	E9	E9
17	3	"1", "4", "3"	"1"," 4"
18	1	"2"	"2"
19	1	"5"	"5"
20	1	"7"	"7"

3.3.3. Identification from 16S rDNA

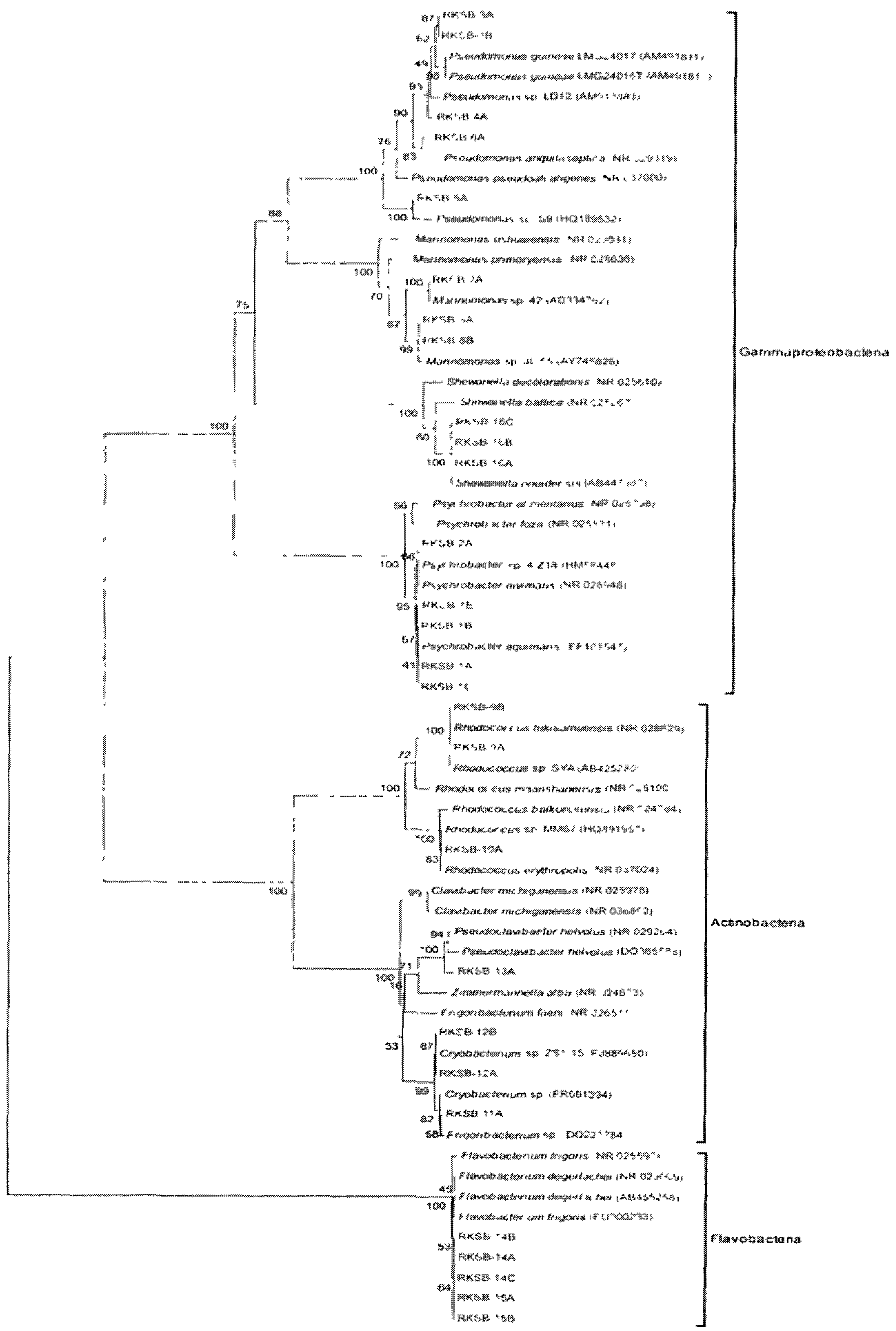
Molecular dereplication of the 16S rDNA using Contig Express resulted in 16 unique ribotypes (**Table 3.5** and **Figure 3.1**). Three different phyla were represented with the *Flavobacteria*, *Actinobacteria* and *Gammaproteobacteria* containing a total of 8 different genera. There were four different *Pseudomonas* species (spp.) and two different groups of *Psychrobacter*, *Marinomonas*, *Rhodococcus* and *Flavobacterium* spp. There was one group of *Frigoribacterium*, *Cryobacterium*, *Pseudoclavibacter*, and *Shewanella* spp. The phylogenetic tree confirms the genera with the insertion of non-redundancy (NR) reference strains. Of interest are the *Frigoribacterium.* The NR reference strain is far removed from the closest matching sequence but is included in the clade which

suggests a lack of a closely related well characterized strain. However, the bootstrap values are quite low indicating poor clade reproduction.

Table 3.5. Basic Local Alignment Search Tool (BLAST) Search Results of Isolates Sharing 97 % Identity of 16S rDNA

I.D	S.F	O.I	S.L	First BLASTn Hit (% Identity)
RKSB-1A-E	B10	G9, "4", "1", C12	1444	*Psychrobacter aquimaris* strain KOPRI24929 (99.65)
RKSB-2A	E3	N/A	1396	*Psychrobacter* sp. 4-Z18 (99.43)
RKSB-3A, 3B	C6	F11	1398	*Pseudomonas guineae* (99.57)
RKSB-4A	"6"	N/A	1398	*Pseudomonas* sp. LD12 (99.43)
RKSB-5A	B7	N/A	1440	*Pseudomonas* sp. S9 (99.31)
RKSB-6A	G10	N/A	1398	*Pseudomonas guineae* (98.43)
RKSB-7A	A4	N/A	1437	*Marinomonas* sp. 42 (99.93)
RKSB-8A, 8B	"5"	A12	1447	*Marinomonas* sp. JL-55 (99.66)
RKSB-9A, 9B	E10	E11	1218	*Rhodococcus* sp. SYA (99.92)
RKSB-10A	E9	N/A	1145	*Rhodococcus* sp. MM67 (100.00)
RKSB-11A	B6	N/A	1156	*Frigoribacterium* sp. PIC-C17 (99.83)
RKSB-12A, 12B	C11	D9	1240	*Cryobacterium* sp. ZS1-15 (99.92)
RKSB-13A	C4	N/A	1226	*Pseudoclavibacter helvolus* strain DSM 20419 (99.18)
RKSB-14A-C	"2"	F3, F9	1231	*Flavobacterium frigoris* strain KOPRI_22311 (99.84)
RKSB-15A, 15B	A1	B8	1232	*Flavobacterium frigoris* strain KOPRI_22311 (99.76)
RKSB-16A-C	D6	A5, "7"	1392	*Shewanella oneidensis* (99.86)

I.D = Isolate identity, S.F = selected for fermentation, O.I = other isolates in group, S.L = sequence length in base pairs



Gammaproteobactena
Actinobactena
Flavobactena

Figure 3.1. Phylogenetic Tree of 28 Bacteria with Closest Match and Reference Strains The phylogenetic tree was constructed using the Neighbor-Joining method. The tree was drawn to scale with the number of substitutions per site indicated at the bottom. The bootstrap values (3000 replicates) are shown at the nodes and indicates the percentage of replicate trees which clustered the taxa together. Accession numbers are in brackets beside isolate sequences retrieved from GenBank. Taxa with accession numbers starting with NR are well characterized reference strains while the remainder are from the closest matching described isolate. RKSB are isolate sequences from the present study. The square brackets indicate the phyla to which the isolates belong.

Flavobacterium spp. are well known marine and terrestrial bacteria which include 95 different species.[164] They have been isolated from many different sources which include soil, sediment, fresh water, salt water, sea ice, and an infected fish.[165] There are 33 different *Psychrobacter* spp.[164] and they have been isolated from many different sources[166] including Arctic sea ice and permafrost. They have also been isolated from Antarctic sea ice, seawater, sediment, and soil.[90, 166, 167] Non marine sources have also been found and include pigeon feces, processed meat, poultry, and an infected lamb.[166]

Pseudoclavibacter spp. contain three different species[164] and have been isolated from soil,[168] ginseng,[169] activated sludge,[170] molinate herbicide,[171] and from an human aortic valve,[172] which indicates that they are primarily terrestrial in origin. *Rhodococcus* spp. are comprised of 44 different species.[164] They have been isolated from soil,[173, 174] plants,[175] and sludge.[176] They have also been isolated from seawater sediment and some have been found to be saltwater obligates[177] which indicate that many have terrestrial origins but there are some from marine environments.

Frigoribacterium spp. include two different species[164] and have been isolated from terrestrial ice cores,[178, 179] alpine permafrost,[180] plants,[181] hay dust,[182] and soil,[182] indicating that they are terrestrial bacteria. *Cryobacterium* spp. include 4 different

species[164] and have been isolated from Antarctic lake sediment,[183] arctic glacier sediment,[184] and soil.[185, 186] It is not clear whether they are marine or terrestrial in origin but it is likely that they can be found in both. *Marinomonas* spp. are marine bacteria that contain 16 different species[164] and have been isolated from seawater[187, 188] and arctic sea ice.[189]

Shewanella spp. have been isolated from many different sources[190] and consist of 55 species.[164] They have been isolated from sediment,[190] seawater,[187] arctic sea ice,[189] and Antarctic sea ice.[90] They have also been isolated from fish,[190] activated sludge,[190] and clinical samples.[190] Some *Shewanella* strains are salt water obligates[90] indicating that some are specific to marine environments.

Many of the isolates are related to ubiquitous bacteria. They have been found from marine sources and from terrestrial sources. Looking at a number of different factors, it's not surprising that some isolates are related to strains which have terrestrial origins. The samples were collected from near shore which can be subject to runoff which is likely for the *Pseudoclavibacter helvolus*. Also, since the isolates were sub-cultured on NA, they would require a low amount of salt for survival. However, since the isolates have been isolated from a marine environment, it is still likely that they may have new secondary metabolites.[57]

3.4. Temperature Growth Range Study of 40 Isolates

Three isolates or less from each RFLP group were used for growth temperature analysis (**Table 3.6**). The temperatures selected for the growth study were 4, 15, 22, 30 and 37 °C. Growth at 4 °C indicated that isolates were cold-adapted and therefore were psychrophilic or psychrotolerant. Growth at 4 and 15 °C indicated that they were psychrophilic and growth at 22 °C indicated that they were psychrotolerant. No growth at 4 °C but growth at higher temperatures indicated isolates were mesophilic. A wide variety of incubation temperatures were selected in order to try to best approximate the optimum growth temperatures and maximum growth temperatures. By Morita's definition of psychrophilic and psychrotolerant bacteria,[77] the lowest temperature should be at least 0 °C, however due to equipment limitations, we selected 4 °C as the lower limit of growth.

No growth was observed for MBI-5 (Malpeque Bay Isolate), MBI-A11, MBI-A5 and MBI-7 likely due to poor viability of the inoculating culture or poor viability on salt poor media. Genera specific growth temperature profiles were observed with all *Psychrobacter*, *Pseudomonas*, *Rhodococcus* and *Frigoribacterium* isolated being psychrotolerant. All *Marinomonas*, *Pseudoclavibacter* and *Shewanella* spp. that grew were mesophilic. *Marinomonas* spp. showed very narrow growth ranges with no growth at 4 or 30 °C. Strain specific growth was observed with *Cryobacterium* and *Flavobacterium* spp. containing psychrophilic strains among psychrotolerant strains.

Flavobacterium spp. are generally cold-adapted bacteria with many strains being isolated from the Arctic and Antarctic.[165] Many different strains have been characterized with some being psychrophilic,[165] psychrotolerant,[165] and mesophilic.[186, 191] *Psychrobacter* spp. are known to be psychrophilic,[90] psychrotolerant,[90, 192] and

mesophilic.[166] They have been isolated from the Arctic, Antarctic, and many other sources.[90, 166, 167] *Pseudoclavibacter* spp. are found in many different areas and are known to be mesophilic.[168, 171] No cold-adapted *Pseudoclavibacter* spp. have been reported.

Rhodococcus spp. have been shown to be psychrotolerant and mesophilic.[193] There are several reports of psychrophilic *Rhodococcus* spp.[194] however, all report growth temperatures above 20 °C and are not considered true psychrophiles as defined by Morita.[77] *Frigoribacterium* spp. have been shown to be psychrophilic,[182] psychrotolerant,[179, 181] and mesophilic.[182] *Cryobacterium* spp. have been classified as psychrophilic,[183, 185] psychrotolerant,[185] and mesophilic.[186] *Marinomonas* spp. have been classified as psychrophilic,[189] psychrotolerant,[189, 195] and mesophilic.[188, 195] *Shewanella* spp. have been reported to be psychrophilic,[90] psychrotolerant,[90, 189] and mesophilic.[190]

Most of the genera associated with the microorganisms isolated have strains that are cold-adapted. The low number of true psychrophiles obtained in the current study agrees with the literature,[77, 88] especially since the source from which the samples were obtained fluctuates seasonally.[132] The isolation method also may not have been rigorous enough to prevent thermal lyses of psychrophiles with lower maximum growth temperatures. Ideally, the sample is always kept cold and all work is performed in a refrigerated area where the temperature never exceeds the maximum growth temperatures desired of isolates. For example, if recovery of isolates with maximum growth temperatures of 10 °C were expected, the incubation temperatures should always be kept below 10 °C. Sub-culturing work was performed at room temperature and equipment and media were not precooled which could have resulted in thermal stress and loss of viability for sensitive isolates.

Table 3.6. Growth Temperature Range of Selected Isolates

Isolate	Genus	Temperature Range (°C)	Est. Optimum Temp. (°C)	Classification
MBI-B10	*Psychrobacter*	4-30	22	Psychrotolerant
MBI-G9	*Psychrobacter*	4-30	22	Psychrotolerant
MBI-B12	*Psychrobacter*	4-30	20	Psychrotolerant
MBI-1	*Psychrobacter*	4-30	22	Psychrotolerant
MBI-4	*Psychrobacter*	4-30	22	Psychrotolerant
MBI-3	*Psychrobacter*	4-30	22	Psychrotolerant
MBI-C12	*Psychrobacter*	4-30	22	Psychrotolerant
MBI-E3	*Psychrobacter*	4-37	27	Psychrotolerant
MBI-G10	*Pseudomonas*	4-30	24	Psychrotolerant
MBI-C6	*Pseudomonas*	4-30	18	Psychrotolerant
MBI-F11	*Pseudomonas*	4-30	27	Psychrotolerant
MBI-6	*Pseudomonas*	4-30	27	Psychrotolerant
MBI-E2	*Pseudomonas*	4-30	27	Psychrotolerant
MBI-C8	*Pseudomonas*	4-30	24	Psychrotolerant
MBI-B7	*Pseudomonas*	4-30	24	Psychrotolerant
MBI-A4	*Marinomonas*	15-22	18	Mesophilic
MBI-A12	*Marinomonas*	15-22	18	Mesophilic
MBI-5	*Marinomonas*	-	-	-
MBI-A11	*Marinomonas*	-	-	-
MBI-E10	*Rhodococcus*	4-30	16	Psychrotolerant
MBI-E11	*Rhodococcus*	4-30	24	Psychrotolerant
MBI-E9	*Rhodococcus*	4-37	28	Psychrotolerant
MBI-B6	*Frigoribacterium*	4-37	22	Psychrotolerant
MBI-D9	*Cryobacterium*	4-22	15	Psychrotolerant
MBI-C11	*Cryobacterium*	4-15	15	Psychrophilic
MBI-E8	*Cryobacterium*	4-22	16	Psychrotolerant
MBI-C4	*Pseudoclavibacter*	15-30	22	Mesophilic
MBI-F3	*Flavobacterium*	4-22	15	Psychrotolerant
MBI-F9	*Flavobacterium*	4-22	18	Psychrotolerant
MBI-H7	*Flavobacterium*	4-15	15	Psychrophilic
MBI-2	*Flavobacterium*	4-22	18	Psychrotolerant
MBI-B8	*Flavobacterium*	4-22	16	Psychrotolerant
MBI-A1	*Flavobacterium*	4-22	18	Psychrotolerant
MBI-E5	*Flavobacterium*	4-15	15	Psychrophilic
MBI-G12	*Flavobacterium*	4-22	17	Psychrotolerant
MBI-D6	*Shewanella*	15-30	22	Mesophilic
MBI-A5	*Shewanella*	-	-	-
MBI-7	*Shewanella*	-	-	-

The time of sample collection and the types of samples collected may also have a large impact. Sea ice has been shown to be a rich source of psychrophilic bacteria.[84, 90] Selecting a sample which is not near the surface and so is not subject to freeze thaws or atmospheric UV radiation may also be enriched in psychrophilic bacteria. Selecting samples in late winter would likely increase the chance for ice to be enriched with psychrophilic bacteria.[81, 90]

3.5. Small Scale Fermentations for Natural Product Production

3.5.1. Selection of Strains and Media Conditions

Representative strains of each sequenced group were selected for small scale fermentations in 96-well plates. To allow for ease in conducting and processing a large number of fermentations, all 17 representative organisms were fermented in 1 mL volumes in 96-well plates. Since the optimum conditions for secondary metabolite production of bacteria are not known,[196] a wide variety of media were chosen based on their richness and their different carbon and nitrogen sources. Different carbon sources included peptone, glycerol, glucose, sodium benzoate, soluble starch and others. Different nitrogen sources included sodium ammonium phosphate tetrahydrate, ammonium nitrate, sodium nitrate and others. Media like *Myxobacterium* Medium and *Ancylobacter Spirosoma* Medium were very lean while others like FF and Czapek Dox Broth were very rich. The incubation temperature of 15 °C was used because psychrotolerant bacteria have an optimum growth temperature above 15 °C and psychrophilic bacteria have an optimum growth temperature below 15 °C. Therefore, 15 °C was the best temperature to grow both types.

3.5.2. Results from Small Scale Fermentation

Analyzing data from the LC-MS analysis of the crude extracts is a time consuming step. Dereplication using the Xcalibur software was performed using 164 unique ions to find the best producer and production media for each ion. When choosing the best isolates, the total number of ions and unique ions were taken into account in choosing the best producers. Also, using the Antibase program, the number of compounds isolated from bacteria with the same genus was determined. This gave a general consensus of the amount of investigation previously associated with related isolates and therefore indicated which isolates have been underexplored for NPs. For production media, media showing most diverse production and providing the most coverage where chosen. See **Table 3.7** for media identification and **Table 3.8** for isolates and fermentation media displaying unique ions.

Table 3.7. Media Designations

Designation	Media
2	ACC Media
3	*Ancylobacter Spirosoma* Medium
4	Benzoate Minimal Salt Medium
5	Cyclohexanone Medium
6	Czapek Dox Broth
7	Emerson YpSs Broth
8	FF Broth
9	Marine Broth
10	MMM
11	MPY Agar
12	Myxobacterium Medium

Table 3.8. LC-MS Data Analysis of 96-Well Plate Fermentations

Isolate, Genus	RTT-Mass	Media*	Antibase Hits of Genus
RKSB-1A, *Psychrobacter*			0
	2.16-176.2	2!, 3, 7, 8, 9, 11, 12	
	2.18-162.2	2!, 3, 7, 8, 11, 12	
	2.24-144.2	2!, 7, 8, 11, 12	
RKSB-2A, *Psychrobacter*			0
	2.16-176.2	2!, 9, 8, 11, 12	
	2.18-162.2	2!, 7, 8, 11, 12	
	2.24-144.2	2!, 7?, 8?, 12?	
	2.25-479.4	11	
	4.05-639.1	9!?	
	3.29-441.0	9!?	
RKSB-3A, *Pseudomonas*			629
	1.92-484.6	2, 8, 12!	
	2.01-491.2	12!	
	2.08-439.3	12!	
	2.14-558.3	12!	
	2.17-218.3		
	2.26-553.4	12!	
	2.72-269.2	10!	
	2.91-269.2	10!	
	2.92-297.2	10!	
	3.11-280.5	2!	
	3.42-695.0	10!	
	3.43-649.6	10!	
	3.44-720.7		
	3.45-651.8	9!, 10!	
	3.58-717.7	10!, 11!	
	3.91-575.5	8!	
	4.34-688.3	2!	
	4.55-702.3	8!	
	4.77-549.5	2!, 8	
	4.80-690.3	2!, 7, 8!	
	4.82-717.7	2!, 7, 8	
	4.83-775.5	8!	

*numbers indicate different media (**Table 3.7**), (!) means a prominent MS peak, (?) means a low intensity peak, RTT-MS= Retention Time and Mass/Charge Ratio (m/z) of an Ion; Continued on next page

Table 3.8. Continuation of LC-MS Data Analysis of 96-Well Plate Fermentations

Isolate, Genus	RTT-Mass	Media (Hits)	Antibase Hits of Genus
RKSB-4A, *Pseudomonas*			629
	2.10-354.3	3, 9!, 11	
	3.42-695.0	10!	
	3.43-649.6	10!	
	3.45-651.8	10!	
	3.58-717.7	10!	
	4.19-553.0	8!	
	4.55-702.3	8!	
	4.80-690.3	7!, 8!	
	4.82-717.7	7, 8	
	4.83-775.5	8!	
RKSB-5A, *Pseudomonas*			629
RKSB-6A, *Pseudomonas*			629
	2.72-269.2	10!, 12, 11, 3, 5, 6	
	2.87-295.2	10!, 11, 12, 9, 3	
	2.91-269.1	10!, 12, 11, 3	
RKSB-7A, *Marinomonas*			2
	2.66-250.4	8!?	
	4.47-663.6	5!?	
RKSB-8A, *Marinomonas*			2
	2.16-176.2	7, 9, 10, 11, 12	
	2.21-215.2	2!, 3, 7, 8	
	3.08-253.5	8, 7, 9, 12?	
RKSB-9A, *Rhodococcus*			24
	2.38-222.2	4!, 8?, 2?	
	2.52-363.2	8!	
	4.37-385.4	8!	
RKSB-10A, *Rhodococcus*			24
	2.38-222.2	7!, 8?	
	4.53-761.6	11!, 8?, 4	
	4.61-787.5	4!, 2?, 11?	
RKSB-11A, *Frigoribacterium*			0
	2.16-176.2	2!?	
	2.18-162.2	2!, 3, 4?, 7?, 8!	
	2.39-216.3	2!, 7!	
	2.65-250.3	8!	
	2.71-329.1	7!	
	4.19-553.0	8!	

Continued on next page

Table 3.8. Continuation of LC-MS Data Analysis of 96-Well Plate Fermentations

Isolate, Genus	RTT-Mass	Media (Hits)	Antibase Hits of Genus
RKSB-12A, *Cryobacterium*			0
	2.39-216.3	2!	
	2.65-315.2	2!, 8?	
RKSB-13A, *Pseudoclavibacter*			0
RKSB-14A, *Flavobacterium*			36
	2.39-216.3	2!	
	2.52-363.2	2!, 3, 7, 8, 9, 11	
	2.60-316.5	2!, 8, 9	
	2.65-250.3	2!	
	2.65-315.2	2	
	2.65-335.0	2!	
	2.67-316.4	9!, 10	
	2.68-320.9	2!	
	2.71-329.1	2!	
	3.06-323.2	2!, 3!, 6?, 7, 8, 9	
	3.45-651.8	6!?	
	2.91-269.1	8!?	
RKSB-15A, *Flavobacterium*			36
	2.30-378.3	2, 7!, 11?, 12	
	2.39-216.3	2!	
	2.52-363.2	2!, 7, 8, 9, 11, 12	
	2.60-316.5	2!, 8, 9	
	2.65-250.3	2!, 8	
	2.65-315.2	2!	
	2.65-335.0	2!	
	2.68-320.9	2!	
	2.69-366.4	2!	
	2.71-329.1	2!	
	2.85-215.3	2!	
RKSB-16A, *Shewanella*			1
	2.30-484.6	2!	
	2.60-316.5	2, 8, 9	
	2.65-250.3	2, 7, 8	
	2.73-282.2	2!, 8, 9	
	3.23-205.2	7!, 12, 3	
	4.40-663.4	3!, 4	

Continued on next page

3.6. Scale-up of Small Scale Fermentation

3.6.1. Selection of Isolates and Media Conditions

Six isolates and two media conditions were chosen to keep the work at a manageable amount but still be able to scale up to 24-well plates and 250 mL Erlenmeyer flasks. Six isolates in two media conditions in triplicate and media blanks, require 48 wells which is the exact amount available from two 24-well plates. Alongside the 5 mL fermentations in 24-well plates were 50 mL fermentations in 250 mL Erlenmeyer flasks in order to provide more material for initial fractionation and bioassays. Scaling up to Erlenmeyer flasks also showed the effects transitioning from a 1 mL well in a plate to a 50 mL Erlenmeyer flask can have which accelerated scale-up. 24-well plates were useful for fermenting a larger volume than available in 96-well plates but they also served as a control when scaling up to Erlenmeyer flasks. Changes in fermentations due to a change in fermentation vessels can be due to differences in size and shape which can affect a many different factors which include but are not limited to, aeration, agitation, heat transfer, and cell density.[197]

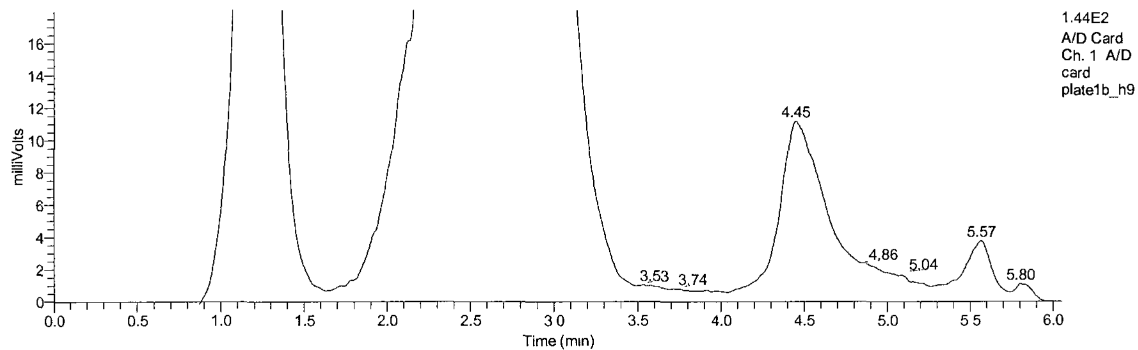
The best production media from the 96-well plate fermentations were generally rich media with the exception of *Ancylobacter Spirosoma* Medium and MMM. FF media in particular is incredibly rich and quite chunky due to the fish meal.

Extractions were performed using HP-20 which is recommended for the extraction of antibiotics and other molecules, and is useful because it can be readily washed for the removal of water soluble components.[198] It is also cheap and disposable. Denatured alcohol was used to elute the organics from the HP-20 resin instead of MeOH because of its more non-polar nature which would also limit the solubility of salt. MeOH was used to

transfer the sample to 96-well plates because the mobile phase in the ALPLC and LC-MS are MeOH. Doing so would limit the amount of material that may irreversibly adhere to the C_{18} columns.

3.6.2. Results from Scale-up of Small Scale Fermentation

Crude extracts were analyzed by LC-ESIMS. The ESI probe was chosen due to its "softer" ionization. The mass spectra were analyzed for new ions however few ions were found. One of the biggest problems in natural products research is the lack of good production of the desired compound. Screening was performed with this in mind so potentially new ions were compared with the ELSD spectra. One ion that had a large corresponding ELSD peak was the 887 ion which had a retention time of 4.3 min (**Figure 3.2**). No corresponding MS or ELSD peaks were observed in the media blank (**Figure 3.3**) so it must have been produced by the bacterium. Combining the remaining extracts from the 50 mL fermentations resulted in enough material to do ALPLC however, the NMR of the corresponding fraction was impure and insufficient for further purification. Searching Antibase and Marinlit revealed numerous hits however, the lack of HR-MS data and inexperience prevented effective dereplication. It was decided to scale up to 1 L in 50 mL fermentations to obtain more of the material for structure elucidation.



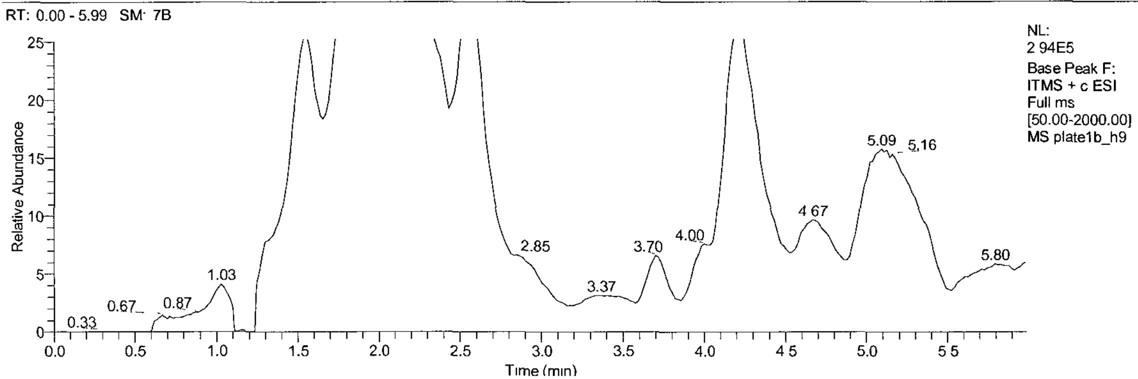
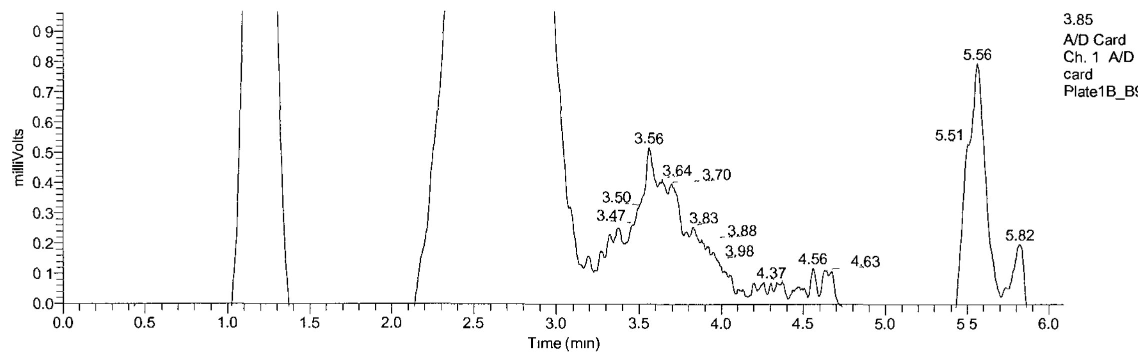


Figure 3.2. ELSD and MS Chromatograms ACC RKSB-11A Crude Extracts



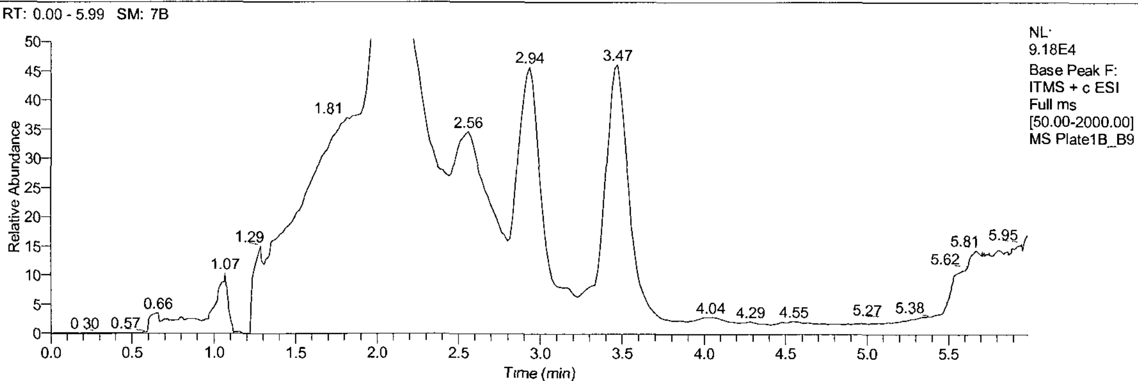


Figure 3.3. ELSD and MS Chromatograms of ACC Media Blank

3.7. RKSB-11A Scale-up and Extraction

3.7.1. Harvesting Optimization

LC-ELSD data indicated that the GGLs were located in the cells. There is a prominent peak at 4.35 min that corresponds to the prominent GGL 887 in the cell pellet sample (**Figure 3.4**), but only a very small peak in the supernatant fraction (**Figure 3.5**). The presence of the small peak at 4.35 could be because some cells did not pellet well and were transferred along with the supernatant during decantation. A definite identification by the MS was not determined because the APCI probe was used and not the ESI probe however, the retention time corresponds to the prominent GGL. What is also observed is that the chromatogram of the cell pellet is greatly simplified due to the absence of the very prominent peak from 0.5-3.0 min which corresponds to the media and other polar compounds. This not only simplifies later fractionation steps due to the lesser complexity but speeds up fractionation due to the greatly reduced quantity of sample needed to be carried forward through the series of fractionations. The presence of the GGLs in the cells also accelerates, simplifies and reduces the cost of the harvesting procedure since HP-20 is not required to obtain the desired product.

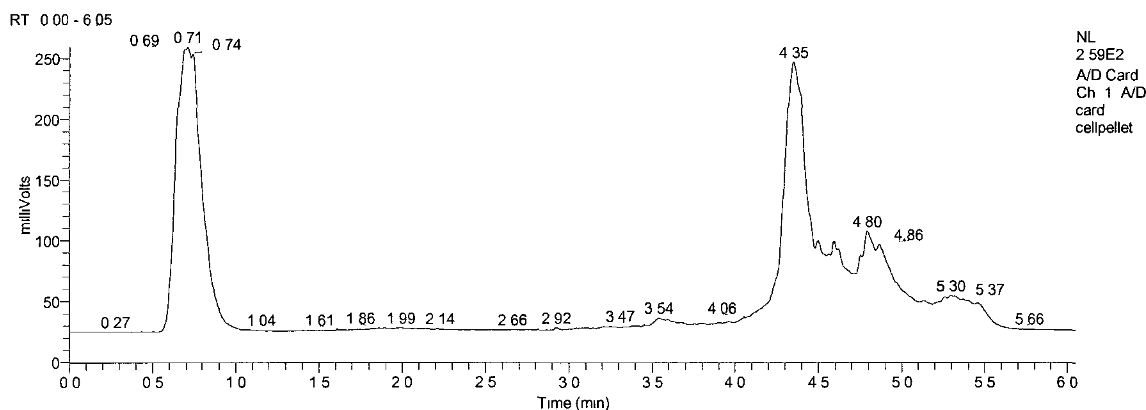


Figure 3.4. ELSD Chromatogram of Crude Extract from Cell Pellet

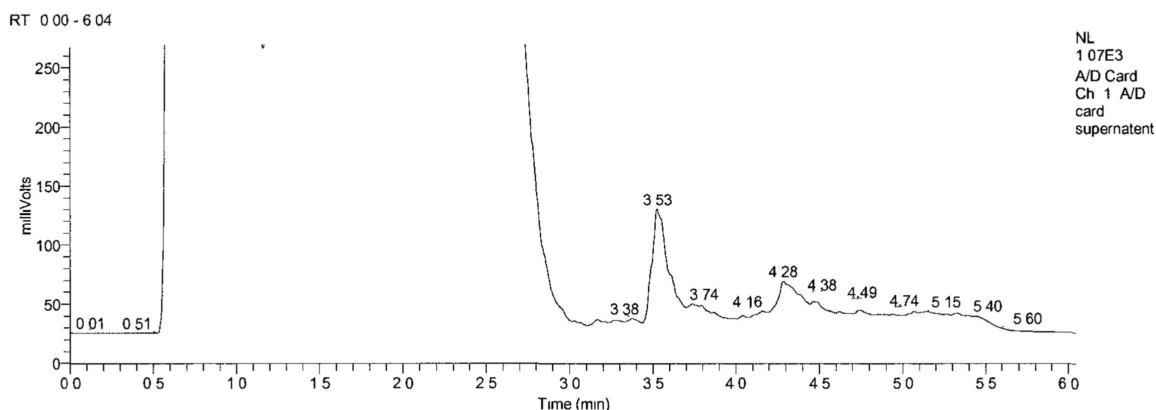


Figure 3.5. ELSD Chromatogram of Crude Extract from Supernatant

3.7.2. Effect of Media on GGL Production

Media supplementation with glycerol and glucose at 20 g/ L has been shown to increase the production of GGLs.[199] The graph in **Figure 3.6** indicates the results obtained. The 10X and 20X correspond to the multiplication of the peak area to keep the data within the dynamic range of the graph. Glycerol supplementation had greatest effect mainly for GGL 887 and both 885 GGLs. The 885 GGLs both have large error bars because of the low quantity, poor peak shape and interference by other compounds which

elute at the same time. Glucose supplementation was best for both GGL 901 and GGL 915. This indicates that glucose supplementations results in GGLs with longer fatty acids. This could be due to the difference in osmotic pressure which results in the increased production of longer fatty acids to increase the melting temperature of the cell walls. A decrease in fatty acid unsaturations was observed for GGLs of *Streptococcus mutans* with increasing sucrose concentration[200] which is another method to increase the melting temperature of the cell walls.[201] We choose to use glucose supplementation because it was observed that there was more diverse and well resolved peaks indicating good production of GGLs earlier in the chromatogram with a reduction in the intensity of the GGL 887 which could complicate fractionation. For the purification and production of GGL 885, it would seem to be better to choose glycerol supplementation. Glycerol supplementation was chosen when scaling up for the 2nd time in order to obtain more of GGL 885 however, the presence of what is believed to be carotenoids complicated purification work.

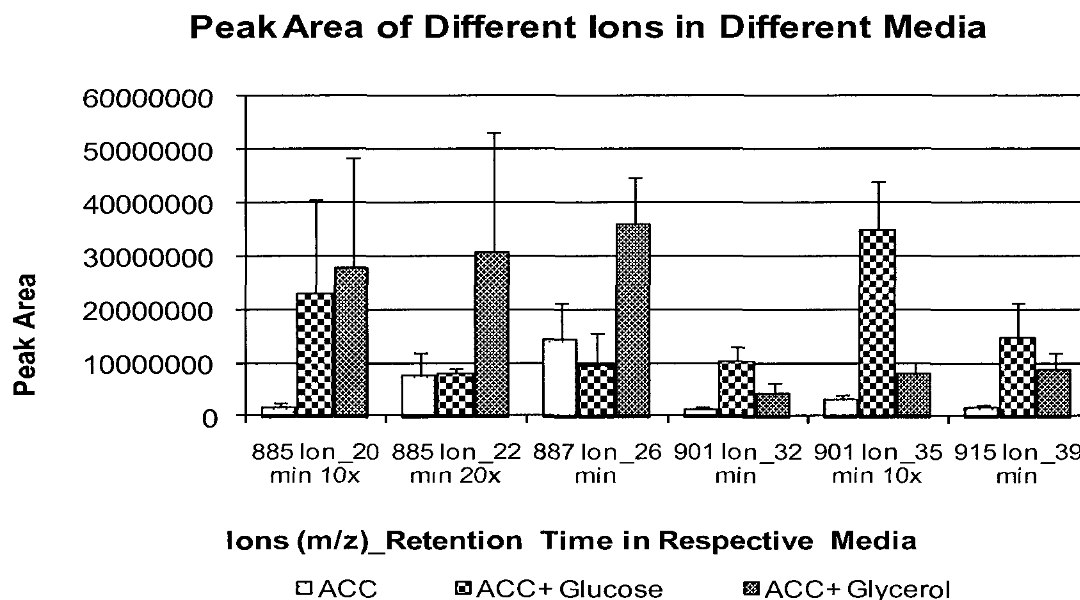


Figure 3.6. Peak Areas of Different Ions Corresponding to GGLs in Three Fermentation Media

3.7.3. Timescale Analysis of Cell Pellet Weight, Cell Density and GGL Production by LC-MS

The data (**Figure 3.7**) showed that cell pellet weight peaked at about day 6 for the 15 °C and RT (21 °C) fermentation however, the 15 °C showed more cell pellet weight overall. This could be due to the mucous that was produced at 15 °C but was not produced in as great a quantity at RT. This would also explain why the OD_{600} for the 15 °C fermentations were lower than the OD_{600} of the RT fermentations for the first 4 days. The OD_{600} peaked between day 2-3 for the RT fermentation and the OD_{600} peaked around day 5 for the 15 °C fermentation. Choosing a compromise between the OD_{600} data and the cell pellet weight data, day 4 would be the optimum time to harvest fermentations at 21 °C. The error bars of the OD_{600} and cell pellet weight are quite small indicating good reproducibility.

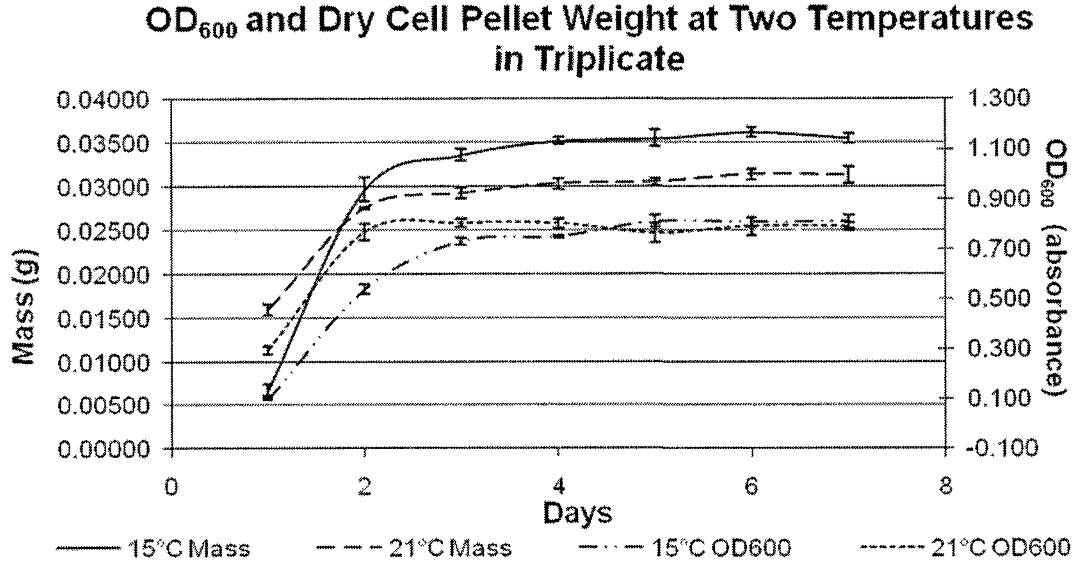


Figure 3.7. Dry Cell Pellet Weights and OD_{600} of Fermentations Incubated at 15 °C and RT in Triplicates

The error bars were quite prominent in the relative quantitation of different GGLs and so were not included in **Figures 3.8** and **3.9**. A trend line for each was included to show the approximate general trends. The peak areas of GGL 885 and 915 were multiplied by a factor of 5 or 10 to bring the data within the dynamic range of the graph. GGL 885 and 915 generally increased in concentration with time at both temperatures, indicating that longer incubation times than those obtained favour their production. At 15 °C, GGL 887 and 917 both drop from higher values from day 1-2 until day 4 at which they recover to less than or equal to day 1 levels which is indicated by the declining or levelling of the trend line. At 22 °C however, GGL 887 and 917 continuously increase indicating that it is a favourable fermentation temperature.

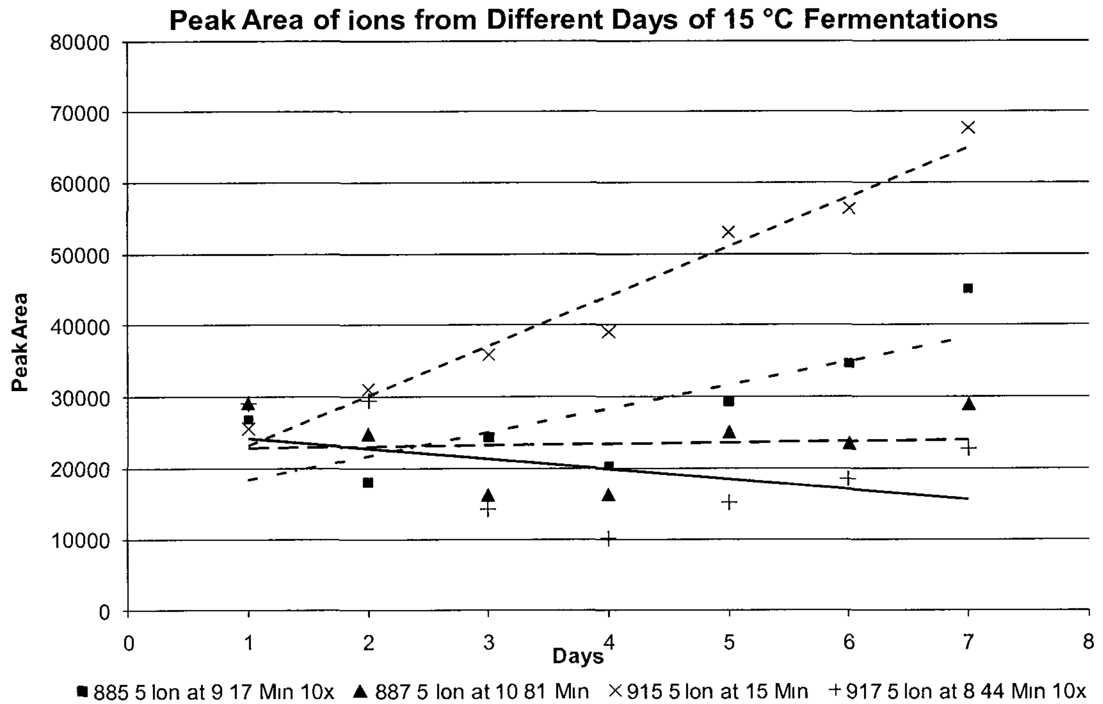


Figure 3.8. Peak Areas of 885, 887, 915 and 917 m/z Ions at Different Days from 15 °C Fermentations

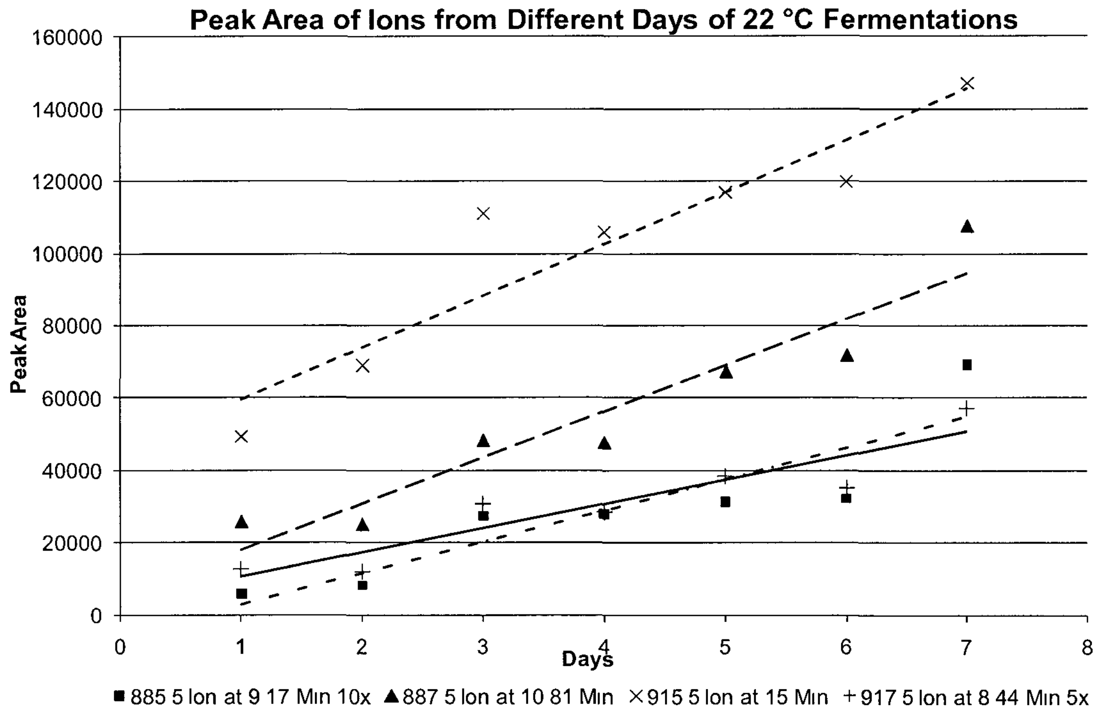


Figure 3.9. Peak Areas of 885, 887, 915 and 917 m/z Ions at Different Days from 21 °C Fermentations

3.8. Chromatographic Purification of GGLs 873, 885, 887 and 915

Preliminary fractionation was performed by ALPLC in order to roughly separate the media components from the desired product. Salts and polar media components like sugars and peptone are separated from more non-polar compounds. Fats and very non-polar compounds are retained on the C_{18} and are eluted in the EtOH wash. The tubes were combined into about 6 fractions using the ALPLC chromatogram as a guide (**Figure 3.10**). They were concentrated under vacuum and the last 3 fractions were analyzed by LC-ESIMS and NMR since the desired compound was generally non polar. Using this approach, the GGLs of interest were located in fraction 5.

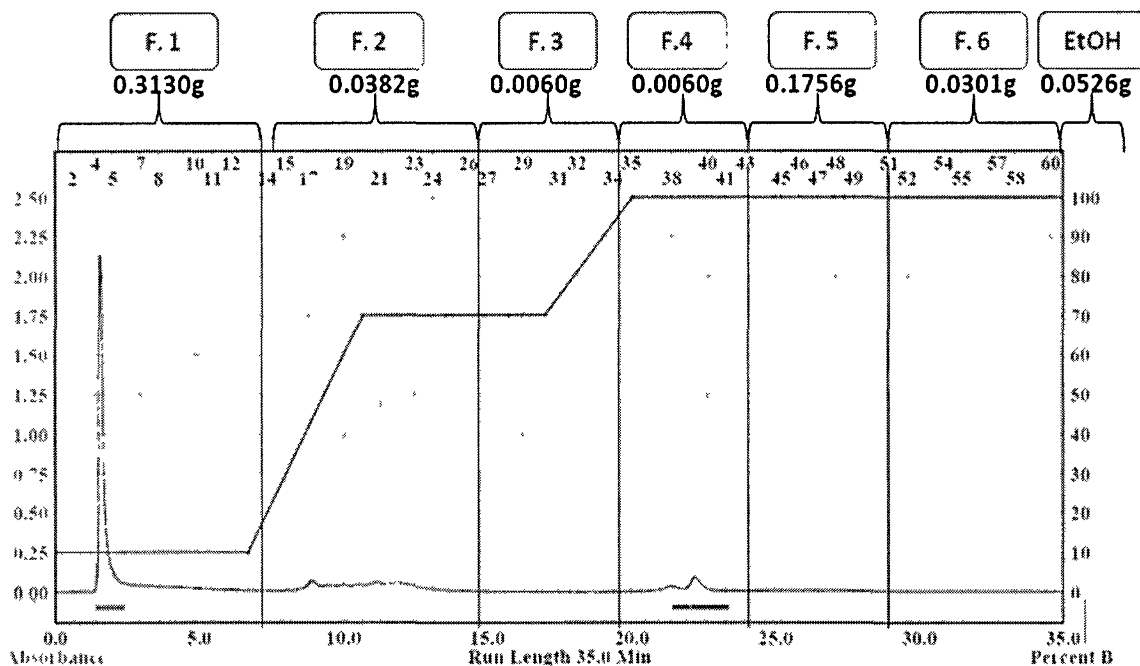


Figure 3.10. ALPLC Chromatogram of Crude Extract from 6 L Fermentation of RKSB-11A in ACC+Glucose Media.
On the x-axis is the retention time in minutes. The wavelength was 214 nm with a threshold of 0.2 AU. The middle line is the water:MeOH gradient with the MeOH percentage at the right and the numbers at the top are the tubes the fractions are being collected in. F.1-F.6 and EtOH are the different fractions.

HPLC fractionation on a C_{18} column was performed using an isocratic method to prevent the need for column re-equilibration after each injection. Known GGLs were generally present in multiple milligram quantities per liter of fermentation culture while unknown GGLs were present in microgram quantities. Nine fractions were collected where the head and tail fractions were collected together and the other 8 fractions were collected separately (**Figure 3.11.**).

Peak 8 had a mass of 915 m/z by LC-ESIMS and has been previously reported.[114, 123, 125] It was purified by HPLC and was characterized by NMR, IR, LC-ESIMS and polarimetry as 1-O-acyl-3-[α-D-mannopyranosyl-(1–3)-(6-O-acyl-α-D-mannopyranosyl)]-*sn*-glycerol. Peak 8 was used as the standard and for procedure development since it was quite abundant. Peak 4 had a mass of 887 m/z by LC-ESIMS and has previously been reported.[114] It was purified by HPLC and characterized by NMR, LC-ESIMS and IR as a second standard. Other GGLs present in multiple milligram quantities were located in peak 5 with a mass of 913 m/z, peak 6 with a mass of 901 m/z and peak 7 with a mass of 901 m/z. GGL 913 has been previously reported and one analogue of GGL 901 has been previously reported.[114]

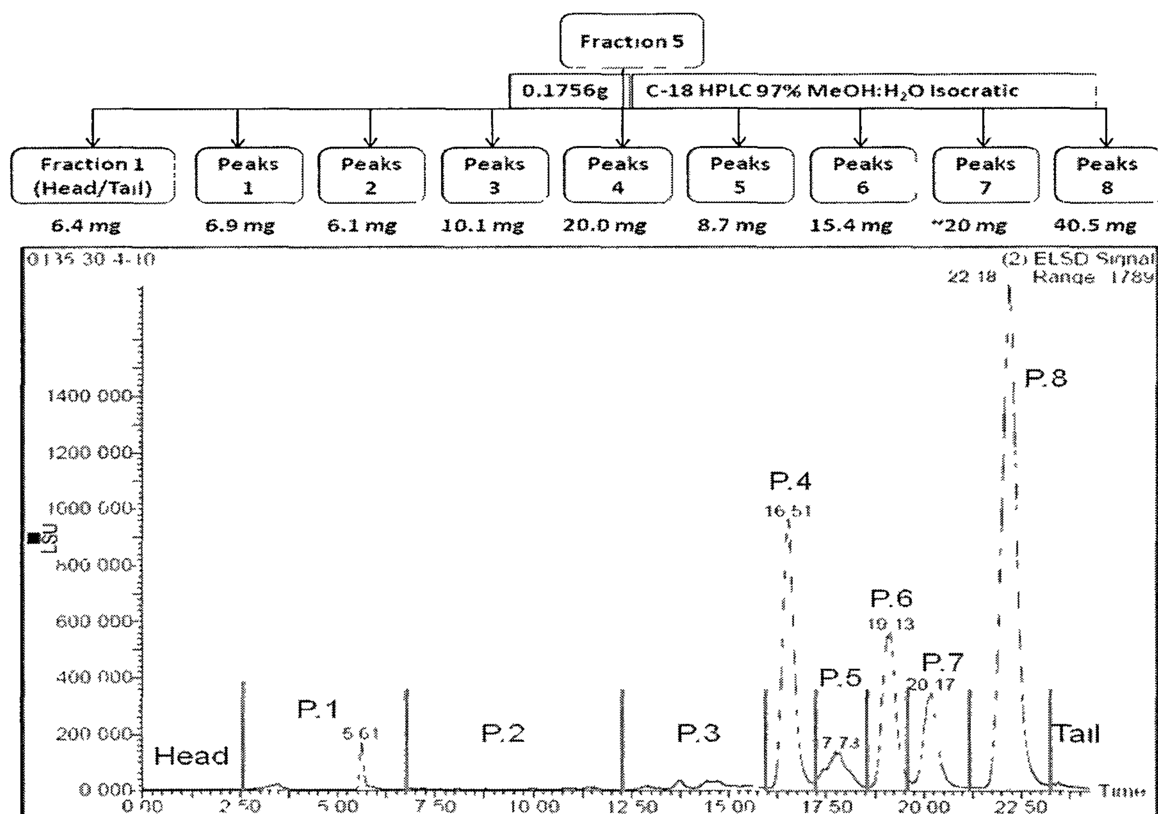


Figure 3.11. Fraction Weights and HPLC Fraction of 6 L Fermentation Fraction 5. The x-axis refers to the retention time in minutes and the y-axis refers to the signal intensity in Light Scattering Units (LSU). LSU can be directly converted to millivolts (mV) in the current settings. Fractions collected are separated by the lines and labelled as Head, P.1-P.8 and Tail.

Peak 3 (**Figure 3.12**) was fractionated by HPLC into six fractions with the head and tail collected together and five other fractions collected separately. Fraction 2 was purified by HPLC and characterized by NMR, IR, LC-ESIMS, polarimetry and HR-MS. Fraction 1 was fractionated but the mass of the resulting fractions were less than 500 µg which is insufficient for NMR characterization. Fraction 3 was fractionated but due to degradation or loss of product during HPLC, only about 500 µg product remained which was still impure. Scale up was required in order to obtain more of Fraction 2 for the surface tension analysis and to try to obtain another new GGL. It was not necessary to

obtain more of fraction 2 after the work was complete because it was discovered that the GGLs did not exhibit surface tension activity when tested with GGL 915 as shown later.

A 12 L fermentation was completed and extracted. Fractionation was performed using ALPLC and HPLC. The ratio of GGLs changed due to the change in supplementation from glucose to glycerol. The ALPLC fraction was fractionated into three fractions to eliminate the polar compounds and the large amount of GGL 887 in the fraction (**Figure 3.13**). A lower yield of Peak 885M compared to Peak 3 was obtained possibly due to harvesting the fermentation on day four instead of day seven,. Peak 885M from the 12 L fermentation and Peak 3 from the 6 L fermentation correspond to the same region in the chromatogram. The desired product was a little higher in ratio compared to the other GGLs in the same fraction when comparing between the glucose and glycerol supplementation. This agrees with our data from the media supplementation study.

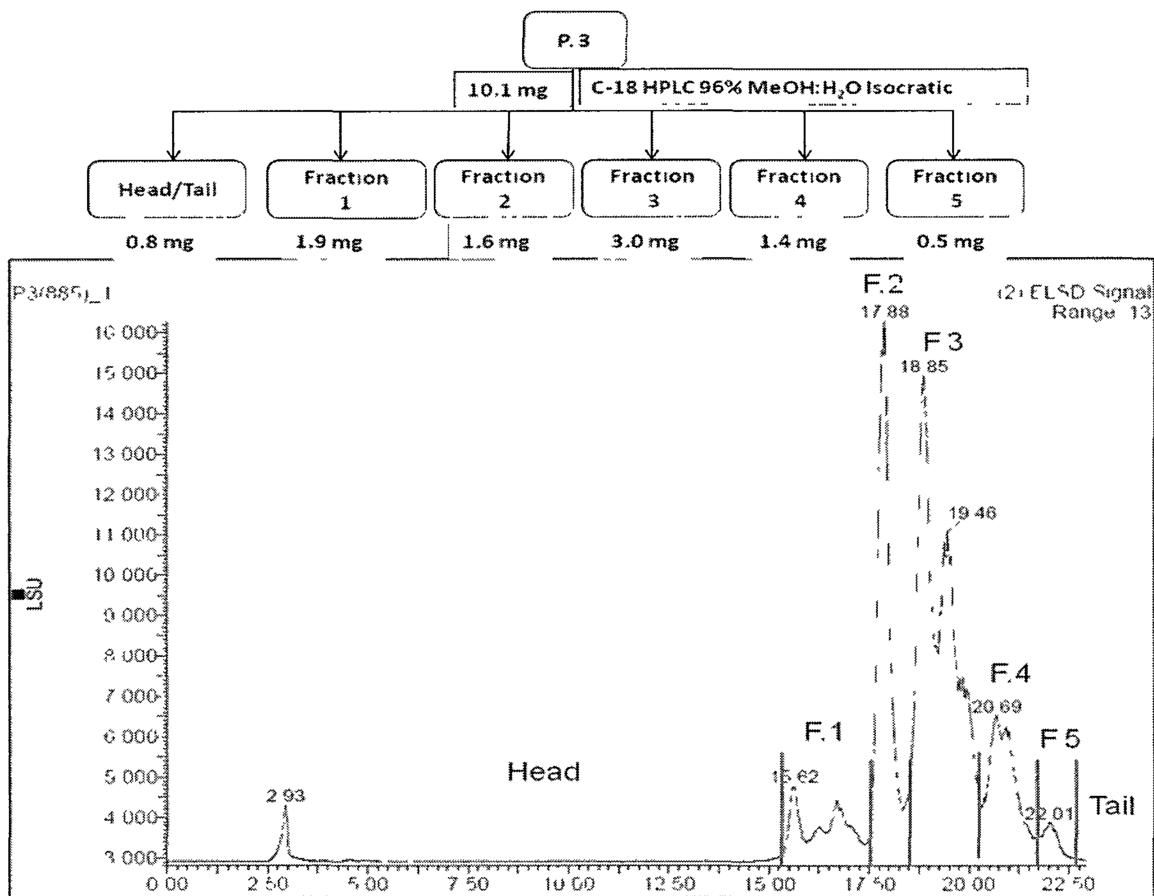


Figure 3.12. Fraction Weights and HPLC Fraction of 6 L Fermentation Fraction P.3

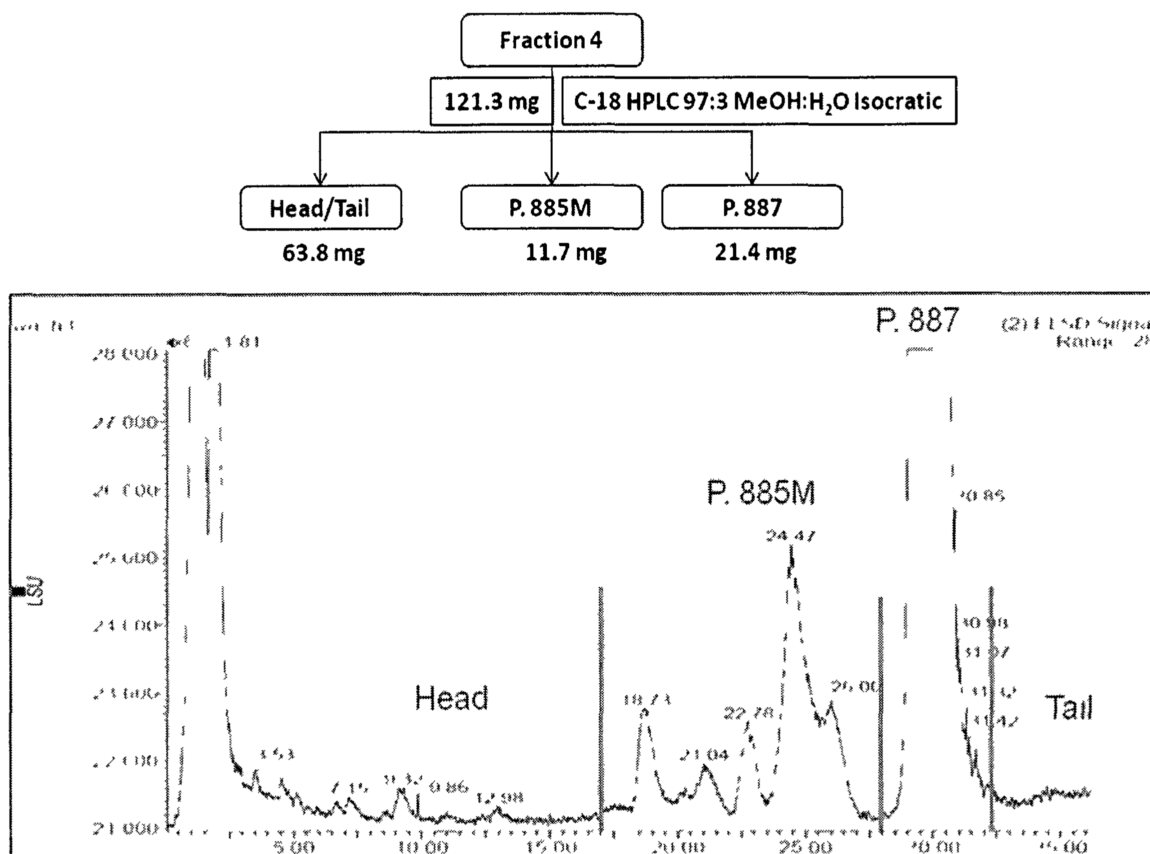


Figure 3.13. Fraction Weights and HPLC Fraction for 12 L Fermentation Fraction 4

Peak 885M was fractionated into seven fractions with the desired fraction being fraction 5 (**Figure 3.14**). Fraction 5 was re-purified by HPLC but was found to have degraded. NMR data indicated that the fatty acids had hydrolyzed from the glycoglycerol backbone and were primarily free fatty acids. This is believed to have occurred possibly due to FA contamination of the MeOH solvent when changing solvents. The compound is believed to be GGL 873 which has been previously observed by MS.[122]

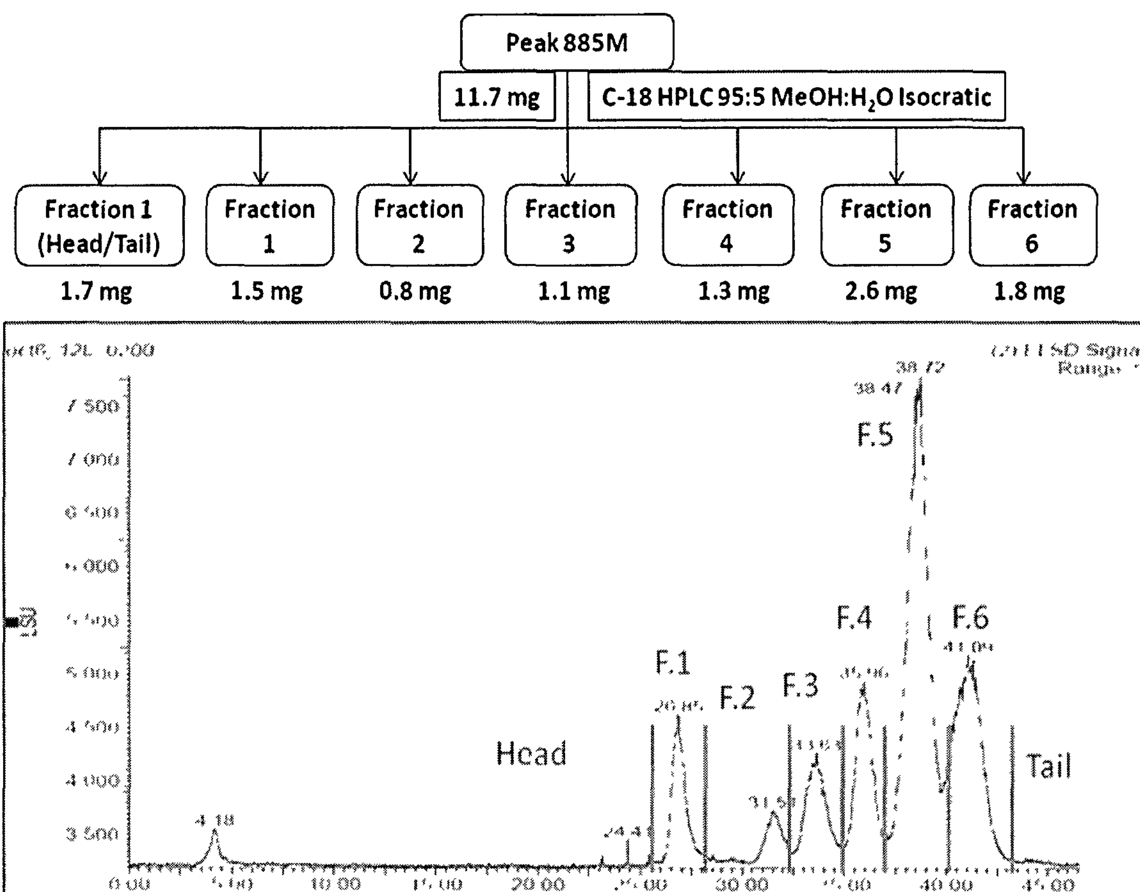


Figure 3.14. Fraction Weights and HPLC Fraction of 12 L Fermentation Fraction P885M

3.9. Spectroscopic Characterization of GGL 915

3.9.1. Analysis of NMR Data

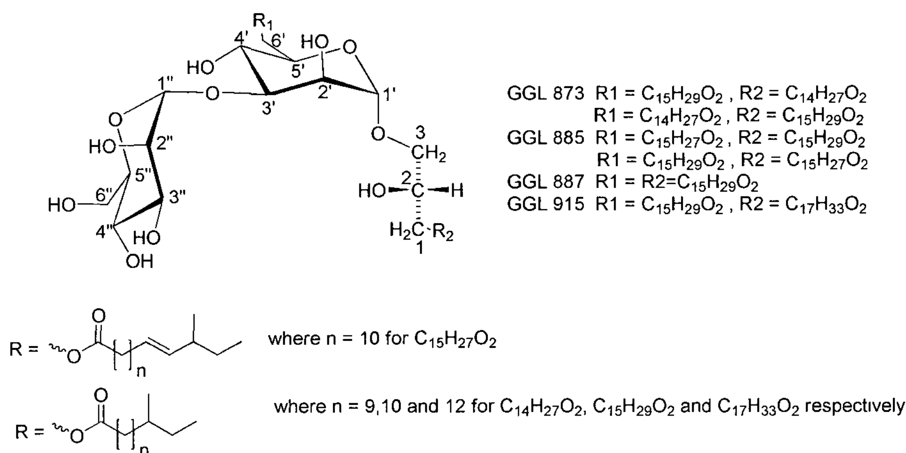


Figure 3.15. Structures of GGLs Isolated from RKSB-11A in ACC Media.

GGL 915 was characterized using 1D and 2D NMR techniques. 1D NMR techniques included ^1H and ^{13}C NMR. 2D NMR techniques included Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple bond Coherence (HMBC), Correlation Spectroscopy (COSY) and Nuclear Overhauser Effect Spectroscopy (NOESY). GGL 915 was compared with literature 1D NMR shifts (**Table 3.9**). The glycerol and sugar NMR shifts were compared with GGL 915 from Niepel *et al.*[114] but not the fatty acids because these authors had miss-assigned the fatty acid tail. The solvent they used was CD_3OD. The fatty acid was compared to that by Wicke[202] however, a noticeable difference is the $^1H_{\omega-2}$ with a 1 ppm difference. The solvent used was $CD_3OD/CDCl_3$ (30:70) however it would not be expected that a tertiary carbon on an alkyl chain would have such a high shift. A typical ^{13}C chemical shift calculated using ChemDraw[203] is about 1.6 ppm. Possible reasons for their assignment could be misinterpretation of the ringing signals of

the α protons on the fatty acid as additional signals and the presence of some additional signals that could be explained as α protons of free fatty acids.

Table 3.9. 1D NMR Data (600 MHz, CD_3OD) of GGL 915 and Literature Reference[114] for Sugars and Literature Reference[202] for Fatty Acids

	GGL 915		Literature References	
Position	δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]
1a	66.4, CH_2	4.14 (dd, 4.5, 11.7)	65.4	4.14 (dd, 4.9, 11.4)
1b		4.09 (dd, 5.6, 11.3)		4.10 (dd, 6.0, 11.4)
2	69.4, CH	3.97 (m)	68.5	3.99 (m)
3a	69.9, CH_2	3.72 (dd, 4.9, 10.2)	69.3	3.76 (m)
3b		3.43 (dd, 6.4, 10.2)		3.44 (dd, 6.6, 10.4)
1'	102.3, CH	4.72 (d, 1.9)	101.2	4.77 (d, 1.8)
2'	71.2, CH	4.07 (dd, 1.8, 3.4)	69.9	4.05 (dd, 1.8, 3.2)
3'	80.2, CH	3.83 (dd, 3.0, 9.4)	79.0	3.84 (m)
4'	67.8, CH	3.74 (m)	66.7	3.77 (m)
5'	72.5, CH	3.75 (m)	71.4	3.76 (m)
6'a	65.1, CH_2	4.29 (dd, 1.5, 11.7)	64.1	4.39 (d, 11.8)
6'b		4.21 (dd, 6.4, 11.7)		4.29 (dd, 5.8, 11.8)
1"	103.9, CH	5.07 (d, 1.5)	102.3	5.09 (d, 1.5)
2"	72.1, CH	3.96 (dd, 1.5, 3.4)	70.7	3.97 (dd, 1.9, 3.3)
3"	72.6, CH	3.79 (dd, 3.4, 9.4)	71.4	3.80 (m)
4"	68.9, CH	3.59 (dd, 9.8, 9.8)	67.8	3.61 (dd, 9.4, 9.4)
5"	75.0, CH	3.76 (m)	73.6	3.75 (m)
6"a	63.0, CH_2	3.84 (dd, 1.8, 11.7)	61.9	3.85 (m)
6"b		3.68 (dd, 6.4, 11.3)		3.73 (m)
C=O	175.5, 175.3, qC		174.9, 174.7	
α	35.1, 35.0, CH_2	2.35, 2.35 (t, 7.5)	34.4	2.35 (m)
β	26.1, 26.1, CH_2	1.62, 1.62 (q, 7.2)	25.2	1.62 (m)
$-CH_2-$	31.2-30.3, 28.2, CH_2	1.35-1.25 (m)	30.3-29.5	1.35-1.23
ω-3a	37.9, CH_2	1.10 (m)	37.0	
ω-3b		1.31 (m)		1.26 (m)
ω-2	35.8, CH	1.30 (m)	34.7	2.35 (m)
ω-2 (CH_3)	19.7, CH_3	0.85 (d, 6.4)	19.4	0.88 (d, 6.8)
ω-1a	~30.5, CH_2	1.14 (dq, 7.5, 14.7)	29.9	1.12 (m)
ω-1b		1.29 (m)		1.26 (m), 1.52 (m)
ω	11.8, CH_3	0.87 (t, 7.2)	11.5	0.86 (t, 6.5)

[a] In ppm, Multiplicity [b] In ppm, multiplicity and J in Hz are in parenthesis; s, singlet; d, doublet; t, triplet; dq, double quintet; m, multiplet

3.9.2. Analysis of MS, IR and Sugar Structure Elucidation

Tandem Mass spectrometry (MS^2) fragmentation data of GGL 915 was obtained (**Figure 3.16**). The 897 ion corresponded to a loss of water. The most prominent ion, 753, corresponded to the loss of the terminal sugar. The 645 and 673 ions corresponded to the loss of a fatty acid at either location on the GGL backbone. The 571 ion corresponded to the loss of the glycerol and the attached fatty acid. Since the fatty acid on the glycerol

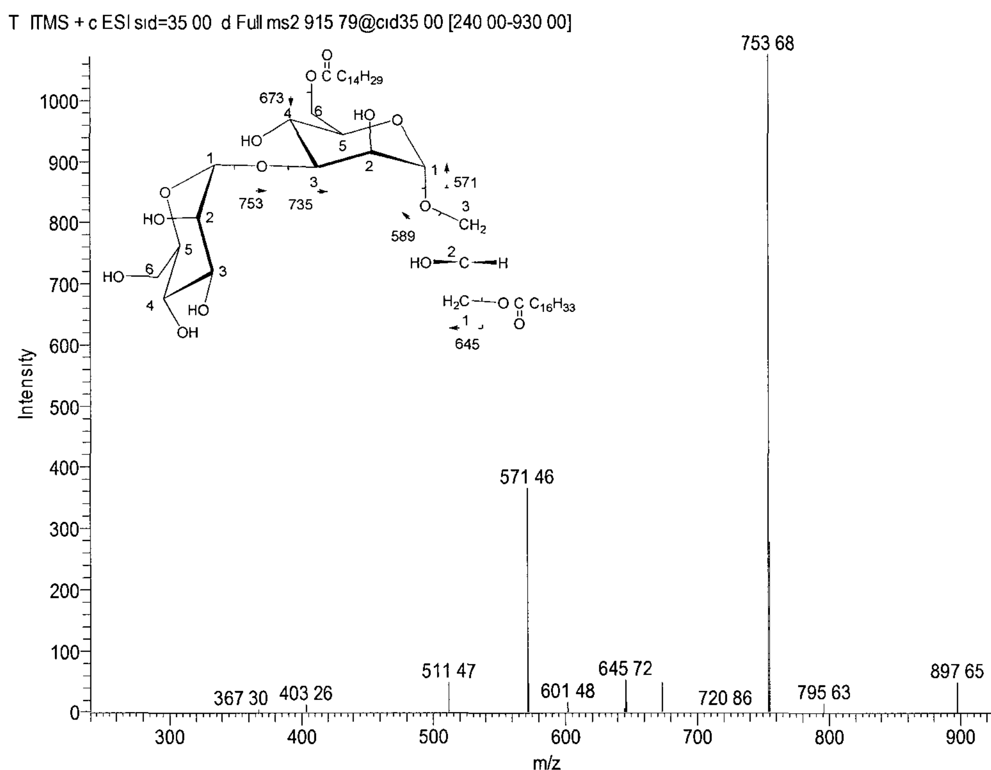


Figure 3.16. Major MS^2 Fragmentation Patterns of 915.79 Ion $[M+Na]^+$

must be lost with the glycerol, it can be used to determine where each fatty acid is located. A 599 ion would be expected if the shorter fatty acid was attached to the glycerol. The 511 ion is the loss of a sugar and the shorter fatty acid. It is interesting that

only the 511 ion is observed and not the 483 ion which would correspond to the loss of a sugar and the longer fatty acid. The 403 ion corresponded to the loss of both fatty acids.

The IR contained the expected signals (cm^{-1}) of 3350 (-OH), 2920 and 2851 (-CH_2-), 1737 (ester C=O), 1465 (-CH_2- bend), 1377 (CH_3 bend) and 1053 (C-O).

For polarimetry, a comparison with the specific rotation obtained by Niepel *et al.*, +16.2°,[114] shows they are similar in sign but are different by 31.2°. This could be due to the low concentrations used, i.e c = 0.11 and 0.53, and also the strong interference of impurities due to the weak optical rotation of GGL 915. When comparing with Yanagi *et al.*, they obtained an optical rotation of +28.2° at a concentration of 7.3.[123] However, they used chloroform as the solvent instead of MeOH.

For sugar identification, two different methods were performed. Method one was hydrolysis of the GGL, the isolation of the monosaccharides, and comparing them to a standard using HPLC and polarimetry. The HPLC column used was not ideal for the identification of hexose monosaccharides due to similar retention times.[204] The column used could however, distinguish glucose from the other monosaccharides and could suggest if a sample was composed of more than one type of monosaccharide based on the peak shape. The retention time of the mannose standard was 12.88 min which was very close to that of the sample which had a retention time of 12.98 min. A single large peak was observed indicating that both sugars were likely the same. Galactose is also commonly found in GGLs[112] and has a retention time close to that of mannose.[204]

By polarimetry, the specific rotation of a D-galactose standard was +78.9°, which is in good agreement with the literature.[205] The specific rotation of D-mannose standard

was +14.4°, which is close to the literature value of +14.5°.[206] The sample had a specific rotation of +9.2° which suggests that the sugars are D-mannose.

The second method to identify the sugars was through the use of coupling constants. The experimental data (**Table 3.10**) was similar to those previously reported.[207] The δ 1.8 Hz $J_{1'-2'}$ and δ 1.5 Hz $J_{1''-2''}$ indicates equatorial-equatorial coupling. The δ 3.4 Hz $J_{2'-3'}$ and $J_{2''-3''}$ indicate equatorial-axial coupling. The δ 9.4 Hz $J_{3'-4'}$ $J_{3''-4''}$ indicate axial-axial coupling. The δ 9.8 Hz $J_{4''-5''}$ indicates an axial-axial coupling. A coupling constant of δ 11.7 Hz is indicative of geminal coupling for $J_{6'a-6'b}$ and $J_{6''a-6''b}$.

The stereochemistry of C-2 on the glycerol can be assigned based on a pattern in the ^{1}H NMR. It has been reported that for the S configuration, the chemical shifts of the protons on C-1 are generally very close i.e 0.01 ppm and the H2-H1 coupling constants for the lowest proton with the lowest chemical shift on C1 is greater than that of the proton with the higher chemical shift. For the R configuration, the C-1 proton chemical shifts are generally farther apart and the H2-H1 coupling constants for the proton with the lowest chemical shift on C1 is less than that of the proton with the higher chemical shift.[208] Based on this trend, the glycerol for GGL 915 would be assigned the R configuration. The same results were obtained by Yangi *et al.* using the same method.[123]

Table 3.10. Coupling Constants of GGL 915 and Mannose Disaccharide

	GGL 915		α-D-Man*p*-(1-3)-α-D-Man*p*-1Me[a207]
Position	δ_H ppm	J[Hz](→H)	J[Hz](→H)
1'	4.72	1.9 (2')	1.7 (2')
2'	4.07	1.8 (1'), 3.4 (3')	1.7 (1'), 3.4 (3')
3'	3.83	3.0 (3'), 9.4 (4')	3.4 (2'), 9.5 (4')
4'	3.74		9.5 (3'), 9.8 (5')
5'	3.75		9.8 (4'), 2.2 (6'a), 6.0 (6'b)
6'a	4.29	1.5 (5'), 11.7 (6'b)	2.2 (5'), 12.2 (6'b)
6'b	4.21	6.4 (5'), 11.7 (6'a)	6.0 (5'), 12.2 (6'a)
1"	5.07	1.5 (2")	1.7 (2")
2"	3.96	1.5 (1"), 3.4 (3")	1.7 (1"), 3.4 (3")
3"	3.79	3.4 (2"), 9.4 (4")	3.4 (2"), 9.5 (4")
4"	3.59	9.8 (3"), 9.8 (5")	9.5 (3"), 9.8 (5")
5"	3.76		9.8 (4"), 2.2 (6"a), 6.0 (6"b)
6"a	3.84	1.8 (5"), 11.7 (6"b)	2.2 (5"), 12.2 (6"b)
6"b	3.68	6.4 (5"), 11.3 (6"a)	6.0 (5"), 12.2 (6"a)

[a]Man*p*, mannopyranosyl

Amorphous solid; $[\alpha]^{20}_D$ +47.4 (*c* 0.11 , MeOH); IR (ATR) ν_{max} 3350, 2920, 2851, 1737, 1053; ^{1}H and ^{13}C NMR data, see **Table 3.9.**

3.10. Spectroscopic Characterization of GGL 887

3.10.1. Analysis of NMR Data

GGL 887 was characterized using 1D and 2D NMR experiments (**Table 3.11**) through comparison to NMR data obtained for GGL 915 (**Table 3.9** and **3.10**).

Table 3.11. 1D and 2D NMR Data (600 MHz, CD_3OD) of GGL 887

Position	δ_C[a]	δ_H[b]	COSY (H→H)	HMBC (H→C)	NOESY (H→H)
1a	66.4, CH_2	4.14 (dd, 4.5, 11.3)	1b, 2	C=O, 2	3b, 2, 1b
1b		4.09 (dd, 5.6, 11.3)	1a, 2	C=O, 2	1a, 2, 3', 3b
2	69.4, CH	3.97 (m)	3a, 3b, 1a, 1b	3	1', 3a, 3b
3a	69.9, CH_2	3.73 (dd, 4.9, 10.2)	3b, 2	1', 3, 2, 2'	1', 2, 3b
3b		3.43 (dd, 6.4, 10.2)	3a, 2	1', 2, 3	1', 3a, 2, 1a, 1b
1'	102.3, CH	4.72 (d, 1.8)	2', 3'	2', 5', 3	3b, 3a, 2',2
2'	71.2, CH	4.07 (dd, 1.8, 3.4)	1', 3'	3, 4', 2	1'
3'	80.2, CH	3.82 (dd, 3.4, 9.0)	2', 1', 4'	2", 4'	1", 1b, 5'
4'	67.8, CH	3.73 (m)	3', 5'	5'	1"
5'	72.5/72.6, CH	3.76 (m)	6'a, 6'b, 4'		6'a, 6'b, 3'
6'a	65.1, CH_2	4.38 (dd, 1.8, 11.2)	6'b, 5'	C=O	6'b, 5'
6'b		4.21 (dd, 6.0, 11.3)	6'a, 5'	C=O, 5'	6'a, 5'
1"	103.9, CH	5.06 (d, 1.5)	2"	3', 5", 3", 2"	2', 3', 2"
2"	72.1, CH	3.96 (dd, 1.5, 3.4)	1", 3"	2', 4"	1", 3"
3"	72.6/72.5, CH	3.79 (dd, 3.4, 9.0)	4", 2"	4", 5"	2", 4"
4"	68.9, CH	3.58 (dd, 9.8, 9.8)	3", 5"	6", 5", 3"	3", 5"
5"	75.6, CH	3.76 (m)	4", 6"b	6"b	4", 6"a, 6"b
6"a	63.0, CH_2	3.84 (dd, 2.3, 11.7)	6"b		6"b, 4"
6"b		3.68 (dd, 6.4, 11.7)	6"a, 5"	5"	6"a, 5"
C=O	175.5, 175.4, qC			α, β, 6'a, 6'b	
α	35.1, 35.0, CH_2	2.35, 2.35 (t, 7.5)	β	C=O	β
β	26.1,26.1, CH_2	1.62, 1.61 (q, 7.2)	α, $-CH_2-$	C=O	α
$-CH_2-$	31.2-30.3, 28.2, CH_2	1.29 (m)	β		
ω-3a	37.9, CH_2	1.09 (m)			
ω-3b		1.29 (m)			
ω-2	35.7, CH	1.29 (m)			
ω-2 (CH_3)	19.7, CH_3	0.85 (d, 6.4)			
ω-1a	~30.5, CH_2	1.29 (m)			
ω-1b		1.13 (dq, 7.5, 14.7)			
ω	11.8, CH_3	0.86 (t, 7.5)			

[a] In ppm, Multiplicity [b] In ppm, multiplicity, J in Hz; s, singlet; d, doublet; t, triplet; dd, double doublet; dq, double quintet; m, multiplet

3.10.2. Analysis of MS, IR and Sugar Structure Elucidation

MS^2 fragmentation data of GGL 887 (**Figure 3.17**) was similar to that for GGL 915. The 869 ion corresponded to a loss of water. The most prominent ion, 725, corresponded to the loss of the terminal sugar. The 645 ion corresponded to the loss of either fatty acid. Since both fatty acids are the same, there is only one signal corresponding with the loss of a fatty acid. The 589 ion corresponded to the loss of a glycerol and fatty acid minus a hydroxyl group. The 571 ion corresponded to the loss of a fatty acid and glycerol. The 483 ion corresponded to the loss of a fatty acid and terminal sugar.

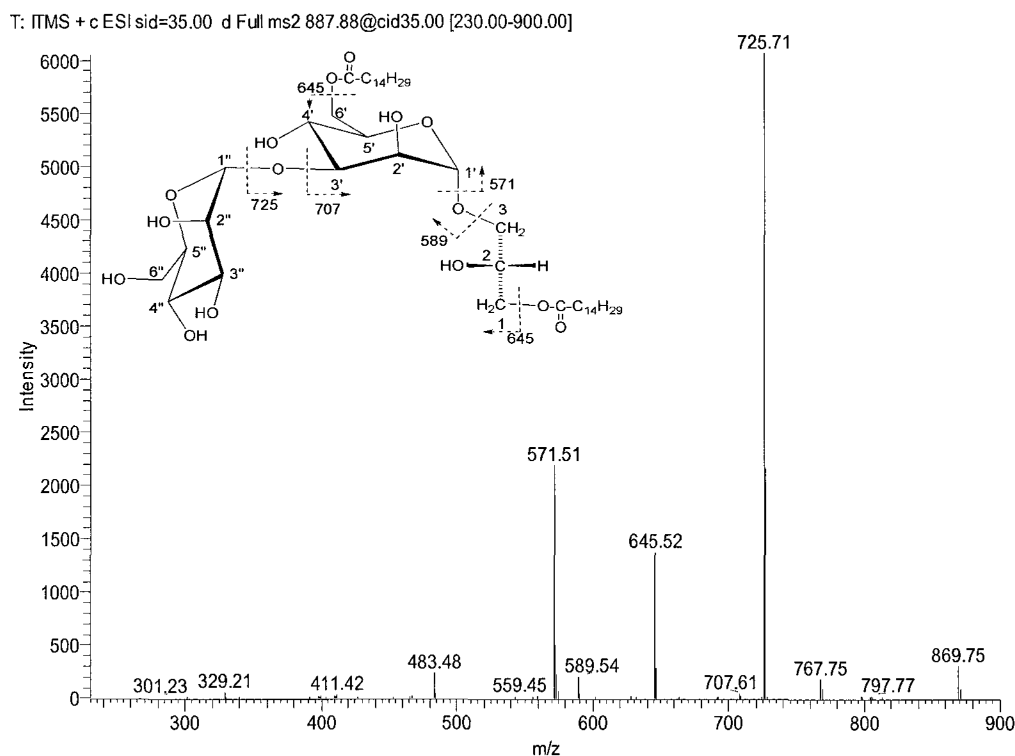


Figure 3.17. Major MS^2 Fragmentation Patterns of 887.88 Ion $[M+Na]^+$

Hydrolysis of GGL 887 was performed and the sugars extracted. The GGL 887 sugar sample had a retention time of 10.56 min which was very close to the mannose standard of 10.49 min using HPLC. Since only a single large peak was observed, this excluded glucose as a possible sugar. Polarimetry of the D-mannose and the sample yielded specific rotations of +14.4 and +14.4 respectively resulting in an exact match suggesting it was D-mannose.

^{1}H NMR in phosphate buffered D_2O was performed. The spectrum was a match with that of D-mannose, supporting D-mannose as the GGL 887 sugar. See Appendix I for spectra.

The coupling constants were the same as previously reported as observed in **Table 3.12**. The δ 1.8 Hz $J_{1'-2'}$ and δ 1.5 Hz $J_{1''-2''}$ indicate equatorial-equatorial coupling. The δ 3.4 Hz $J_{2'-3'}$ and $J_{2''-3''}$ indicate equatorial-axial coupling. The δ 9.0 Hz $J_{3'-4'}$ and $J_{3''-4''}$

Table 3.12. Coupling Constants of GGL 887 and Mannose Disaccharide

	GGL 887		α-D-Man*p*-(1-3)-α-D-Man*p*-1Me[207]
Position	δ_H ppm	J[Hz](→H)	J[Hz](→H)
1'	4.72	1.8 (2')	1.7 (2')
2'	4.07	1.8 (1'), 3.4 (3')	1.7 (1'), 3.4 (3')
3'	3.82	3.4 (2'), 9.0 (4')	3.4 (2'), 9.5 (4')
4'	3.73		9.5 (3'), 9.8 (5')
5'	3.76		9.8 (4'), 2.2 (6'a), 6.0 (6'b)
6'a	4.38	1.8 (5'), 11.2 (6'b)	2.2 (5'), 12.2 (6'b)
6'b	4.21	6.0 (5'), 11.3 (6'a)	6.0 (5'), 12.2 (6'a)
1"	5.06	1.5 (2")	1.7 (2")
2"	3.96	1.5 (1"), 3.4 (3")	1.7 (1"), 3.4 (3")
3"	3.79	3.4 (2"), 9.0 (4")	3.4 (2"), 9.5 (4")
4"	3.58	9.8 (3"), 9.8 (5")	9.5 (3"), 9.8 (5")
5"	3.76		9.8 (4"), 2.2 (6"a), 6.0 (6"b)
6"a	3.84	2.3 (5"), 11.7 (6"b)	2.2 (5"), 12.2 (6"b)
6"b	3.68	6.4 (5"), 11.7 (6"a)	6.0 (5"), 12.2 (6"a)

indicate axial-axial coupling. The δ 9.8 $J_{4''-5''}$ indicates an axial-axial coupling. A coupling constant of δ 11.3 Hz and δ 11.7 Hz is indicative of germinal coupling for $J_{6'a-6'b}$ and $J_{6''a-6''b}$ respectively. Based on the trend as explained in the **Section 3.9**, the glycerol is assigned the R configuration.

Amorphous solid; ^{1}H, ^{13}C, COSY, NOESY, and HMBC NMR data, see **Table 3.11**.

3.11. Spectroscopic Characterization of GGL 885

3.11.1. Analysis of NMR Data

GGL 885 was characterized using 1D and 2D NMR techniques which include Rotating-frame Overhauser Effect Spectroscopy (ROESY) as shown in **Table 3.13.** HSQC was used to assign the ^{1}H signals to the ^{13}C signals. For the ^{1}H NMR spectrum, the CD_3OH solvent peak was referenced at 4.84 ppm and the CHD_2OD solvent peak referenced at 3.31 ppm. For the ^{13}C NMR spectrum, the CD_3OD solvent peak was referenced at 49.05 ppm. The multiplicities were determined by HSQC.

The terminal methyl groups of the fatty acids were easily determined and had the ^{1}H and ^{13}C chemical shifts farthest upfield. They also had a characteristic splitting pattern where the terminal protons form a triplet and the protons on the methyl side chain formed a doublet. The tertiary carbon connected to the branched methyl was readily distinguished from the background of secondary carbons. The prominent ^{1}H signal at 1.6 ppm indicated long alkyl chains. The fatty acid signals overlap in the ^{1}H spectrum and integrations were used to confirm purity of the GGLs and determine if hydrolysis or contamination had occurred. The ^{13}C signals at 175 ppm were quaternary carbons belonging to carbonyl

groups. The vinyl protons were observed at 129 ppm and 137 ppm that corresponded to the double bond on the fatty acid.

Anomeric protons and carbons were easily distinguishable with being downfield from the rest of the sugar signals. The small coupling constants indicated that both sugars were alpha.[209] The protons attached to the carbons adjacent to the attached fatty acids were easily distinguished since they were located on the two secondary carbons and possessed chemical shifts farther downfield than the protons adjacent to the anomeric protons. Using COSY data and starting at the anomeric protons, the ^{1}H assignments from C-1 to C-6 for the sugars were determined.

COSY correlations were used to link the three carbons on the glycerol. COSY correlations were able to link the alpha, beta and the -CH2- protons on the fatty acids. Using COSY correlations, the unsaturated fatty acid linkage was determined starting at the vinyl protons and extending out two carbons to $H_{\omega-6'}$ and $H_{\omega-2\ (CH3)'}$. COSY correlations of H_{ω} to $H_{\omega-1a}$ to $H_{\omega-2}$ helped with the assignment of the terminal region of the saturated fatty acid. HMBC correlations from the carbonyl on the fatty acids to the secondary carbon protons on the glycerol and sugar supported the attachment locations of the fatty acids. NMR was not able to determine the location of the fatty acids however, that was determined by LC-MS as described later.

HMBC correlations connected the carbonyls to the alpha and beta protons. HMBC correlations also determined that the fatty acids had ante-iso tails since H_{ω} and $H_{\omega-2\ (CH3)}$ both correlated with $H_{\omega-2}$. HMBC correlations linked the monosaccharides in a 1-3 linkage. They also connected the glycerol to the first monosaccharide in a 1-3 linkage.

HMBC correlations also linked C1' to C5' and C1" to C5" indicating that the sugars were cyclized.

Table 3.13. 1D and 2D NMR Data (600 MHz, CD_3OD) of GGL 885

Position	δ_C [a]	δ_H [b]	ROESY (H→H)	HMBC (H→C)	COSY (H→H)
1a	66.4, CH_2	4.14 (dd, 4.5, 11.3)	2, 1b	C=O, 3, 2	1b, 2
1b		4.09 (dd, 5.6, 11.3)	3a, 2	C=O, 3, 2	1a, 2
2	69.4, CH	3.97 (dddd, 5.2-5.6)	1a, 3a	3, 1	1a, 1b, 3b, 3a
3a	69.9, CH_2	3.73 (dd, 4.9, 10.2)	3b, 2	1', 2, 1	2
3b		3.44 (dd, 6.4, 10.2)	3a	1', 2, 1	2
1'	102.3, CH	4.73 (d, 1.5)		3', 5', 2', 3, 2	2'
2'	71.2, CH	4.07 (dd, 1.8, 3.4)		3'	1', 3'
3'	80.2, CH	3.83 (dd, 3.8, 9.8)		5', 2', 4'	2', 4'
4'	67.8, CH	3.74 (m)		3', 5', 4'	3', 5'
5'	72.6, CH	3.75 (m)	6'b, 6'a		6'b, 6'a, 4'
6'a	65.0, CH_2	4.39 (dd, 1.1, 11.2)	6'b, 5'	C=O	6'b, 5'
6'b		4.22 (dd, 6.4, 11.3)	6'a, 5'	5'	6'a, 5'
1"	103.9, CH	5.07 (d, 1.5)	2", 3"	3', 5", 3"	2"
2"	72.1, CH	3.96 (dd, 1.8, 3.0)	1"	4"	1", 3"
3"	72.5, CH	3.79 (dd, 3.4, 9.4)	1"	4"	2", 4"
4"	68.9, CH	3.59 (dd, 9.8, 9.8)	5"	6"	3", 5"
5"	75.0, CH	3.77 (m)	4"		4", 6"b
6"a	63.0, CH_2	3.84 (dd, 2.3, 11.6)			6"b
6"b		3.68 (dd, 6.4, 11.7)		5"	6"a, 5"

[a] In ppm, multiplicity [b] In ppm, multiplicity, J in Hz; s, singlet; d, doublet; t, triplet; dd, double doublets; dt, double triplet; m, multiplet; Continued next page

Table 3.13. Continuation of 1D and 2D NMR Data (600 MHz, CD_3OD) of GGL 885

Position	δ_C[a]	δ_H[b]	ROESY (H→H)	HMBC (H→C)	COSY (H→H)
C=O	175.5, 175.3, qC				
α	35.1, 35.0, CH_2	2.35 (t, 7.5)	β, -CH_2-	C=O, β	β
β	26.1, 26.1, CH_2	1.62 (m)	α, -CH_2-	C=O, α	α, -CH_2-
-CH_2-	31.2-30.1, 28.2, CH_2	1.62		ω-3', β	B
ω-3a	37.8, CH_2	1.10 (m)			
ω-3b		1.31 (m)			
ω-2	35.7, CH	1.30 (m)			ω-1a
ω-2 (CH_3)	19.7, CH_3	0.85 (d, 6.0)		ω-2, ω-3, ω-1	
ω-1a	30.7, CH_2	1.14 (m)		ω-2	ω, ω-2
ω-1b		1.30 (m)			
ω	11.8, CH_3	0.87 (t, 7.5)		ω-2, ω-1	ω-1a
ω-6'	31.2-30.2, CH_2	1.35 (m)			ω-5'
ω-5'	33.6, CH_2	1.98 (m)		ω-3', ω-4', CH2	ω-4', ω-6'
ω-4'	129.9, CH_2	5.35 (dt, 6.8, 15.4)	ω-3', ω-2 (CH_3)', -CH_2-, ω-5',	ω-2', ω-5'	ω-3', ω-5',
ω-3'	137.4, CH_2	5.23 (dd, 7.9, 15.1)	ω-4', ω-2 (CH_3)', -CH_2-, ω-2'	ω-5', ω-2 (CH_3)'	ω-4', ω-2'
ω-2'	39.9, CH	1.94 (m)	-CH_2-	ω', ω-3'	ω-3', ω-2 (CH_3)', ω-1'
ω-2 (CH_3)'	21.2, CH_3	0.94 (d, 6.8)		ω-3', ω-2', -CH_2-	ω-2'
ω-1'	31.2-30.2, CH_2	1.25 (m)			ω-2'
ω'	12.3, CH_3	0.85 (t, 7.5)		ω-2'	

[a] In ppm, multiplicity [b] In ppm, multiplicity, J in Hz; s, singlet; d, doublet; t, triplet; dd, double doublets; dt, double triplet; m, multiplet

3.11.2. Analysis of MS, IR and Sugar Structure Elucidation

MS^2 fragmentation data of GGL 885 was obtained (**Figure 3.18**) and was similar to GGL 915. The 867 ion corresponded to a loss of water. The most prominent 723 ion corresponds to the loss of the terminal sugar. The 643 and 645 ions corresponded to the

loss of either fatty acid. The 571 and 569 ions corresponded to the loss of the glycerol and fatty acid. Since two different ions are present, it indicates that GGL 885 is a mixture of two compounds where the double bond is located on the fatty acid directly attached to the sugar in one compound and the other compound has the double bond located on the fatty acid attached to the glycerol. The 483 and 481 ions indicated the loss of the end sugar and a fatty acid. The 329 ion corresponded to the disaccharide.

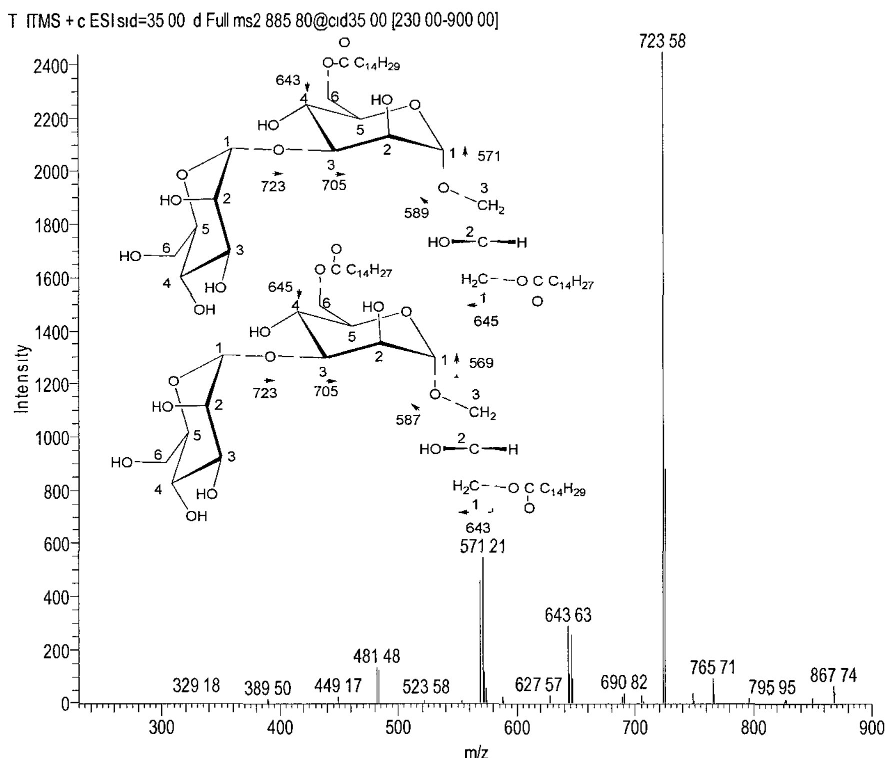


Figure 3.18. Major MS^2 Fragmentation Patterns of 885.80 Ion $[M+Na]^+$

The specific rotation was 34.6° which is a little lower than 47.4° for GGL 915. The error for the specific rotations may be larger due to the low concentration used and the weak optical activity of the compounds.

The coupling constants were the same as previously reported as observed in **Table 3.14**. The δ 1.5 Hz $J_{1'-2'}$ and $J_{1''-2''}$ indicate equatorial-equatorial coupling. The δ 3.4 Hz $J_{2'-}$

3' and δ 3.0 Hz $J_{2''-3''}$ indicate equatorial-axial coupling. The δ 9.4 Hz $J_{3''-4''}$ indicates axial-axial coupling. The δ 9.8 $J_{4''-5''}$ indicates an axial-axial coupling. A coupling constant of δ 11.2 Hz and δ 11.6 Hz is indicative of germinal coupling for $J_{6'a-6'b}$ and $J_{6''a-6''b}$ respectively.

Based upon the trend as explain at the end of **Section 3.9**, the glycerol was assigned the R configuration.

Table 3.14. Coupling Constants of GGL 885 and Mannose Disaccharide

	GGL 885		α-D-Man*p*-(1-3)-α-D-Man*p*-1Me[207]
Position	δ_H ppm	J[Hz](→H)	J[Hz](→H)
1'	4.72	1.5 (2')	1.7 (2')
2'	4.07	1.8 (1'), 3.4 (3')	1.7 (1'), 3.4 (3')
3'	3.82	3.8 (2'), 9.8 (4')	3.4 (2'), 9.5 (4')
4'	3.73		9.5 (3'), 9.8 (5')
5'	3.76		9.8 (4'), 2.2 (6'a), 6.0 (6'b)
6'a	4.38	1.1 (5'), 11.2 (6'b)	2.2 (5'), 12.2 (6'b)
6'b	4.21	6.4 (5'), 11.3 (6'a)	6.0 (5'), 12.2 (6'a)
1"	5.06	1.5 (2")	1.7 (2")
2"	3.96	1.8 (1"), 3.0 (3")	1.7 (1"), 3.4 (3")
3"	3.79	3.4 (2"), 9.4 (4")	3.4 (2"), 9.5 (4")
4"	3.58	9.8 (3"), 9.8 (5")	9.5 (3"), 9.8 (5")
5"	3.76		9.8 (4"), 2.2 (6"a), 6.0 (6"b)
6"a	3.84	2.3 (5"), 11.6 (6"b)	2.2 (5"), 12.2 (6"b)
6"b	3.68	6.4 (5"), 11.7 (6"a)	6.0 (5"), 12.2 (6"a)

The IR contained the expected signals (cm^{-1}) of 3362 (-OH), 2923 and 2853 ($-CH_2-$), 1738 (ester C=O), 1675 (C=C), 1462 ($-CH_2-$ bend), 1377 (CH_3 bend) and 1056 (C-O).

High resolution MS obtained a mass of 885.5510 which had a mean error of 3.44 when compared to the calculated mass for $[C_{45}H_{82}O_{15} + Na]^+$ of 885.5546.

Amorphous solid; $[\alpha]^{20}_D$ 34.6 (*c* 0.07, MeOH); IR (ATR) v_{max} 3362, 2923, 2853, 1738, 1675, 1462, 1377, 1056; ^1H, ^{13}C, COSY, ROESY and HMBC NMR data, see **Table 3.13**; HRESITOFMS *m/z* 885.5515 $[M+Na]^+$ (calcd for $C_{45}H_{82}O_{15}$ + Na, 885.5546)

3.12. Spectroscopic Characterization of GGL 873

3.12.1. Analysis of NMR Data

GGL 873 was tentatively characterized using 1D and 2D NMR techniques as observed in **Table 3.15**. The NMR data was selected from a mixture of what is believed to contain carotenoids. Carotenoids are believed to be the contaminant due to their orange-yellowish color and the presence of double bonds. ESI could not readily detect the contaminating compound but APCI was capable, likely due to the lack of readily ionisable groups on the contaminants. Further purification resulted in hydrolysis of the fatty acids as determined by NMR. Due to time constraints, another fermentation could not be completed and extracted.

Fatty acid tails were tentatively identified as iso and ante-iso from the characteristic chemical shifts[202] from the hydrolyzed product. The characteristic ^{13}C signal at 23.3 ppm matched that of the terminal methyl groups on an iso fatty acid. The characteristic ^{13}C signal at 19.9 ppm matched that of the ω-2 methyl group on an ante-iso fatty acid however, the expected ^{13}C signal of about 11 ppm for the terminal methyl group was not observed. Looking at the NMR data from the mixture, signals for a mixture of the two different fatty acids could be observed however, it is possible that one or more of the signals belonged to the contaminating compounds.

The sample was run in DMSO because solubility was very poor in CD_3OD. The chemical shifts are very close to the GGL 915 standard however, signals were generally quite broad resulting in the loss of resolution needed to obtain most coupling constant information. The most significant difference between the two compounds is the shift in the 4' signal in the HSQC spectrum. This could be due to the contaminants or the

difference in concentrations of the GGLs in solution. The protons on the fatty acids are consistently different by 0.1 ppm which could be due to different concentrations of fatty acid.

Table 3.15. NMR Data (600 MHz, DMSO-d_6) of GGL 873 Mixture and GGL 915 Standard

	GGL 873 Mixture				GGL 915	
Posit.	δ_C[a]	δ_H[b]	COSY (H→H)	HMBC (H→C)	δ_C[a]	δ_H[b]
1a	64.7, CH_2	3.97 (dd, 4.5, 11.3)	2		64.9, CH_2	3.90 (m)
1b		3.95 (m)	2			3.88 (m)
2	66.7, CH	3.82 (m)	1a, 1b, 3a, 3b		67.6, CH	3.79 (m)
3a	67.8, CH_2	3.54 (m)	2, 3b		68.9, CH_2	3.52 (m)
3b		3.32 (m)	2, 3a			3.24 (m)
1’	100.0, CH	4.59 (s)	2’	3’, 5’	100.6, CH	4.56 (s)
2’	68.9, CH	3.85 (m)	1’, 3’		69.4, CH	3.85 (m)
3’	78.6, CH	3.56 (m)	2’		78.8, CH	3.57 (m)
4’	65.2, CH	3.54 (m)	5’		66.2, CH	3.44 (m)
5’	70.6, CH	3.57 (m)	6’a, 6’b, 4’		70.9, CH	3.57 (m)
6’a	63.3, CH_2	4.28 (d, 11.7)	6’b		64.2, CH_2	4.28 (d, 10)
6’b		4.04 (dd, 5.6, 10.9)	6’a, 5’			3.98 (m)

[a] In ppm, mulitiplicity [b] In ppm, multiplicity, J in Hz; s, singlet; d, doublet; m, multiplet;
Continued on Next Page

Table 3.15. Continuation of NMR Data (600 MHz, DMSO-d_6) of GGL 873 Mixture and GGL 915 Standard

	GGL 873 Mixture				GGL 915	
Posit.	δ_C[a]	δ_H[b]	COSY (H→H)	HMBC (H→C)	δ_C[a]	δ_H[b]
1”	102.1, CH	4.84 (s)	2”	3’, 5”, 2”, 3”	102.4, CH	4.83 (s)
2”	69.9, CH	3.74 (m)	1”, 3”		70.2, CH	3.75 (m)
3”	70.6, CH	3.57 (m)	4”		70.9, CH	3.57 (m)
4”	66.7, CH	3.41 (m)	5”, 3”		67.0, CH	3.40 (m)
5”	73.3, CH	3.58 (m)	4”		73.6, CH	3.57 (m)
6”a	60.7, CH_2	3.64 (m)	6”b		61.0, CH_2	3.61 (m)
6”b		3.46 (m)	6”a			3.48 (m)
C=O	172.6, qC				172.9, qC	
α	33.0, CH_2	2.28 (m)	β	C=O, -CH2-, β	33.8, CH_2	2.18 (m)
β	24.1, CH_2	1.51 (m)	α, -CH2-	-CH2-	24.6, CH_2	1.44 (m)
-CH2-	28.6, CH_2	1.28 (m)	β		29.8, CH_2	1.15 (m)
ω-4a	26.2, CH_2	1.26 (m)			27.0, CH_2	1.16 (m)
ω-4b		1.21 (m)				1.11 (m)
ω-3a	35.8, CH_2	1.06 (m)	ω-2(Me)	ω-2	36.6, CH_2	0.95 (m)
ω-3b		1.25 (m)				1.19 (m)
ω-2	33.3, CH	1.27 (m)	ω-2(Me)		34.2, CH	1.16 (m)
ω-2(Me)	18.8, CH_3	0.82 (m)	ω-3a, ω-2	ω-1, ω-3	19.0, CH_3	0.70 (m)
ω-1a	28.6, CH_2	1.10 (m)	ω	ω-2	29.2, CH_2	0.98 (m)
ω-1b		1.29 (m)		ω-2		1.15 (m)
ω	10.7, CH_3	0.83 (m)	ω-1a	ω-2, ω-1	11.0, CH_3	0.71 (m)
ω-1’	26.9, CH	1.05 (m)		ω’		
ω’	22.3, CH_3	0.83(m)		ω-1’		

[a] In ppm, mulitiplicity [b] In ppm, multiplicity, J in Hz; s, singlet; d, doublet; m, multiplet

3.12.2. Analysis of MS, IR and Sugar Structure Elucidation

MS^2 fragmentation data of GGL 873 was obtained (**Figure 3.19**) and was similar to GGL 887. The 855 ion corresponded to a loss of water. The most prominent ion, 711, corresponded to the loss of the end sugar. The 631 and 645 ions corresponded to the loss of either fatty acid. The 571 and 557 ions corresponded to the loss of the glycerol and fatty acid. Since two different ions are present, this indicates that GGL 873 is a mixture of

two compounds where the shorter fatty acid is directly attached to the sugar for one compound and the other compound has the shorter fatty acid attached to the glycerol. The 483 ion and a very weak 469 ion indicated the loss of the end sugar and a fatty acid. Because there is believed to be two different fatty acid ends, iso and ante-iso, there is as many as 8 different possible analogues. These analogues could have fatty acid tails of ante-iso/ante-iso, ante-iso/iso, iso/ante-iso and iso/iso with the different fatty acids at either location.

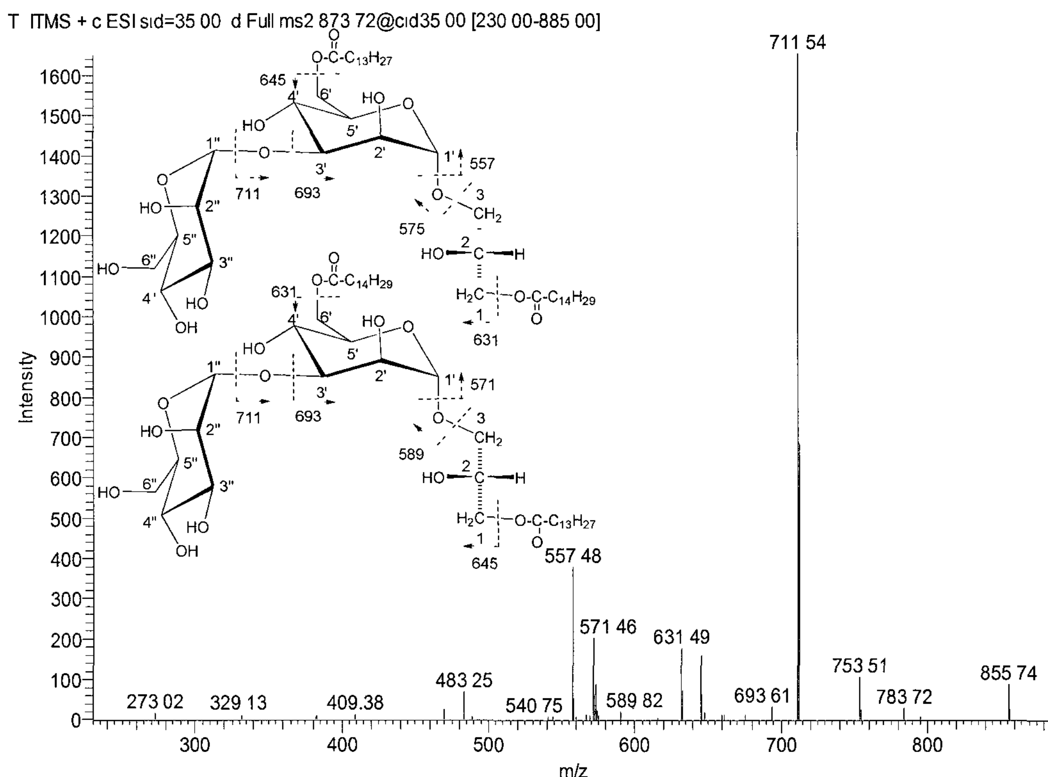


Figure 3.19. Major MS^2 Fragmentation Patterns of 873.72 Ion $[M+Na]^+$

3.13. Discussion of Structure Elucidation Methods for GGLs

Structure elucidation of GGLs generally require a variety of techniques. LC-MS is a good technique to determine the different masses of GGLs.[210] The preferred method of

ionization is ESI since APCI is not as soft and does not display the parent ion. LC-ESIMS also displays the fragmentation patterns and general locations of attachment for the different parts of GGLs. This is especially useful for the determination of the general locations of the fatty acids and the general locations of each individual fatty acid if the fatty acid masses are different. For GGL mixtures with different fatty acid tails but the same molecular weight, selective hydrolysis of a fatty acid using an enzyme[211] could be used to simplify the GGL mixture. This combined with fatty acid methyl esters (FAME) analysis, a well known technique for fatty acid determination,[212, 213] fatty acids attached at one location on the GGL backbone could be identified. NMR or derivatization and GC-MS analysis can be used to confirm successful selective hydrolysis.

The technique used for the structure elucidation of fatty acids was NMR and LC-MS. By the characteristic splitting patterns and chemical shifts of the fatty acid tails, the fatty acid structures can be deduced[202] and with the MS^2 fragmentation data, the molecular weights of the fatty acids can be deduced. Characterization of FAME is a good alternative method to determine the structures of fatty acids. Some GC-MS analysis of FAME was completed however, some difficulty was observed with the loss of product during transfers. For the characterization of mixtures of GGLs, FAME would be the best method to determine the fatty acid composition.

For sugar analysis, there are a number of different methods used for characterization. Acid hydrolysis followed by HPLC and polarimetry was selected due to its simplicity. Readily available was a RHM-monosaccharide column which could exclude glucose as a sugar however, ideally, the experiments would have been completed using a RPM-monosaccharide column which gives good separation for glucose, mannose

and galactose.[204] Also, attempts to synthesize derivatives suitable for GC-MS analysis were unsuccessful. Another good method would be GC-MS analysis of derivatives due to the small amount required and the good resolution.[214-216] It also provides information about whether the sugars are the D or L enantiomers which is not determined by HPLC analysis unless a chiral column is used. However, to determine which anomer is present in the GGL, NMR needs to be performed due to epimerization which occurs after the hydrolysis of the GGLs.

The stereochemistry of the glycerol can be quite difficult to obtain. We identified the stereochemistry based on the fingerprint pattern obtained after derivitization and circular dichroism analysis of GGLs. The original article was published in a Japanese journal[217] however, the method has been used by a number of different authors for the determination of the glycerol stereochemistry.[218-221] An alternative method is by HPLC analysis using a chiral column.[222]

3.14. Surface Tension Analysis of GGL 915

The Critical Micelle Concentration (CMC) was determined of SDS for method validation. The results obtained **(Figure 3.20)** using a petri dish were close to the literature CMC of 8.08 ± 0.12 mM.[223] The results obtained using the 1 mL vessel were lower than the literature value perhaps due to the small volume used, differences in time of measurement or contamination.

GGL 915 was analyzed and found to exert a minimal effect on the surface tension **(Figure 3.21)**. A problem at higher concentrations was the insolubility of the material in water. The 1 mg/mL solution of GGL 915 was heated to 55 °C for 5-15 min and the

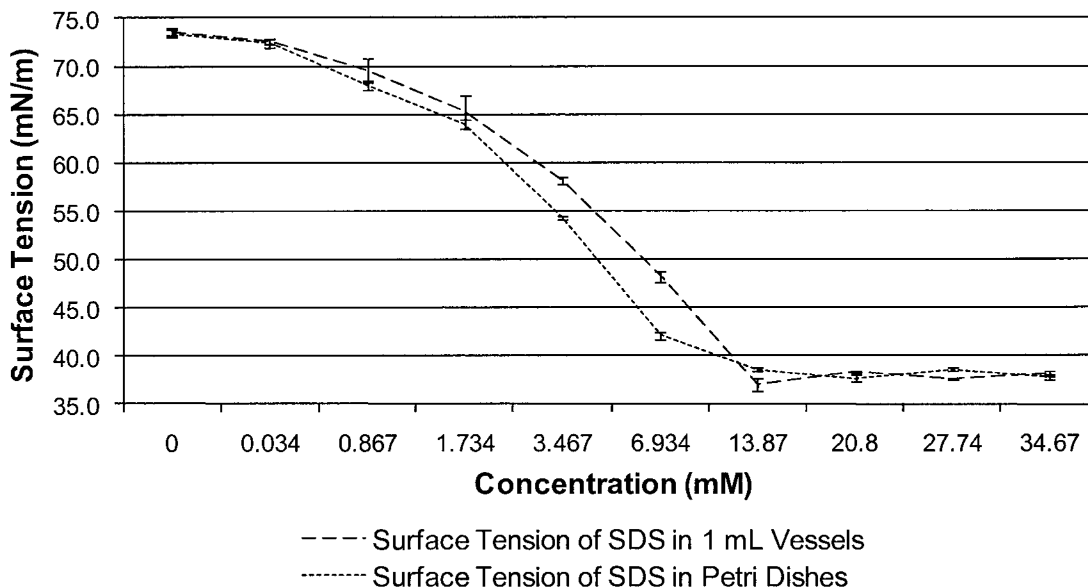


Figure 3.20. Surface Tension of SDS at Different Concentrations

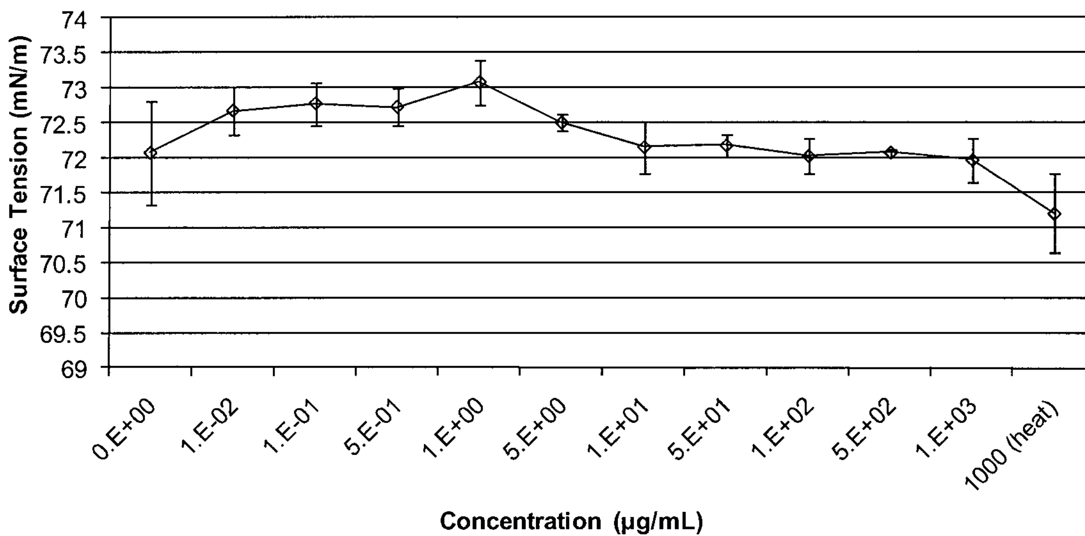


Figure 3.21. Surface Tension of GGL 915 at 10 Different Dilutions

surface tension measured after letting it cool. However, no significant decrease in surface tension was observed. The general decrease in surface tension observed was likely due to an elevated temperature of the solution during measurement.

Surface tension measurements were performed due to the report that 1-O-acyl-3-[α-glycopyranosyl-(1-3)-(6-O-acyl-α-mannopyranosyl)]-*sn*-glycerol had surface active properties.[202] They reported that a mixture of GGLs displayed good surface tension activity. A concern is that if a mixture of GGLs were used, other co-eluting compounds may be obtained in the same fraction which may display potent surface tension activity. The predominant compound in their fraction was determined to be GGL 915[202] which differs from GGL 915 reported in this thesis by the terminal sugar unit. To confirm the difference in surface activity that replacing the terminal sugar unit would produce, both compounds would have to be re-tested.

3.15. Antimicrobial Assays

3.15.1. Antimicrobial Assay of Crude extracts from 50 mL Fermentations

Crude extracts from the 50 mL fermentations were tested at concentrations of 1 mg/mL in order to determine any potential bioactivity to prioritize crude extracts for fractionation. The crude extracts were tested against *S. aureus*, *E. faecalis*, *P. aeruginosa* and *C. albicans*.

The media controls were active against both gram-positive bacteria. Increased activity was primarily observed with isolates containing FF media, indicating the presence of an antimicrobial agent in one of the media reagents. The large number of hits observed in the *S. aureus* assay indicated that the sensitivity range was not likely

appropriate. Less extract would need to be screened in order to be able to better differentiate between poorly active and very active extracts. In order to try to get a better understanding of the activity associated with the fermentation media, two media were selected for fermentation with corresponding media blanks. They were then fractionated and assayed at two different concentrations to determine the general distribution of active material in the media and to determine the optimum concentration for antimicrobial analysis.

Table 3.16. Antimicrobial Assay of 5 μL Extracts against *S. aureus* (Averaged Duplicates)

		Eluting Conditions from C_{18}					
Isolate	Media	20% MeOH	40% MeOH	60% MeOH	80% MeOH	100% MeOH	50% MeOH/DCM
Blank (1)	FF	□	□	□	■	■	▩
Blank (2)	FF	□	□	□	■	■	□
Blank (1)	MPY	□	▦	□	□	□	□
Blank (2)	MPY	□	▦	□	□	□	□
RKSB-14A (1)	FF	▦	□	■	■	■	▦
RKSB-14A (2)	FF	▦	□	▦	■	■	▦
RKSB-11A (1)	FF	■	□	□	▦	□	▦
RKSB-11A (2)	FF	■	□	□	□	□	□

□< 20% Inhibition, 20% <▦< 50% inhibition, 50% <▩< 80% inhibition, 80% inhibition <■

Table 3.17. Antimicrobial Assay of 5 μL Extracts against *E. faecalis* (Averaged Duplicates)

Isolate	Media	Eluting Conditions from C_{18}					
		20% MeOH	40% MeOH	60% MeOH	80% MeOH	100% MeOH	50% MeOH/DCM
Blank (1)	FF				■	■	▦
Blank (2)	FF					■	▦
Blank (1)	MPY			▦			
Blank (2)	MPY				■		
RKSB-14A (1)	FF				▦	▦	
RKSB-14A (2)	FF			▦	▦	▦	▦
RKSB-11A (1)	FF	▦					▦
RKSB-11A (2)	FF	▩		▦	▦	▦	▦

□< 20% Inhibition, 20% <▦< 50% inhibition, 50% <▩< 80% inhibition, 80% inhibition <■

Table 3.18. Antimicrobial Assay of 5 μL Extracts against *C. albicans* (Averaged Duplicates)

Isolate	Media	Eluting Conditions from C_{18}					
		20% MeOH	40% MeOH	60% MeOH	80% MeOH	100% MeOH	50% MeOH/DCM
Blank (1)	FF				▩	▦	
Blank (2)	FF				▩	▩	▦
Blank (1)	MPY					▦	
Blank (2)	MPY				▦		
RKSB-14A (1)	FF	▦		▦			
RKSB-14A (2)	FF	▦			▦	▦	
RKSB-11A (1)	FF	▦					
RKSB-11A (2)	FF	▦					

□< 20% Inhibition, 20% <▦< 50% inhibition, 50% <▩< 80% inhibition, 80% inhibition <■

Table 3.19. Antimicrobial Assay of 5 µL Extracts against *P. aeruginosa* (Averaged Duplicates)

Isolate	Media	Eluting Conditions from C_{18}					
		20% MeOH	40% MeOH	60% MeOH	80% MeOH	100% MeOH	50% MeOH/DCM
Blank (1)	FF					[black]	[grid]
Blank (2)	FF					[dotted]	
Blank (1)	MPY						
Blank (2)	MPY						
RKSB-14A (1)	FF						
RKSB-14A (2)	FF				[grid]		
RKSB-11A (1)	FF	[grid]					
RKSB-11A (2)	FF	[grid]					

[ ] < 20% Inhibition, 20% < [grid] < 50% inhibition, 50% < [dotted] < 80% inhibition, 80% inhibition < [black]

Table 3.20. Antimicrobial Assay of 50 µL Extracts against *S. aureus* (Averaged Duplicates)

Isolate	Media	Eluting Conditions from C_{18}					
		20% MeOH	40% MeOH	60% MeOH	80% MeOH	100% MeOH	50% MeOH/DCM
Blank (1)	FF			[black]		[black]	
Blank (2)	FF			[grid]	[black]	[grid]	
Blank (1)	MPY						
Blank (2)	MPY						
RKSB-14A (1)	FF	[dotted]	[black]	[black]	[black]	[black]	
RKSB-14A (2)	FF		[grid]	[black]	[black]	[black]	
RKSB-11A (1)	FF	[black]	[black]	[black]	[black]	[black]	
RKSB-11A (2)	FF	[black]	[grid]	[black]	[black]	[grid]	

[ ] < 20% Inhibition, 20% < [grid] < 50% inhibition, 50% < [dotted] < 80% inhibition, 80% inhibition < [black]

Table 3.21. Antimicrobial Assay of 50 μL Extracts against *E. faecalis* (Averaged Duplicates)

Isolate	Media	Eluting Conditions from C_{18}					
		20% MeOH	40% MeOH	60% MeOH	80% MeOH	100% MeOH	50% MeOH/DCM
Blank (1)	FF			■	▦	■	
Blank (2)	FF			▦	■	▦	
Blank (1)	MPY						
Blank (2)	MPY				■		
RKSB-14A (1)	FF	■	▦	■	■	■	
RKSB-14A (2)	FF			■	■	■	
RKSB-11A (1)	FF	■		■	■	■	
RKSB-11A (2)	FF	■		■	■	■	

☐ < 20% Inhibition, 20% < ▦ < 50% inhibition, 50% < ▩ < 80% inhibition, 80% inhibition < ■

Table 3.22. Antimicrobial Assay of 50 μL Extracts against *C. albicans* (Averaged Duplicates)

Isolate	Media	Eluting Conditions from C_{18}					
		20% MeOH	40% MeOH	60% MeOH	80% MeOH	100% MeOH	50% MeOH/DCM
Blank (1)	FF						
Blank (2)	FF						
Blank (1)	MPY					▦	
Blank (2)	MPY			■	▦		
RKSB-14A (1)	FF				▦		▦
RKSB-14A (2)	FF				▦		
RKSB-11A (1)	FF			▦			
RKSB-11A (2)	FF	▦		▦			▦

☐ < 20% Inhibition, 20% < ▦ < 50% inhibition, 50% < ▩ < 80% inhibition, 80% inhibition < ■

Table 3.23. Antimicrobial Assay of 50 µL Extracts against *P. aeruginosa* (Averaged Duplicates)

Isolate	Media	Eluting Conditions from C_{18}					
		20% MeOH	40% MeOH	60% MeOH	80% MeOH	100% MeOH	50% MeOH/DCM
Blank (1)	FF						
Blank (2)	FF						
Blank (1)	MPY						
Blank (2)	MPY						
RKSB-14A (1)	FF						
RKSB-14A (2)	FF						
RKSB-11A (1)	FF						
RKSB-11A (2)	FF						

< 20% Inhibition, 20% < < 50% inhibition, 50% < < 80% inhibition, 80% inhibition <

The FF media blanks generally displayed activity against gram-positive bacteria in fractions 3, 4 and 5. The FF fermentation media usually displayed strong activity in fractions 1, 3, 4 and 5. The presence of activity in fraction 1 could be due to the enzymatic modification or degradation of more non-polar bioactive compounds. The assay results indicate that FF would be a very poor media to use for bioassay-guided fractionation using gram-positive bacteria since bioactive compounds from the media would be present in many of the fractions. There are many possible sources of bioactive compounds from FF media since it is prepared with fish meal. Some well known bioactive compounds from fish are antimicrobial peptides which could contribute to the interfering bioactivity.[224, 225] The MPY media showed activity in some fractions but the activity was not observed in the first MPY media blank. Overall, this indicates that care is needed when following up bioactivity from crude extracts due to possible media influences.

3.15.2. Antimicrobial assay of GGL 915

GGL 915 was tested against MRSA, VRE, *C. albicans* and *P. aeruginosa*. Concentrations tested ranged up to 1 mg/mL in order to try to determine an IC_{50} value. GGLs 1-O-acyl-3-[α-D-mannopyranosyl-(1–3)-(6-O-acyl-α-D-mannopyranosyl)]-sn-glycerol have previously been reported not to have antimicrobial activity.[114] Complete inhibition was observed for VRE and about 70% inhibition was observed for MRSA at 1 mg/mL. No inhibition was observed for *C. albicans* or *P. aeruginosa*. GGL 915 cannot be considered to display relevant activity since antibiotics are generally required to display activity below 30 μg/mL.

3.16. Cytotoxic Assay

Putative toxicity in the MTT assay was defined as cell activity or formazan production deviating by ±25% from the norm. This can either be -25% which is indicative of decreased cell viability, activity, population or reduction potential, or +25% which is indicative of increased formazan production which has been associated to efflux protein inhibition,[226] cell cycle arrest,[227-229] and has been shown to mask growth inhibition by interferons[230] and tyrosine kinase inhibitor STI571.[157] The increased production of formazan at 40 μM for HeLa cells; 40 and 50 μM for Huh7 cells with GGL 915 were a non-indicative indication of toxicity (**Figure 3.22**). This indicated that concentrations used for antiviral assays should be lower than 40 μM inorder to avoid any toxic events. No cytotoxicity was determined which is consistent with previous reports.[114]

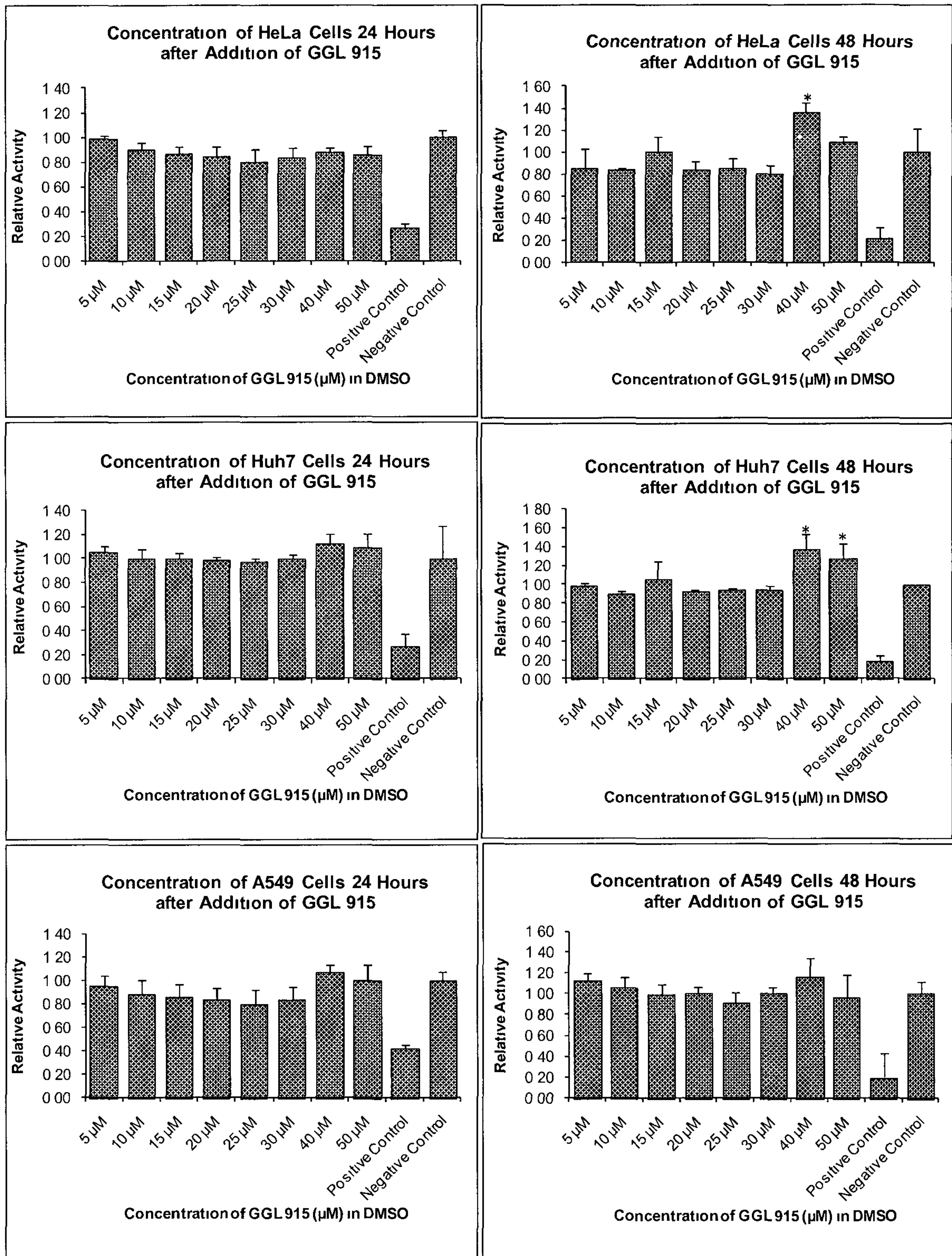


Figure 3.22. Cytotoxic Activity of GGL 915 Results

3.17. Antiviral Assays

GGLs 885, 887 and 915 were tested against the *Vaccinia* virus. A previous publication indicated that GGL 915 had viral binding activity against Influenza A.[130] The initial concentrations tested were 0 μM, 5 μM, 10 μM, 20 μM and 30 μM using GGLs 885, 887 and 915 against Huh7 and HeLa cell lines. It was apparent that the only viral replication inhibition present was likely due to cell toxicity for GGLs 885 and 887 (**Figure 3.23**). GGL 915 showed potential inhibition of viral replication and was therefore repeated twice but ultimately displayed no apparent antiviral activity. These results are not surprising considering that the mode of action proposed for antiviral activity is due to the ability of GGLs to mimic sialic acid.[130] Sialic acids are receptors on cells which the influenza virus binds to via hemagglutinin proteins. Neuraminidases are enzymes which cleave the receptors from the cells and therefore free the virus particle. Both activities are necessary in order to have a fully functioning virus particle.[131] GGLs with are believed to be able to mimic the sialic acid and therefore attach to the hemagglutinin while being different enough so that the neuraminidases cannot hydrolyse them.[130] Saturating the hemagglutinin proteins would prevent their ability to bind to cells and therefore prevent infection. The cell toxicity observed in the antiviral assay but not present in the MTT assay is due to the increased stress the cells were subject to. In the MTT assay, DMSO was used as the vehicle and the compound being tested are stressors for the cells. In the antiviral assay, the DMSO vehicle, the compounds tested and the virus are stressors which result in a greater stress level for the cells. This increased stress level makes the cells more fragile and leads to cell death.

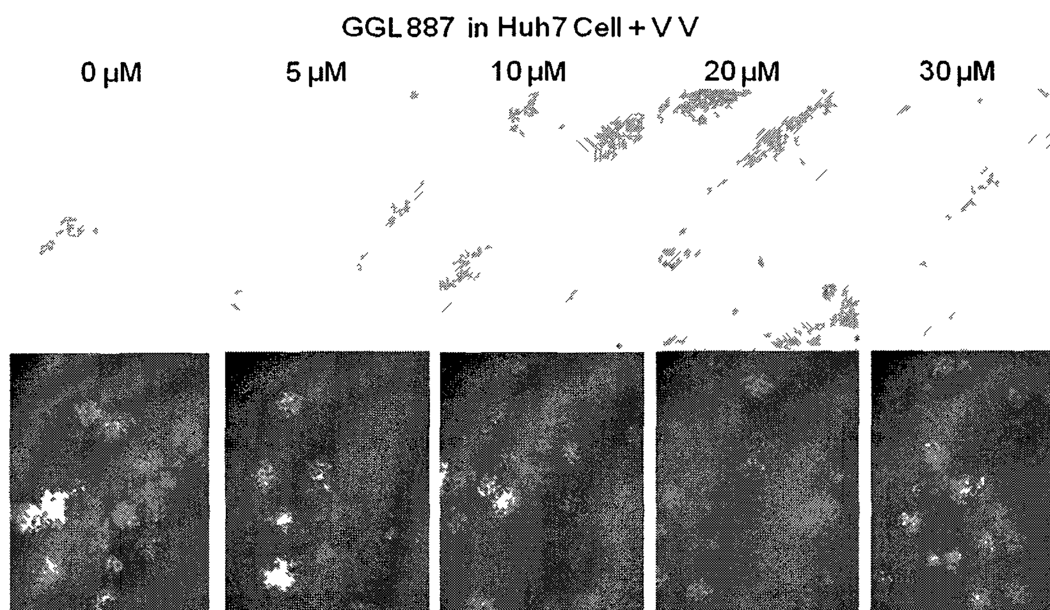


Figure 3.23. GGL 887 with Huh7 Cells and *Vaccinia* Virus (VV). Top Images are of the cell monolayer. Bottom images are fluorescent microscopy images where light images are of green fluorescent protein (GFP) transformed *Vaccinia* Virus.

The GGLs 885, 887 and 915 were tested against influenza A (**Figure 3.24**). Increased cell deaths were observed for trial 2 due to the increased stress caused by TPCK-Trypsin. GGL 885 and GGL 915 displayed no antiviral activity which is surprising since it was reported that GGL 915 was active. However, different assay conditions were used. The current assay scored cell monolayer health after infection. The reported results were based on GGL binding to viral particles on a Thin Layer Chromatography(TLC) plate, inhibition of hemolysis activity from rinses of GGL coated gauze dipped in a viral suspension, and released cytosolic lactate dehydrogenase (LDH) activity from pretreated MDCK monolayer cells after infection. Since the assays used are so different, little can be said about the different results.

GGL 887 displayed modest activity but more work would be required to confirm the activity. The activity displayed is on the border of the error range or significance of

the assay of which is described to be ±0.25. Due to the limited amount of material, replicates of the assay were not performed indicating that the results would need to be repeated.

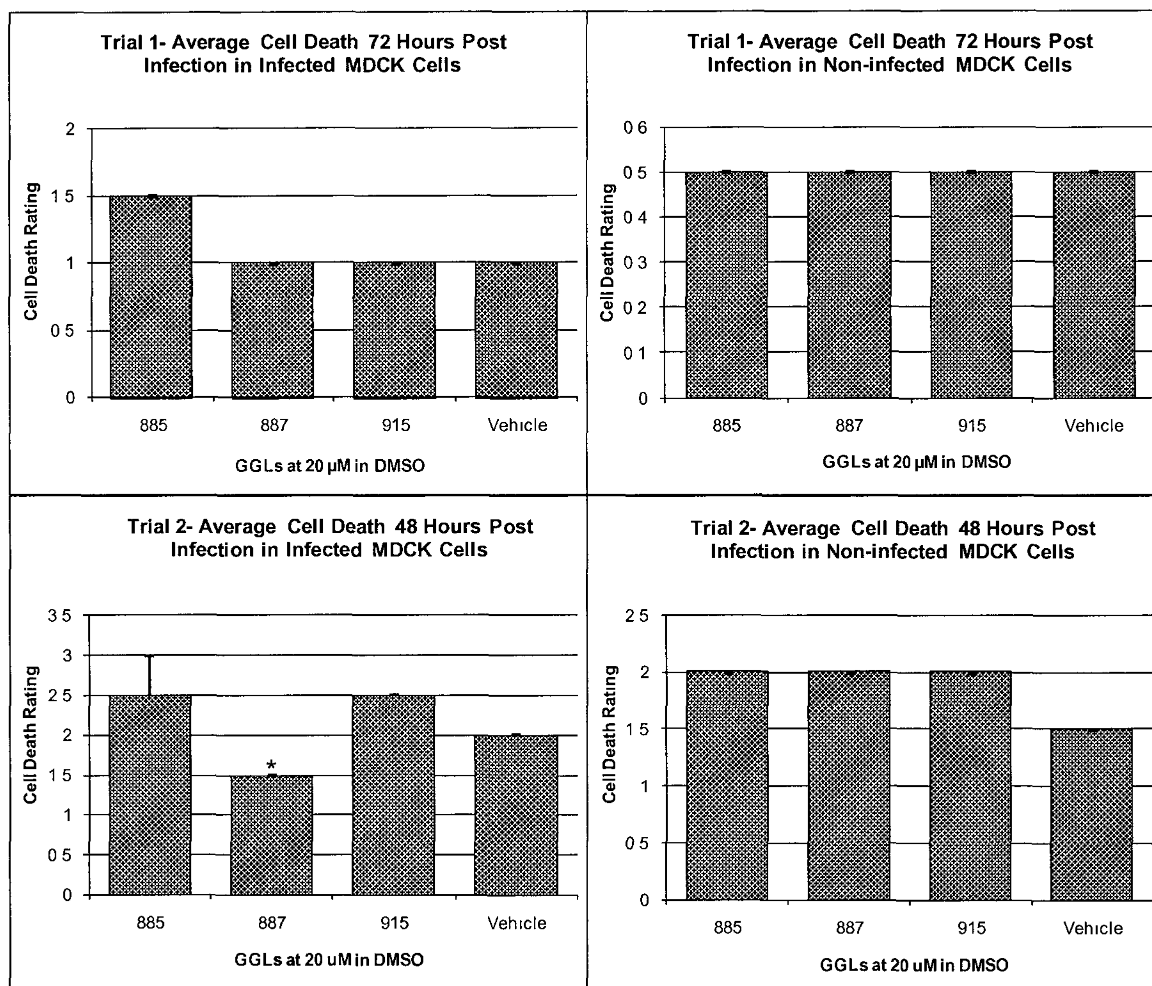


Figure 3.24. Trial 1 and 2 of MDCK Cells Infected with H1N1 Virus with Controls of Uninfected Cells

3.18. Antifouling Assay

No antifouling activity was displayed for GGL 915 against *Ciona intestinalis.* GGL 915 displayed less anti-settling activity than the seawater control **(Table 3.24)**.

There has been no previous antifouling assays reported using dimannoglycerolipids. A different GGL has been shown to exhibit antimicrofouling activity and a relative has shown larval settlement induction in sea urchins. Galactoglycerolipids were shown to display antimicrofouling activity however the product was a mixture containing a complex mixture of different fatty acids so a specific analogue was not purified.[231] A digalactoglycerolipid showed induction of sea urchin *Strongylocentrotus intermedius* larval settlement and metamorphosis however specific analogues were also not purified.[232] As well, galactoglycerolipids have been shown to be feeding stimulants for sea urchins.[233] It's interesting that fatty acids have been shown to have antifouling activity. Ante-iso fatty acid 12-methyl dodecanoic acid, which is the same fatty acid that is contained in all of the isolated GGLs in this thesis, has been shown to display good antifouling activity against *Hydroides elegans* with a settlement inhibition concentration of 50% larvae (EC_{50}) at 0.6 μg/mL and a lethal dose concentration of 50% of larvae (LD_{50}) of 80 μg/mL.[234] This suggests that if significant hydrolysis of the GGL had occurred in the antifouling assay, it would likely have displayed antifouling activity.

Table 3.24. Antifouling Assay of GGL 915

Plate#	Replicate	Settle (+)	Swim (+)	Change (-)	Dead (-)	Total
1A	MeOH Control 1	5	2	2	1	10
1B	MeOH Control 2	7	0	0	1	8
1C	MeOH Control 3	4	2	4	0	10
2A	GGL 915 1	6	1	0	2	9
2B	GGL 915 2	7	2	0	0	9
2C	GGL 915 3	6	3	0	0	9
3A	Seawater Control 1	2	6	0	0	8
3B	Seawater Control 2	7	1	1	1	10
3C	Seawater Control 3	5	4	0	2	11

CHAPTER 4-CONCLUSION

There is a diverse array of different cold-adapted bacteria from Malpeque Bay, P.E.I. Sixteen different bacteria were isolated which cover 8 different genera. Of these genera, many isolates in the literature have been isolated from permanently cold regions of the earth. A growth temperature study indicated that 3 isolates were psychrophilic, 27 were psychrotolerant and 4 were mesophilic. These results agreed with the general results obtained in the literature. Further investigation of these bacteria could yield many different bioactive NPs with applications to human health.

Extraction and fermentation studies were completed to optimize the extraction and production of GGLs. GGLs were observed to be localized in the bacterial cells which eliminated the need use HP20 resins for extractions. Media supplementation experiments with glycerol and glucose were completed, which showed selective improved production of different GGL analogues. Cell concentration and cell weight were studied at 15 °C and 21 °C which determined that maximum cell concentrations were achieved by day 4 for both temperatures. Maximum cell weight generally plateaued at day 3. Temperature was found to have an impact on GGL production with a general increase in production at 21 °C. Also, GGL 885 and 917 production generally declined or was stable over the 7 days at 15 °C but not at 21 °C.

There are a variety of different methods to characterize GGLs. The GGLs characterized in this thesis were 915 and 887, which have previously been reported; which were subsequently used as standards. They were characterized by NMR, LC-ESIMS/MS, Polarimetry, IR and HR-MS. The new GGLs were a mixture of two new analogues of GGL 885 and were characterized by NMR, LC-ESIMS/MS, polarimetry, IR

and HR-MS. GGL 873 was tentatively characterized from a mixture of compounds and was a mixture of at least two analogues of which have not yet been reported. GGL 873 was characterized by NMR and LC-ESIMS/MS.

No surface tension activity was observed for GGL 915 however, as a mixture, the glucosylmannosyl glycerolipid has been reported to have good surface activity.
No antimicrobial activity was observed for GGL 915 until 1 mg/mL concentrations in which activity was observed against VRE and MRSA. No antifouling activity was observed with GGL 915 however, the fatty acids attached have been reported to have good activity. No cytotoxic activity was observed for GGL 915, however, some increased metabolic activity was observed at 40 and 50 μg/mL in Huh7 and HeLa cell lines. No antiviral activity was observed for GGL 885, 887 and 915 against the *Vaccinia* virus that could not be explained by cytotoxic effects. Some activity was observed with GGL 887 against the H1N1 virus, however, follow up experiments would have to be completed to ensure activity was present.

REFERENCES

(1) Cragg, G. M.; Grothaus, P. G.; Newman, D. J. Impact of Natural Products on Developing New Anti-Cancer Agents. *Chem. Rev.* **2009**, *109*, 3012-3043.

(2) Wright, A. Marine Natural Products Drug Discovery, Harbor Branch Oceanographic Institute, Florida Atlantic University, 2010.

(3) Newman, D. J.; Cragg, G. M. Natural Product Scaffolds as Leads to Drugs. *Future Med. Chem.* **2009**, *1*, 1415-1427.

(4) Koehn, F. E.; Carter, G. T. The Evolving Role of Natural Products in Drug Discovery. *Nat. Rev. Drug Discov.* **2005**, *4*, 206-220.

(5) Costantino, V.; Fattorusso, E.; Menna, M.; Taglialatela-Scafati, O. Chemical Diversity of Bioactive Marine Natural Products: An Illustrative Case Study. *Curr. Med. Chem.* **2004**, *11*, 1671-1692.

(6) Young, R. N. Importance of Biodiversity to the Modern Pharmaceutical Industry. *Pure Appl. Chem.* **1999**, *71*, 1655-1661.

(7) Newman, D. J.; Cragg, G. M.; Battershill, C. N. Therapeutic Agents from the Sea: Biodiversity, Chemo-Evolutionary Insight and Advances to the End of Darwin's 200th Year. *Div. Hyper. Med.* **2009**, *39*, 216-225.

(8) Suckling, C. J. Chemical Approaches to the Discovery of New Drugs. *Sci. Prog.* **1991**, *75*, 323-359.

(9) Butler, M. S. Natural Products to Drugs: Natural Product-Derived Compounds in Clinical Trials. *Nat. Prod. Rep.* **2008**, *25*, 475-516.

(10) Ganesan, A. The Impact of Natural Products upon Modern Drug Discovery. *Curr. Opin. Chem. Biol.* **2008**, *12*, 306-317.

(11) McGraw-Hill, Ed.; In *McGraw-Hill Dictionary of Scientific and Technical Terms.* McGraw-Hill: New York, 2003; Vol. 6th Ed., pp 2380.

(12) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv. Drug Deliv. Rev.* **2001**, *46*, 3-26.

(13) Zhang, M.-Q.; Wilkinson, B. Drug Discovery Beyond the 'Rule-Of-Five'. *Curr. Opin. Biotechnol.* **2007**, *18*, 478-488.

(14) Newman, D. J.; Cragg, G. M.; Snader, K. M. The Influence of Natural Products upon Drug Discovery. *Nat. Prod. Rep.* **2000**, *17*, 215-234.

(15) Facklam, M.; Facklam, H.; Grady, S. M. In *Modern Medicines : The Discovery and Development of Healing Drugs;* Facts On File: New York, USA, 2004; , pp 226.

(16) Kremers, E.; Urdang, G.; Sonnedecker, G. In *Kremers and Urdang's History of Pharmacy.* Lippincott: Philadelphia, 1986.

(17) Musallam, B. Avicenna. Encylopaedia Iranica. Online Edition. http://www.iranica.com/articles/avicenna-x (accessed March 16, 2011).

(18) Hughes, C. C.; Fenical, W. Antibacterials from the Sea. *Chem. Eur. J.* **2010**, *16*, 12512-12525.

(19) Singh, S. B.; Barrett, J. F. Empirical Antibacterial Drug Discovery - Foundation in Natural Products. *Biochem. Pharmacol.* **2006**, *71*, 1006-1015.

(20) Butler, M. S.; Buss, A. D. Natural Products - The Future Scaffolds for Novel Antibiotics? *Biochem. Pharmacol.* **2006**, *71*, 919-929.

(21) Kingston, D. G. I.; Newman, D. J. Mother Nature's Combinatorial Libraries; their Influence on the Synthesis of Drugs. *Curr. Opin. Drug Discov. Devel.* **2002**, *5*, 304-316.

(22) Singh, B. K.; Macdonald, C. A. Drug Discovery from Uncultivable Microorganisms. *Drug Discov. Today.* **2010**, *15*, 792-799.

(23) Chakraborty, C.; Hsu, C.-H.; Wen, Z.-H.; Lin, C.-S. Anticancer Drugs Discovery and Development from Marine Organisms. *Curr. Top. Med. Chem.* **2009**, *9*, 1536-1545.

(24) Bhatnagar, I.; Kim, S.-K. Marine Antitumor Drugs: Status, Shortfalls and Strategies. *Mar. Drugs.* **2010**, *8*, 2702-2720.

(25) Jimeno, J.; Faircloth, G.; Sousa-Faro, J. M.; Scheuer, P.; Rinehart, K. New Marine Derived Anticancer Therapeutics - A Journey from the Sea to Clinical Trials. *Mar. Drugs.* **2004**, *2*, 14-29.

(26) Newman, D. J.; Cragg, G. M. Marine Natural Products and Related Compounds in Clinical and Advanced Preclinical Trials. *J. Nat. Prod.* **2004**, *67*, 1216-1238.

(27) Duffy, E. J.; Stachowicz, J. J. Why Biodiversity is Important to Oceanography: Potential Roles of Genetic, Species, and Trophic Diversity in Pelagic Ecosystem Processes. *Mar. Ecol. Prog. Ser.* **2006**, *311*, 179-189.

(28) Jensen, P. R.; Fenical, W. Marine Bacterial Diversity as a Resource for Novel Microbial Products. *J. Ind. Microbiol. Biotechnol.* **1996**, *17*, 346-351.

(29) Costello, M. J.; Coll, M.; Danovaro, R.; Halpin, P.; Ojaveer, H.; Miloslavich, P. A Census of Marine Biodiversity Knowledge, Resources, and Future Challenges. *PLoS ONE.* **2010**, *5*, e12110.

(30) Hill, R. T.; Fenical William, W. Pharmaceuticals from Marine Natural Products: Surge or Ebb? *Curr. Opin. Biotechnol.* **2010**, *21*, 777-779.

(31) Munro, M. H. G.; Blunt, J. W.; Dumdei, E. J.; Hickford, S. J. H.; Lill, R. E.; Li, S.; Battershill, C. N.; Duckworth, A. R. The Discovery and Development of Marine Compounds with Pharmaceutical Potential. *J. Biotechnol.* **1999**, *70*, 15-25.

(32) Ocean Explorer. The Aquarius Underwater Laboratory: America's "Inner Space" Station. *http://oceanexplorer.noaa.gov/technology/diving/aquarius/aquarius.html* (accessed March 17, 2011).

(33) Molinski, T. F.; Dalisay, D. S.; Lievens, S. L.; Saludes, J. P. Drug Development from Marine Natural Products. *Nat. Rev. Drug Discov.* **2009**, *8*, 69-85.

(34) Monterey Bay Aquarium Research Institute. Ship Rates. *http://www.mbari.org/dmo/ship_rates.htm* (accessed March 17, 2011).

(35) Görög, S. In *Identification and Determination of Impurities in Drugs;* Elsevier: Amsterdam; New York, 2000; .

(36) CRC Press In *Combined Chemical Dictionary;* CHEMnetBASE, Ed.; Taylor and Francis Group: 2011; Vol. CCD 14.2, pp http://ccd.chemnetbase.com/dictionary-search.do?method=view&id=783364&si=.

(37) Mayer, A. M. S. Marine Pharmacology: The Clinical Pipeline. *http://marinepharmacology.midwestern.edu/clinPipeline.htm* (accessed March 18, 2011).

(38) Mayer, A. M. S.; Glaser, K. B.; Cuevas, C.; Jacobs, R. S.; Kem, W.; Little, R. D.; McIntosh, J. M.; Newman, D. J.; Potts, B. C.; Shuster, D. E. The Odyssey of Marine Pharmaceuticals: A Current Pipeline Perspective. *Trends Pharmacol. Sci.* **2010**, *31*, 255-265.

(39) US Department of Health and Human Services. FDA Approves New Treatment Option for Late - Stage Breast Cancer. *http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm233863.htm* (accessed June 13, 2011).

(40) Francisco, J. A.; Cerveny, C. G.; Meyer, D. L.; Mixan, B. J.; Klussman, K.; Chace, D. F.; Rejniak, S. X.; Gordon, K. A.; DeBlanc, R.; Toki, B. E.; Law, C.; Doronina, S. O.; Siegall, C. B.; Senter, P. D.; Wahl, A. F. cAC10-vcMMAE, an Anti-CD30-Monomethyl Auristatin E Conjugate with Potent and Selective Antitumor Activity. *Blood.* **2003**, *102*, 1458-1465.

(41) Seattle Genetics. Monoclonal Antibody Therapies for Cancer. http://www.seagen.com/index.php (accessed April 1, 2011).

(42) Celldex Therapeutics. CDX-011: Clinical Program. http://www.celldextherapeutics.com/wt/page/cdx_011_breast (accessed April 1, 2011).

(43) Lingappa, Y.; Lockwood, J. L. A Chitin Medium for Isolation, Growth and Maintenance of Actinomycetes. *Nature.* **1961**, *189*, 158-159.

(44) PharmaMar. Research and Develpment Areas of PharmaMar. http://www.pharmamar.com/products-professionals.aspx (accessed March 12, 2011).

(45) Ocio, E. M.; Maiso, P.; Chen, X. Zalypsis: A Novel Marine-Derived Compound with Potent Antimyeloma Activity that Reveals High Sensitivity of Malignant Plasma Cells to DNA Double-Strand Breaks. *Blood.* **2010**, *116*, 151.

(46) Kuznetsov, G.; TenDyke, K.; Towle, M. J.; Cheng, H.; Liu, J.; Marsh, J. P.; Schiller, S. E. R.; Spyvee, M. R.; Yang, H.; Seletsky, B. M.; Shaffer, C. J.; Marceau, V.; Yao, Y.; Suh, E. M.; Campagna, S.; Fang, F. G.; Kowalczyk, J. J.; Littlefield, B. A. Tubulin-based Antimitotic Mechanism of E7974, a Novel Analogue of the Marine Sponge Natural Product Hemiasterlin. *Mol. Cancer Ther.* **2009**, *8*, 2852-2860.

(47) Nereus Pharmaceuticals Inc. Nereus Pharmaceuticals Initiates Combination Clinical Trial for Proteasome Inhibitor Npi-0052 in Solid Tumors. http://www.nereuspharm.com/news_041808.shtml (accessed March 14, 2011).

(48) Bergmann, W.; Burke, D. C. Contributions to the Study of Marine Products. XXXIX. The Nucleosides of Sponges. III. Spongothymidine and Spongouridine. *J. Org. Chem.* **1955**, *20*, 1501-1507.

(49) Howard, J. P. Cytosine Arabinoside (NSC-63878) in Acute Leukemia in Children. *Cancer Chemother. Rep.* **1966**, *50*, 287-291.

(50) National Institute of Health. Cytarabine. http://www.cancer.gov/cancertopics/druginfo/cytarabine (accessed March 13, 2011).

(51) Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Keifer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. Ecteinascidins 729, 743, 745, 759A, 759B, and 770: Potent

Antitumor Agents from the Caribbean Tunicate *Ecteinascidia turbinata*. *J. Org. Chem.* **1990**, *55*, 4512-4515.

(52) Wright, A. E.; Forleo, D. A.; Gunawardana, G. P.; Gunasekera, S. P.; Koehn, F. E.; McConnell, O. J. Antitumor Tetrahydroisoquinoline Alkaloids from the Colonial Ascidian *Ecteinascidia turbinata*. *J. Org. Chem.* **1990**, *55*, 4508-4512.

(53) Cuevas, C.; Francesch, A. Development of Yondelis (Trabectedin, ET-743). A Semisynthetic Process Solves the Supply Problem. *Nat. Prod. Rep.* **2009**, *26*, 322-337.

(54) Haefner, B. Drugs from the Deep: Marine Natural Products as Drug Candidates. *Drug Discov. Today.* **2003**, *8*, 536-544.

(55) Proksch, P.; Edrada, R. A.; Ebel, R. Drugs from the Seas - Current Status and Microbiological Implications. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 125-134.

(56) Schmidtko, A.; Lötsch, J.; Freynhagen, R.; Geisslinger, G. Ziconotide for Treatment of Severe Chronic Pain. *Lancet.* **2010**, *375*, 1569-1577.

(57) Jensen, P. R.; Fenical, W. In *Marine Microorganisms and Drug Discovery: Current Status and Future Potential;* Fusetani, N., Ed.; Drugs from the Sea; Karger: Basel, 2000; pp 6-29.

(58) Zhang, L.; An, R.; Wang, J.; Sun, N.; Zhang, S.; Hu, J.; Kuai, J. Exploring Novel Bioactive Compounds from Marine Microbes. *Curr. Opin. Microbiol.* **2005**, *8*, 276-281.

(59) Azam, F.; Worden, A. Z. Microbes, Molecules, and Marine Ecosystems. *Science.* **2004**, *303*, 1622-1624.

(60) Fenical, W.; Jensen, P. R. Developing a New Resource for Drug Discovery: Marine Actinomycete Bacteria. *Nat. Chem. Biol.* **2006**, *2*, 666-673.

(61) Synnes, M. Bioprospecting of Organisms from the Deep Sea: Scientific and Environmental Aspects. *Clean Techn. Environ. Policy.* **2007**, *9*, 53-59.

(62) Penesyan, A.; Kjelleberg, S.; Egan, S. Development of Novel Drugs from Marine Surface Associated Microorganisms. *Mar. Drugs.* **2010**, *8*, 438-459.

(63) Bhatnagar, I.; Kim, S.-K. Immense Essence of Excellence: Marine Microbial Bioactive Compounds. *Mar. Drugs.* **2010**, *8*, 2673-2701.

(64) Vignesh, S.; Raja, A.; Arthur James, R. Marine Drugs: Implication and Future Studies. *Int. J. Pharmacol.* **2011**, *7*, 22-30.

(65) Thakur, N. L.; Thakur, A. N.; Müller, W. E. G. Marine Natural Products in Drug Discovery. *Nat. Prod. Rad.* **2005**, *4*, 471-477.

(66) Rahman, H.; Austin, B.; Mitchell, W. J.; Morris, P. C.; Jamieson, D. J.; Adams, D. R.; Spragg, A. M.; Schweizer, M. Novel Anti-Infective Compounds from Marine Bacteria. *Mar. Drugs.* **2010**, *8*, 498-518.

(67) Penesyan, A.; Marshall-Jones, Z.; Holmstrom, C.; Kjelleberg, S.; Egan, S. Antimicrobial Activity Observed Among Cultured Marine Epiphytic Bacteria Reflects their Potential as a Source of New Drugs: Research Article. *FEMS Microbiol. Ecol.* **2009**, *69*, 113-124.

(68) König, G. M.; Kehraus, S.; Seibert, S. F.; Abdel-Lateff, A.; Müller, D. Natural Products from Marine Organisms and Their Associated Microbes. *Chem. Bio. Chem.* **2006**, *7*, 229-238.

(69) Newman, D. J.; Hill, R. T. New Drugs from Marine Microbes: The Tide is Turning. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 539-544.

(70) Andrianasolo, E. H.; Gross, H.; Goeger, D.; Musafija-Girt, M.; McPhail, K.; Leal, R. M.; Mooberry, S. L.; Gerwick, W. H. Isolation of Swinholide A and Related Glycosylated Derivatives from Two Field Collections of Marine Cyanobacteria. *Org. Lett.* **2005**, *7*, 1375-1378.

(71) Waters, A. L.; Hill, R. T.; Place, A. R.; Hamann, M. T. The Expanding Role of Marine Microbes in Pharmaceutical Development. *Curr. Opin. Biotechnol.* **2010**, *21*, 780-786.

(72) Feling, R. H.; Buchanan, G. O.; Mincer, T. J.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. Salinosporamide A: A Highly Cytotoxic Proteasome Inhibitor from a Novel Microbial Source, a Marine Bacterium of the New Genus *Salinospora*. *Angew. Chem. Int. Ed.* **2003**, *42*, 355-357.

(73) Fenical, W.; Jensen, P. R.; Palladino, M. A.; Lam, K. S.; Lloyd, G. K.; Potts, B. C. Discovery and Development of the Anticancer Agent Salinosporamide A (NPI-0052). *Bioorg. Med. Chem.* **2009**, *17*, 2175-2180.

(74) Wilson, Z. E.; Brimble, M. A. Molecules Derived from the Extremes of Life. *Nat. Prod. Rep.* **2009**, *26*, 44-71.

(75) Rothschild, L. J.; Mancinelli, R. L. Life in Extreme Environments. *Nature.* **2001**, *409*, 1092-1101.

(76) Skropeta, D. Deep-Sea Natural Products. *Nat. Prod. Rep.* **2008**, *25*, 1131-1166.

(77) Morita, R. Y. Psychrophilic Bacteria. *Bacteriol. Rev.* **1975**, *39*, 144-167.

(78) Adams, M. R.; Moss, M. O. In *Food Microbiology;* RSC Pub.: Cambridge, UK, 2008; .

(79) Russell, N. J.; Cowan, D. A. Handling of Psychrophilic Microorganisms. *Method Microbiol.* **2006**, *35*, 371-393.

(80) Gounot, A.-M. Psychrophilic and Psychotrophic Microorganisms. *Experientia.* **1986**, *42*, 1192-1197.

(81) Helmke, E.; Weyland, H. Psychrophilic Versus Psychrotolerant Bacteria - Occurrence and Significance in Polar and Temperate Marine Habitats. *Cell. Mol. Biol.* **2004**, *50*, 553-561.

(82) Margesin, R.; Feller, G. Biotechnological Applications of Psychrophiles. *Environ. Technol.* **2010**, *31*, 835-844.

(83) University of California. Glossary: General Circulation of the Ocean. *http://earthguide.ucsd.edu/virtualmuseum/Glossary_Climate/gloss_g-l.shtml* (accessed April 8, 2011).

(84) Thomas, D. N.; Dieckmann, G. S. Antarctic Sea Ice - A Habitat for Extremophiles. *Science.* **2002**, *295*, 641-644.

(85) Russell, N. J. Molecular Adaptations in Psychrophilic Bacteria: Potential for Biotechnological Applications. *Adv. Biochem. Eng. Biotechnol.* **1998**, *61*, 1-21.

(86) Parkes, R. J.; Cragg, B. A.; Bale, S. J.; Getlifff, J. M.; Goodman, K.; Rochelle, P. A.; Fry, J. C.; Weightman, A. J.; Harvey, S. M. Deep Bacterial Biosphere in Pacific Ocean Sediments. *Nature.* **1994**, *371*, 410-413.

(87) Feller, G.; Gerday, C. Psychrophilic Enzymes: Hot Topics in Cold Adaptation. *Nat. Rev. Microbiol.* **2003**, *1*, 200-208.

(88) Bowman, J. P. Methods for Psychrophilic Bacteria. *Method Microbiol.* **2001**, *30*, 591-614.

(89) Stanley, S. O.; Rose, A. H. Bacteria and Yeasts from Lakes on Deception Island. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **1967**, *252*, 199-207.

(90) Bowman, J. P.; Mccammon, S. A.; Brown, M. V.; Nichols, D. S.; Mcmeekin, T. A. Diversity and Association of Psychrophilic Bacteria in Antarctic Sea Ice. *Appl. Environ. Microbiol.* **1997**, *63*, 3068-3078.

(91) Ray, M. K.; Kumar, G. S.; Janiyani, K.; Kannan, K.; Jagtap, P.; Basu, M. K.; Shivaji, S. Adaptation to Low Temperature and Regulation of Gene Expression in Antarctic Psychrotrophic Bacteria. *J. Biosci.* **1998**, *23*, 423-435.

(92) Isaksen, M. F.; Jørgensen, B. O. B. Adaptation of Psychrophilic and Psychrotrophic Sulfate-Reducing Bacteria to Permanently Cold Marine Environments. *Appl. Environ. Microbiol.* **1996**, *62*, 408-414.

(93) Russell, N. J. Cold Adaptation of Microorganisms. *Philos. Trans. R. Soc. Lond. B* **1990**, *326*, 595-611.

(94) Helmke, E.; Weyland, H. Bacteria in Sea Ice and Underlying Water of the Eastern Weddell Sea in Midwinter. *Mar. Ecol. Prog. Ser.* **1995**, *117*, 269-288.

(95) Harder, W.; Veldkamp, H. Competition of Marine Psychrophilic Bacteria at Low Temperatures. *Antonie Van Leeuwenhoek.* **1971**, *37*, 51-63.

(96) O'Brien, A.; Sharp, R.; Russell, N. J.; Roller, S. Antarctic Bacteria Inhibit Growth of Food-borne Microorganisms at Low Temperatures. *FEMS Microbiol. Ecol.* **2004**, *48*, 157-167.

(97) Lebar, M. D.; Heimbegner, J. L.; Baker, B. J. Cold-Water Marine Natural Products. *Nat. Prod. Rep.* **2007**, *24*, 774-797.

(98) Sánchez, L. A.; Gómez, F. F.; Delgado, O. D. Cold-Adapted Microorganisms as a Source of New Antimicrobials. *Extremophiles.* **2009**, *13*, 111-120.

(99) Mitova, M.; Tutino, M. L.; Infusini, G.; Marino, G.; De Rosa, S. Exocellular Peptides from Antarctic Psychrophile *Pseudoalteromonas haloplanktis*. *Mar. Biotechnol.* **2005**, *7*, 523-531.

(100) Gustafson, K.; Roman, M.; Fenical, W. The Macrolactins, a Novel Class of Antiviral and Cytotoxic Macrolides from a Deep-Sea Marine Bacterium. *J. Am. Chem. Soc.* **1989**, *111*, 7519-7524.

(101) Schumacher, R. W.; Davidson, B. S.; Montenegro, D. A.; Bernan, V. S. Î-Indomycinone, a New Pluramycin Metabolite from a Deep-Sea Derived Actinomycete. *J. Nat. Prod.* **1995**, *58*, 613-617.

(102) Davidson, B. S.; Schumacher, R. W. Isolation and Synthesis of Caprolactins A and B, New Caprolactams from a Marine Bacterium. *Tetrahedron.* **1993**, *49*, 6569-6574.

(103) Jagannadham, M. V.; Rao, V. J.; Shivaji, S. The Major Carotenoid Pigment of a Psychotrophic *Micrococcus roseus* Strain: Purification, Structure, and Interaction with Synthetic Membranes. *J. Bacteriol.* **1991**, *173*, 7911-7917.

(104) Schouten, S.; Bowman, J. P.; Rijpstra, W. I. C.; Sinninghe Damsté, J. S. Sterols in a Psychrophilic Methanotroph, *Methylosphaera hansonii* . *FEMS Microbiol. Lett.* **2000**, *186*, 193-195.

(105) Fong, N. J. C.; Burgess, M. L.; Barrow, K. D.; Glenn, D. R. Carotenoid Accumulation in the Psychrotrophic Bacterium *Arthrobacter agilis* in Response to Thermal and Salt Stress. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 750-756.

(106) Lu, Y.; Wang, L.; Xue, Y.; Zhang, C.; Xing, X.-H.; Lou, K.; Zhang, Z.; Li, Y.; Zhang, G.; Bi, J.; Su, Z. Production of Violet Pigment by a Newly Isolated Psychrotrophic Bacterium from a Glacier in Xinjiang, China. *Biochem. Eng. J.* **2009**, *43*, 135-141.

(107) Hamana, K.; Niitsu, M. Large Production of an Aromatic Amine, 2-Phenylethylamine, in a Psychrophilic Marine Bacterium, *Psychroflexus torquis*. *J. Gen. Appl. Microbiol.* **2001**, *47*, 103-105.

(108) Shaw, N. Lipid Composition as a Guide to the Classification of Bacteria. *Adv. Appl. Microbiol.* **1974**, *17*, 63-108.

(109) Fahy, E.; Subramaniam, S.; Brown, H. A.; Glass, C. K.; Merrill, A. H.; Murphy, R. C.; Raetz, C. R. H.; Russell, D. W.; Seyama, Y.; Shaw, W.; Shimizu, T.; Spener, F.; van Meer, G.; VanNieuwenhze, M. S.; White, S. H.; Witztum, J. L.; Dennis, E. A. A Comprehensive Classification System for Lipids. *J. Lipid Res.* **2005**, *46*, 839-862.

(110) Fahy, E.; Subramaniam, S.; Murphy, R. C.; Nishijima, M.; Raetz, C. R. H.; Shimizu, T.; Spener, F.; van Meer, G.; Wakelam, M. J. O.; Dennis, E. A. Update of the LIPID MAPS Comprehensive Classification System for Lipids. *J. Lipid Res.* **2009**, *50*, S9-S14.

(111) Nature Publishing Group. The LIPID MAPS–Nature Lipidomics Gateway. http://www.lipidmaps.org/ (accessed April 14, 2011).

(112) Hölzl, G.; Dörmann, P. Structure and Function of Glycoglycerolipids in Plants and Bacteria. *Prog. Lipid Res.* **2007**, *46*, 225-243.

(113) Shaw, N. Bacterial Glycolipids. *Bacteriol. Rev.* **1970**, *4*, 365-377.

(114) Niepel, T.; Meyer, H.; Wray, V.; Abraham, W.-R. A New Type of Glycolipid, 1-[α-Mannopyranosyl-(1α-3)-(6-O-Acyl-α-Mannopyranosly)]-3-O-Acylglycerol, from *Arthrobacter atrocyaneus*. *Tetrahedron.* **1997**, *53*, 3593-3602.

(115) Lennarz, W. J.; Talamo, B. The Chemical Characterization and Enzymatic Synthesis of Mannolipids in *Micrococcus lysodeikticus*. *J. Biol. Chem.* **1966**, *241*, 2707-2719.

(116) Walker, R. W.; Bastl, C. P. The Glycolipids of *Arthrobacter globiformis* . *Carbohydr. Res.* **1967**, *4*, 49-54.

(117) Shaw, N. Lipid Composition of some Species of *Arthrobacter*. *J. Bacteriol.* **1971**, *107*, 130-133.

(118) Khuller, G. K.; Brennan, P. J. Further Studies on the Lipids of Corynebacteria. The Mannolipids of *Corynebacterium aquaticum*. *Biochem. J.* **1972**, *127*, 369-373.

(119) Shaw, N. The Lipid Composition of *Microbacterium facticum*. *Biochim. Biophys. Acta.* **1968**, *152*, 427-428.

(120) Shaw, N. A Study of the Lipid Composition of *Microbacterium thermosphactum* as a Guide to its Taxonomy. *J. Appl. Bacteriol.* **1970**, *33*, 470-473.

(121) Bultel-Poncé, V.; Debitus, C.; Blond, A.; Cerceau, C.; Guyot, M. Lutoside: An Acyl-1-(Acyl-6'-Mannobiosyl)-3-Glycerol Isolated from the Sponge-Associated Bacterium *Micrococcus luteus*. *Tetrahedron Lett.* **1997**, *38*, 5805-5808.

(122) Paściak, M.; Sanchez-Carballo, P.; Duda-Madej, A.; Lindner, B.; Gamian, A.; Holst, O. Structural Characterization of the Major Glycolipids from *Arthrobacter globiformis* and *Arthrobacter scleromae*. *Carbohydr. Res.* **2010**, *345*, 1497-1503.

(123) Yanagi, H.; Matsufuji, M.; Nakata, K.; Nagamatsu, Y.; Ohta, S.; Yoshimoto, A. A New Type of Glycoglycerolipids from *Corynebacterium aquaticum*. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 424-427.

(124) Paściak, M.; Ekiel, I.; Grzegorzewicz, A.; Mordarska, H.; Gamian, A. Structure of the Major Glycolipid from *Rothia dentocariosa*. *Biochim. Biophys. Acta.* **2002**, *1594*, 199-205.

(125) Gamian, A.; Mordarska, H.; Ekiel, I.; Ulrich, J.; Szponar, B.; Defaye, J. Structural Studies of the Major Glycolipid from *Saccharopolyspora* Genus. *Carbohydr. Res.* **1996**, *296*, 55-67.

(126) Akasaka, K.; Shichijyukari, S.; Meguro, H.; Ohrui, H. Determination of the Absolute Configurations of the Anteiso Acid Moieties of Glycoglycerolipid S365A Isolated from *Corynebacterium aquaticum* . *Biosci. Biotechnol. Biochem.* **2002**, *66*, 1719-1722.

(127) Mordarska, H.; Pasciak, M. A Simple Method for Differentiation of *Propionibacterium acnes* and *Propionibacterium propionicum*. *FEMS Microbiol. Lett.* **1994**, *123*, 325-330.

(128) Gupta, P.; Chaturvedi, P.; Pradhan, S.; Delille, D.; Shivaji, S. *Marinomonas polaris* sp. nov., a Psychrohalotolerant Strain Isolated from Coastal Sea Water Off the Subantarctic Kerguelen Islands. *Int. J. Syst. Evol. Microbiol.* **2006**, *56*, 361-364.

(129) Van Trappen, S.; Vandecandelaere, I.; Mergaert, J.; Swings, J. *Flavobacterium degerlachei* sp. nov., *Flavobacterium frigoris* sp. nov. and *Flavobacterium micromati* sp. nov., Novel Psychrophilic Bacteria Isolated from Microbial Mats in Antarctic Lakes. *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 85-92.

(130) Nakata, K.; Guo, C.-T.; Matsufuji, M.; Yoshimoto, A.; Inagaki, M.; Higuchi, R.; Suzuki, Y. Influenza A Virus-binding Activity of Glycoglycerolipids of Aquatic Bacteria. *J. Biochem.* **2000**, *127*, 191-198.

(131) Norkin, L. C. In *Virology :Molecular Biology and Pathogenesis;* ASM Press: Washington, D.C., 2010; , pp 725.

(132) Fisheries and Oceans Canada Area 4Tj. *http://www2.mar.dfo-mpo.gc.ca/science/ocean/coastal_temperature/Areas/4Tj.html* (accessed April 6, 2011).

(133) Atlas, R. M. In *Handbook of Media for Environmental Microbiology;* CRC Press: Boca Raton, 1995; pp 540.

(134) Simon, A.; Ridge, E. H. The Use of Ampicillin in a Simplified Selective Medium for the Isolation of Fluorescent Pseudomonads. *J. Appl. Bacteriol.* **1974**, *37*, 459-460.

(135) DSMZ. *Ancylobacter-Spirosoma* Medium. *http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium7.pdf.*

(136) Solano, F.; Lucas-Elío, P.; Fernández, E.; Sanchez-Amat, A. *Marinomonas mediterranea* MMB-1 Transposon Mutagenesis: Isolation of a Multipotent Polyphenol Oxidase Mutant. *J. Bacteriol.* **2000**, *182*, 3754-3760.

(137) Hayakawa, M. Selective Isolation Methods and Distribution of Soil Actinomycetes. *Actinomycetol.* **1990**, *4*, 103-112.

(138) Atlas, R. M. In *Handbook of Microbiological Media for the Examination of Food;* Taylor and Francis Group: Boca Raton, 2006; pp 446.

(139) Crawford, D. L.; Lynch, J. M.; Whipps, J. M.; Ousley, M. A. Isolation and Characterization of Actinomycete Antagonists of a Fungal Root Pathogen. *App. Environ. Micriobiol.* **1993**, *59*, 3899-3905.

(140) Bredholt, H.; Fjaervik, E.; Johnsen, G.; Zotchev, S. B. Actinomycetes from Sediments in the Trondheim Fjord, Norway: Diversity and Biological Activity. *Mar. Drugs.* **2008**, *6*, 12-24.

(141) Sambrook, J. and Russell, D.W. In *Molecular Cloning: A Laboratory Manual. 3rd Ed.* Cold Spring Harbor Laboratory Press: NY, USA, 2001; Vol. 1, pp 2344.

(142) Hayakawa, M.; Nonomura, H. A New Method for the Intensive Isolation of Actinomycetes from Soil. *Actinomycetol.* **1989**, *3*, 95-104.

(143) Van Huynh, N.; De Backer, O.; Decleire, M.; Colson, C. A Procedure for the Preparation of Bacterial DNA that Employs Dimethyl Sulfoxide to Induce the Lysis of Cells. *Anal. Biochem.* **1989**, *176*, 464-467.

(144) Invitrogen. Vector NTI Advance 10. Contig Express. **2007**, *10.3.1*.

(145) Benson, D. A.; Karsch-Mizrachi, I.; Lipman, D. J.; Ostell, J.; Wheeler, D. L. GenBank. *Nucleic Acids Res.* **2008**, *36*, D25-D30.

(146) Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. *Nucleic Acids Res.* **1997**, *25*, 3389-3402.

(147) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596-1599.

(148) Saitou, N.; Nei, M. The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol.* **1987**, *4*, 406-425.

(149) Tamura, K.; Nei, M.; Kumar, S. Prospects for Inferring Very Large Phylogenies by Using the Neighbor-joining Method. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 11030-11035.

(150) Thermo Electron Corporation. Xcalibur. **2006**, *2.0 SR2*.

(151) Laatsch, H. In *Antibase 2007: The Natural Product Identifier;* Wiley VCH: May 18, 2007; .

(152) Blunt, J. W.; Munro, M. H. G. In *MarinLit: A Comprehensive Database of the Literature for Marine Natural Products.* University of Canterbury: Christ Church, New Zealand, 2007.

(153) Poli, A.; Manca, M. C.; De Giulio, A.; Strazzullo, G.; De Rosa, S.; Nicolaus, B. Bioactive Exopolysaccharides from the Cultured Cells of Tomato, *Lycopersicon esculentum* var. San Marzano. *J. Nat. Prod.* **2006**, *69*, 658-661.

(154) Mestrelab Research. MestRec. **2005**, *4.7.0.0*.

(155) Scifinder. Chemical Abstracts Service: Columbus, OH. https://scifinder.cas.org (accessed June 20, 20011).

(156) Clinical and Laboratory Standards Institute. In *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, Approved Standard - Sixth Edition;* CLSI Document M7-A6; Clinical and Laboratory Standards Institute: USA: Wayne, P.A, 2003; Vol. 23, pp 47.

(157) Smith, K. A.; Colvin, C. J.; Weber, P. S. D.; Spatz, S. J.; Coussens, P. M. High Titer Growth of Human and Avian Influenza Viruses in an Immortalized Chick Embryo Cell Line Without the Need for Exogenous Proteases. *Vaccine* **2008**, *26*, 3778-3782.

(158) Cirino, P.; Toscano, A.; Caramiello, D.; Macina, A.; Miraglia, V.; and Monte, A. Laboratory Culture of the Ascidian *Ciona intestinalis* (L.): A Model System for Molecular Developmental Biology Research. *Mar. Mod. Elec. Rec. [Online]*. **2002.**, January 12, 2011-(Accessed at http://www.mbl.edu/BiologicalBulletin/MMER/cirino/CirTit.html).

(159) Hayakawa, M. Studies on the Isolation and Distribution of Rare Actinomycetes in Soil. *Actinomycetol.* **2008**, *22*, 12-19.

(160) Hsu, S. C.; Lockwood, J. L. Powdered Chitin Agar as a Selective Medium for Enumeration of Actinomycetes in Water and Soil. *J. Appl. Microbiol.* **1975**, *29*, 422-426.

(161) Qiu, D.; Ruan, J.; Huang, Y. Selective Isolation and Rapid Identification of Members of the Genus *Micromonospora* . *Appl. Environ. Microbiol.* **2008**, *74*, 5593-5597.

(162) Porter, J. N.; Wilhelm, J. J.; Tresner, H. D. Method for the Preferential Isolation of Actinomycetes from Soils. *Appl. Microbiol.* **1960**, *8*, 174-178.

(163) Bull, A. T.; Ward, A. C.; Goodfellow, M. Search and Discovery Strategies for Biotechnology: The Paradigm Shift. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 573-606.

(164) Euzeby, J. P. List of Bacterial Names with Standing in Nomenclature: A Folder Available on the Internet. *Int. J. Syst. Bacteriol.* **1997**, *47*, 590-592.

(165) Miyashita, M.; Fujimura, S.; Nakagawa, Y.; Nishizawa, M.; Tomizuka, N.; Nakagawa, T.; Nakagawa, J. *Flavobacterium algicola* sp. nov., Isolated from Marine Algae. *Int. J. Syst. Evol. Microbiol.* **2010**, *60*, 344-348.

(166) Baik, K. S.; Park, S. C.; Lim, C. H.; Lee, K. H.; Jeon, D. Y.; Kim, C. M.; Seong, C. N. *Psychrobacter aestuarii* sp. nov., Isolated From a Tidal Flat Sediment. *Int. J. Syst. Evol. Microbiol.* **2010**, *60*, 1631-1636.

(167) Brinkmeyer, R.; Knittel, K.; Jurgens, J.; Weyland, H.; Amann, R.; Helmke, E. Diversity and Structure of Bacterial Communities in Arctic versus Antarctic Pack Ice. *Appl. Environ. Microbiol.* **2003**, *69*, 6610-6619.

(168) Kim, M. K.; Jung, H. *Pseudoclavibacter soli* sp. nov., a Beta-Glucosidase-producing Bacterium. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 835-838.

(169) Cho, K.; Hong, S.; Lee, S.; Kim, Y.; Kahng, G.; Lim, Y.; Kim, H.; Yun, H. Endophytic Bacterial Communities in Ginseng and their Antifungal Activity Against Pathogens. *Microb. Ecol.* **2007**, *54*, 341-351.

(170) Cho, S.; Jung, M. Y.; Park, M.; Chang, Y.; Yoon, J.; Myung, S. C.; Kim, W. *Pseudoclavibacter chungangensis* sp. nov., Isolated from Activated Sludge. *Int. J. Syst. Evol. Microbiol.* **2010**, *60*, 1672-1677.

(171) Manaia, C. M.; Nogales, B.; Weiss, N.; Nunes, O. C. *Gulosibacter molinativorax* gen. nov., sp. nov., a Molinate-Degrading Bacterium, and Classification of '*Brevibacterium helvolum*' DSM 20419 as *Pseudoclavibacter helvolus* gen. nov., sp. nov. *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 783-789.

(172) Mages, I. S.; Frodl, R.; Bernard, K. A.; Funke, G. Identities of *Arthrobacter* spp. and *Arthrobacter*-Like Bacteria Encountered in Human Clinical Specimens. *J. Clin. Microbiol.* **2008**, *46*, 2980-2986.

(173) Fujii, K.; Takagi, K.; Hiradate, S.; Iwasaki, A.; Harada, N. Biodegradation of Methylthio-s-Triazines by *Rhodococcus* sp. strain FJ1117YT, and Production of the Corresponding Methylsulfinyl, Methylsulfonyl and Hydroxy Analogues. *Pest Manag. Sci.* **2007**, *63*, 254-260.

(174) Wang, Y.; Wang, H.; Zhang, Y.; Xu, L.; Jiang, C.; Li, W. *Rhodococcus kunmingensis* sp. nov., an Actinobacterium Isolated from a Rhizosphere Soil. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 1467-1471.

(175) Leigh, M. B.; Prouzova, P.; Mackova, M.; Macek, T.; Nagle, D. P.; Fletcher, J. S. Polychlorinated Biphenyl (PCB)-Degrading Bacteria Associated with Trees in a PCB-Contaminated Site. *Appl. Environ. Microbiol.* **2006**, *72*, 2331-2342.

(176) Ghosh, A.; Paul, D.; Prakash, D.; Mayilraj, S.; Jain, R. K. *Rhodococcus imtechensis* sp. nov., a Nitrophenol-Degrading Actinomycete. *Int. J. Syst. Evol. Microbiol.* **2006**, *56*, 1965-1969.

(177) Engelhardt, K.; Degnes, K. F.; Kemmler, M.; Bredholt, H.; Fjaervik, E.; Klinkenberg, G.; Sletta, H.; Ellingsen, T. E.; Zotchev, S. B. Production of a New Thiopeptide Antibiotic, TP-1161, by a Marine *Nocardiopsis* Species. *Appl. Environ. Microbiol.* **2010**, *76*, 4969-4976.

(178) Zhang, X.; Yao, T.; Tian, L.; Xu, S.; An, L. Phylogenetic and Physiological Diversity of Bacteria Isolated from Puruogangri Ice Core. *Microb. Ecol.* **2008**, *55*, 476-488.

(179) Miteva, V. I.; Sheridan, P. P.; Brenchley, J. E. Phylogenetic and Physiological Diversity of Microorganisms Isolated from a Deep Greenland Glacier Ice Core. *Appl. Environ. Microbiol.* **2004**, *70*, 202-213.

(180) Bai, Y.; Yang, D.; Wang, J.; Xu, S.; Wang, X.; An, L. Phylogenetic Diversity of Culturable Bacteria from Alpine Permafrost in the Tianshan Mountains, Northwestern China. *Res. Microbiol.* **2006**, *157*, 741-751.

(181) Sheng, H.; Gao, H.; Xue, L.; Ding, S.; Song, C.; Feng, H.; An, L. Analysis of the Composition and Characteristics of Culturable Endophytic Bacteria Within Subnival Plants of the Tianshan Mountains, Northwestern China. *Curr. Microbiol.* **2011**, *62*, 923-932.

(182) Dastager, S. G.; Lee, J.; Ju, Y.; Park, D.; Kim, C. *Frigoribacterium mesophilum* sp. nov., a Mesophilic Actinobacterium Isolated from Bigeum Island, Korea. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 1869-1872.

(183) Shivaji, S.; Kumari, K.; Kishore, K. H.; Pindi, P. K.; Rao, P. S.; Radha Srinivas, T. N.; Asthana, R.; Ravindra, R. Vertical Distribution of Bacteria in a Lake Sediment from Antarctica by Culture-Independent and Culture-Dependent Approaches. *Res. Microbiol.* **2011**, *162*, 191-203.

(184) Vardhan Reddy, P. V.; Shiva Nageswara Rao, S. S.; Pratibha, M. S.; Sailaja, B.; Kavya, B.; Manorama, R. R.; Singh, S. M.; Radha Srinivas, T. N.; Shivaji, S. Bacterial Diversity and Bioprospecting for Cold-Active Enzymes from Culturable Bacteria Associated with Sediment from a Melt Water Stream of Midtre Loenbreen Glacier, an Arctic Glacier. *Res. Microbiol.* **2009**, *160*, 538-546.

(185) Bajerski, F.; Ganzert, L.; Mangelsdorf, K.; Lipski, A.; Wagner, D. *Cryobacterium arcticum* sp. nov., a Novel Psychrotolerant Species from Store Koldewey, Northeast-Greenland. *Int. J. Syst. Evol. Microbiol. [Online Early Access]*. DOI:

10.1099/ijs.0.027128-0, Published Online: September 10, 2010, http://www.ncbi.nlm.nih.gov/pubmed (Accessed June 20, 2011).

(186) Dastager, S. G.; Lee, J.; Ju, Y.; Park, D.; Kim, C. *Cryobacterium mesophilum* sp. nov., a Novel Mesophilic Bacterium. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 1241-1244.

(187) Du, H.; Jiao, N.; Hu, Y.; Zeng, Y. Diversity and Distribution of Pigmented Heterotrophic Bacteria in Marine Environments. *FEMS Microbiol. Ecol.* **2006**, *57*, 92-105.

(188) Ivanova, E. P.; Onyshchenko, O. M.; Christen, R.; Lysenko, A. M.; Zhukova, N. V.; Shevchenko, L. S.; Kiprianova, E. A. *Marinomonas pontica* sp. nov., Isolated from the Black Sea. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 275-279.

(189) Yu, Y.; Li, H.; Zeng, Y.; Chen, B. Extracellular Enzymes of Cold-Adapted Bacteria from Arctic Sea Ice, Canada Basin. *Polar Biol.* **2009**, *32*, 1539-1547.

(190) Verma, P.; Pandey, P. K.; Gupta, A. K.; Kim, H. J.; Baik, K. S.; Seong, C. N.; Patole, M. S.; Shouche, Y. S. *Shewanella indica* sp. nov., Isolated from the Sediment of Arabian Sea. *Int. J. Syst. Evol. Microbiol. [Online Early Access]*. DOI: 10.1099/ijs.0.026310-0, Published Online: September 17, 2010, http://www.ncbi.nlm.nih.gov/pubmed (Accessed June 20, 2011).

(191) Kim, J.; Jin, H. M.; Lee, H. J.; Jeon, C. O.; Kanaya, E.; Koga, Y.; Takano, K.; Kanaya, S. *Flavobacterium banpakuense* sp. nov. Isolated from Leaf-and-Branch Compost. *Int. J. Syst. Evol. Microbiol. [Online Early Access]*. DOI:10.1099/ijs.0.022467-0, Published Online: August 6, 2010, http://www.ncbi.nlm.nih.gov/pubmed (Accessed June 20, 2011).

(192) Yumoto, I.; Hirota, K.; Kimoto, H.; Nodasaka, Y.; Matsuyama, H.; Yoshimune, K. *Psychrobacter piscatorii* sp. nov., a Psychrotolerant Bacterium Exhibiting High Catalase Activity Isolated from an Oxidative Environment. *Int. J. Syst. Evol. Microbiol.* **2010**, *60*, 205-208.

(193) Li, J.; Zhao, G.; Chen, H.; Qin, S.; Xu, L.; Jiang, C.; Li, W. *Rhodococcus cercidiphylli* sp. nov., a New Endophytic Actinobacterium Isolated from a *Cercidiphyllum japonicum* Leaf. *Syst. Appl. Microbiol.* **2008**, *31*, 108-113.

(194) Rapp, P.; Gabriel-Jurgens, L. H. E. Degradation of Alkanes and Highly Chlorinated Benzenes, and Production of Biosurfactants, by a Psychrophilic *Rhodococcus* sp. and Genetic Characterization of Its Chlorobenzene Dioxygenase. *Microbiology.* **2003**, *149*, 2879-2890.

(195) Espinosa, E.; Marco-Noales, E.; Gomez, D.; Lucas-Elio, P.; Ordax, M.; Garcias-Bonet, N.; Duarte, C. M.; Sanchez-Amat, A. Taxonomic Study of *Marinomonas* Strains Isolated from the Seagrass *Posidonia oceanica*, with Descriptions of *Marinomonas balearica* sp. nov. and *Marinomonas pollencensis* sp. nov. *Int. J. Syst. Evol. Microbiol.* **2010**, *60*, 93-98.

(196) Knight, V.; Sanglier, J.-J.; DiTullio, D.; Braccili, S.; Bonner, P.; Waters, J.; Hughes, D.; Zhang, L. Diversifying Microbial Natural Products for Drug Discovery. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 446-458.

(197) Fernandes, P.; Cabral, J. M. S. Microlitre/Millilitre Shaken Bioreactors in Fermentative and Biotransformation Processes - A Review. *Biocatal. Biotransformation.* **2006**, *24*, 237-252.

(198) Sigma-Aldrich. Diaion HP20. *http://www.sigmaaldrich.com/catalog/ProductDetail.do?D7=0&N5=SEARCH_CONCAT_PNO%7CBRAND_KEY&N4=13607%7CSUPELCO&N25=0&QS=ON&F=SPEC* (accessed May 30, 2011).

(199) Lang, S.; Beil, W.; Tokuda, H.; Wicke, C.; Lurtz, V. Improved Production of Bioactive Glucosylmannosyl-Glycerolipid by Sponge-Associated *Microbacterium* Species. *Mar. Biotechnol.* **2004**, *6*, 152-156.

(200) Sato, M.; Tsuchiya, H.; Kato, M.; Yamamoto, K.; Kanematsu, N.; Takagi, N.; Namikawa, I. Sucrose Concentrations in the Growth Medium Influence the Membrane Lipids of *Streptococcus mutans*. *Lett. Appl. Microbiol.* **1988**, *6*, 69-73.

(201) Chattopadhyay, M. K. Mechanism of Bacterial Adaptation to Low Temperature. *J. Biosci.* **2006**, *31*, 157-165.

(202) Wicke, C.; Hüners, M.; Wray, V.; Nimtz, M.; Bilitewski, U.; Lang, S. Production and Structure Elucidation of Glycoglycerolipids from a Marine Sponge-Associated *Microbacterium* Species. *J. Nat. Prod.* **2000**, *63*, 621-626.

(203) CambridgeSoft. ChemBioDraw Ultra. **2007**, *11.0.1*.

(204) Phenomenex. REZEX: HPLC Columns for Carbohydrate, Oligosaccharide, and Organic Acid Analysis. *http://chromservis.cz/media/0/05_pdf/Bochure_Rezex_UK090422.pdf* (accessed May 27, 2011).

(205) Mukherjee, S.; Srivastava, H. C. The Structure of Neem Gum. *J. Am. Chem. Soc.* **1955**, *77*, 422-423.

(206) Prestegard, J. H.; Miner, V. W.; Tyrell, P. M. Deuterium NMR as a Structural Probe for Micelle-Associated Carbohydrates: D-Mannose. *Proc. Natl. Acad. Sci. U. S. A.* **1983**, *80*, 7192-7196.

(207) Winnik, F. M.; Brisson, J.; Carver, J. P.; Krepinsky, J. J. Syntheses of Model Oligosaccharides of Biological Significance. Synthesis of Methyl 3,6-DI-O-(α-d-Mannopyranosyl)-α-d-Mannopyranoside and the Corresponding Mannobiosides. *Carbohydr. Res.* **1982**, *103*, 15-28.

(208) Rho, M.; Matsunaga, K.; Yasuda, K.; Ohizumi, Y. A Novel Monogalactosylacylglycerol with Inhibitory Effect on Platelet Aggregation from the Cyanophyceae Oscillatoria rosea. *J. Nat. Prod.* **1996**, *59*, 308-309.

(209) Dabrowski, J.; Hanfland, P.; Egge, H. Structural Analysis of Glycosphinoglipids by High-Resolution ^{1}H Nuclear Magnetic Resonance Spectroscopy. *Biochemistry* **1980**, *19*, 5652-5658.

(210) Han, X. Shotgun Lipidomics: Electrospray Ionization Mass Spectrometric Analysis and Quantitation of Cellular Lipidomes Directly from Crude Extracts of Biological Samples. *Mass Spectrom. Rev.* **2005**, *24*, 367-412.

(211) Murakami, N.; Imamura, H.; Morimoto, T.; Ueda, T.; Nagai, S. -I.; Sakakibara, J.; Yamada, N. Selective Preparation of SN-1 and SN-2 Lysogalactolipids by Enzymatic Hydrolysis Using Lipase (from *Rhizopus arrhizus*). *Tetrahedron Lett.* **1991**, *32*, 1331-1334.

(212) Ichihara, K.; Fukubayashi, Y. Preparation of Fatty Acid Methyl Esters for Gas-Liquid Chromatography. *J. Lipid Res.* **2010**, *51*, 635-640.

(213) Ruiz-Rodriguez, A.; Reglero, G.; Ibañez, E. Recent Trends in the Advanced Analysis of Bioactive Fatty Acids. *J. Pharm. Biomed. Anal.* **2010**, *51*, 305-326.

(214) Sigma-Aldrich. Bulletin 909A: Guide to Derivatization Reagents for GC. *http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/Application_Notes/4537.Par.0001.File.tmp/4537.pdf* (accessed May 27, 2011).

(215) MacLeod, J. K.; Ward, A.; Oelrichs, P. B. Structural Investigation of Resin Glycosides from *Ipomoea lonchophylla*. *J. Nat. Prod.* **1997**, *60*, 467-471.

(216) Pieri, V.; Schwaiger, S.; Ellmerer, E. P.; Stuppner, H. Iridoid Glycosides from the Leaves of *Sambucus ebulus*. *J. Nat. Prod.* **2009**, *72*, 1798-1803.

(217) Uzawa, H. Determination of Absolute Configuration of Natural Glycerols. *Kagaku to Seibutsu.* **1991**, *29*, 394.

(218) Hiraga, Y.; Shikano, T.; Widianti, T.; Ohkata, K. Three New Glycolipids with Cytolytic Activity from Cultured Marine Dinoflagellate *Heterocapsa circularisquama*. *Nat. Prod. Res.* **2008**, *22*, 649-657.

(219) Bultel-Poncé, V.; Debitus, C.; Berge, J.-P.; Cerceau, C.; Guyot, M. Metabolites from the Sponge-Associated Bacterium *Micrococcus luteus*. *J. Mar. Biotechnol.* **1998**, *6*, 233-236.

(220) Kwon, H. C.; Zee, O. P.; Lee, K. R. Two New Monogalactosylacylglycerols from *Hydrocotyle ramiflora*. *Planta Med.* **1998**, *64*, 477-479.

(221) Rho, M.-C.; Matsunaga, K.; Yasuda, K.; Ohizumi, Y. A Novel Inhibitor of Platelet Aggregation from the Cyanophyceae *Oscillatoria rosea* (NIES-208). *Planta Med.* **1996**, *62*, 473-474.

(222) Takahashi, Y.; Itabashi, Y.; Suzuki, M.; Kuksis, A. Determination of Stereochemical Configuration of the Glycerol Moieties in Glycoglycerolipids by Chiral Phase High-Performance Liquid Chromatography. *Lipids* **2001**, *36*, 741-748.

(223) Fuguet, E.; Ràfols, C.; Rosés, M.; Bosch, E. Critical Micelle Concentration of Surfactants in Aqueous Buffered and Unbuffered Systems. *Anal. Chim. Acta.* **2005**, *548*, 95-100.

(224) Jia, X.; Patrzykat, A.; Devlin, R. H.; Ackerman, P. A.; Iwama, G. K.; Hancock, R. E. W. Antimicrobial Peptides Protect Coho Salmon from *Vibrio anguillarum* Infections. *Appl. Environ. Microbiol.* **2000**, *66*, 1928-1932.

(225) Patrzykat, A.; Gallant, J. W.; Seo, J.; Pytyck, J.; Douglas, S. E. Novel Antimicrobial Peptides Derived from Flatfish Genes. *Antimicrob. Agents Chemother.* **2003**, *47*, 2464-2470.

(226) Vellonen, K.; Honkakoski, P.; Urtti, A. Substrates and Inhibitors of Efflux Proteins Interfere with the MTT Assay in Cells and May Lead to Underestimation of Drug Toxicity. *Eur. J. Pharm. Sci.* **2004**, *23*, 181-188.

(227) Holian, O.; Walter, R. J. Resveratrol Inhibits the Proliferation of Normal Human Keratinocytes *in vitro*. *J. Cell. Biochem.* **2001**, *81*, 55-62.

(228) Pagliacci, M. C.; Spinozzi, F.; Migliorati, G.; Fumi, G.; Smacchia, M.; Grignani, F.; Riccardi, C.; Nicoletti, I. Genistein Inhibits Tumour Cell Growth in vitro but Enhances Mitochondrial Reduction of Tetrazolium Salts: A Further Pitfall in the Use of the MTT Assay for Evaluating Cell Growth and Survival. *Eur. J. Cancer* **1993**, *29*, 1573-1577.

(229) Es Saady, D. MCF-7 Cell Cycle Arrested at G1 Through Ursolic Acid, and Increased Reduction of Tetrazolium Salts. *Anticancer Res.* **1996**, *16*, 481-486.

(230) Jabbar, S. A. B.; Twentyman, P. R.; Watson, J. V. The MTT Assay Underestimates the Growth Inhibitory Effects of Interferons. *Br. J. Cancer* **1989**, *60*, 523-528.

(231) Plouguerné, E.; Ioannou, E.; Georgantea, P.; Vagias, C.; Roussis, V.; Hellio, C.; Kraffe, E.; Stiger-Pouvreau, V. Anti-Microfouling Activity of Lipidic Metabolites from the Invasive Brown Alga *Sargassum muticum* (Yendo) Fensholt. *Mar. Biotechnol.* **2010**, *12*, 52-61.

(232) Takahashi, Y.; Itoh, K.; Ishii, M.; Suzuki, M.; Itabashi, Y. Induction of Larval Settlement and Metamorphosis of the Sea Urchin *Strongylocentrotus intermedius* by Glycoglycerolipids from the Green Alga *Ulvella lens*. *Mar. Biol.* **2002**, *140*, 763-771.

(233) Fusetani, N. Biofouling and Antifouling. *Nat. Prod. Rep.* **2004**, *21*, 94-104.

(234) Fusetani, N. Antifouling Marine Natural Products. *Nat. Prod. Rep.* **2011**, *28*, 400-410.

APPENDIX I

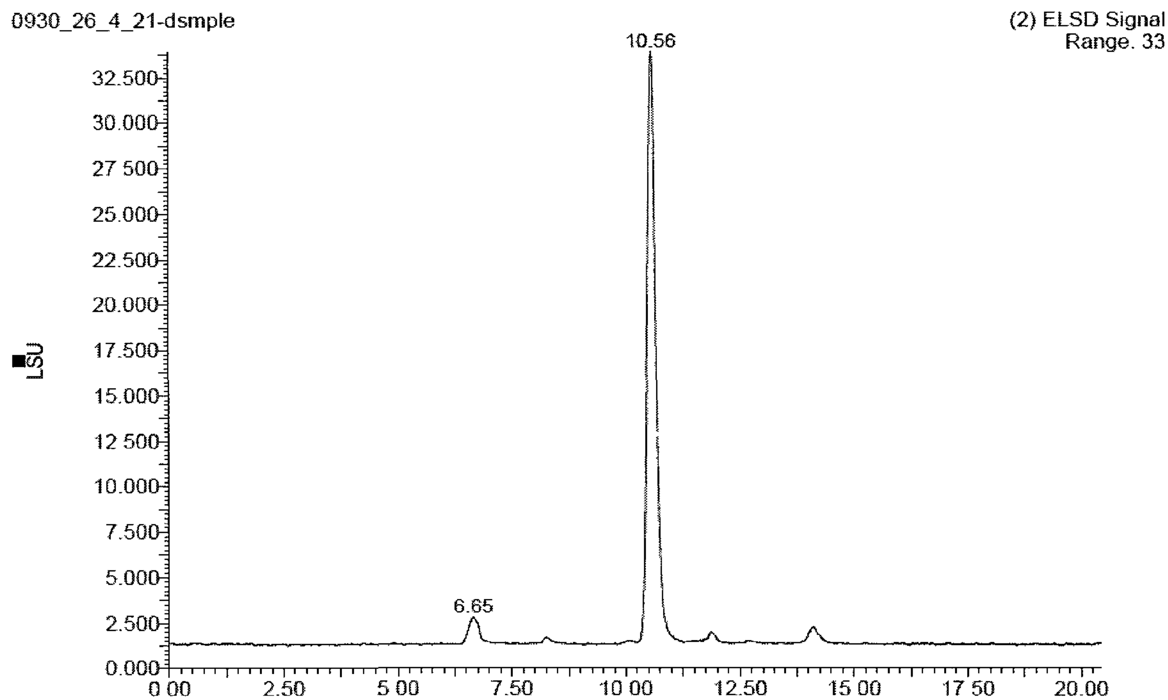


Figure S1. HPLC Chromatogram of Sugars from GGL 887 Hydrolysis

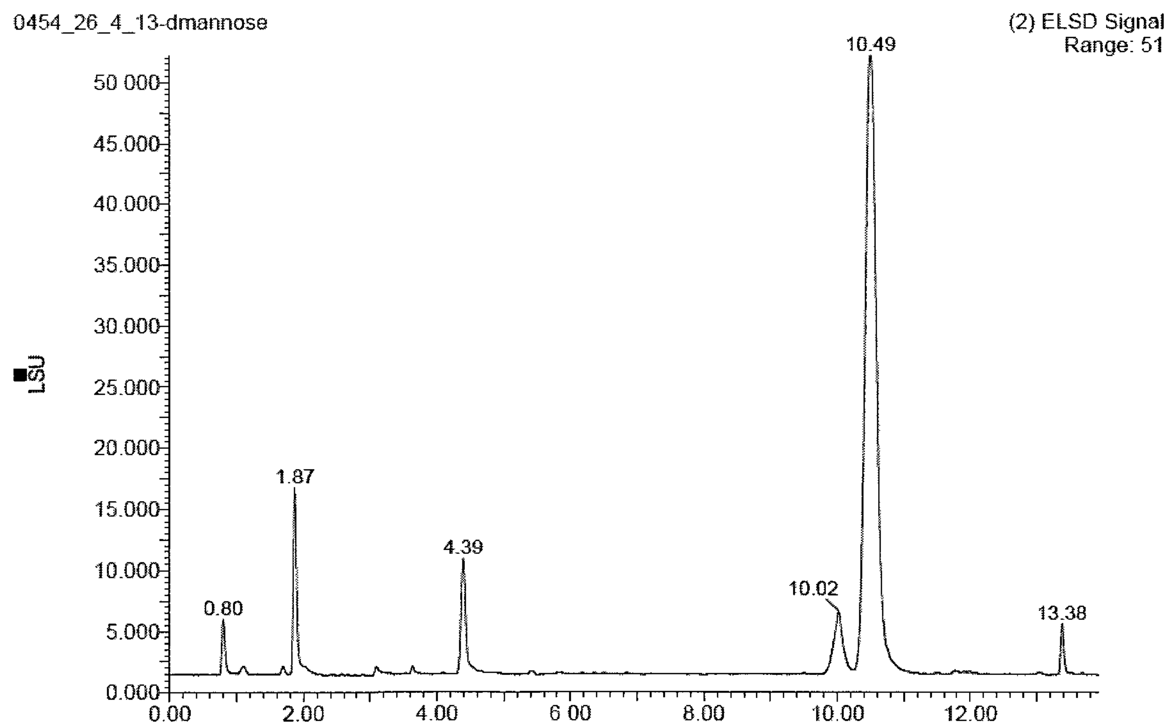


Figure S2. HPLC Chromatogram of D-Mannose Standard

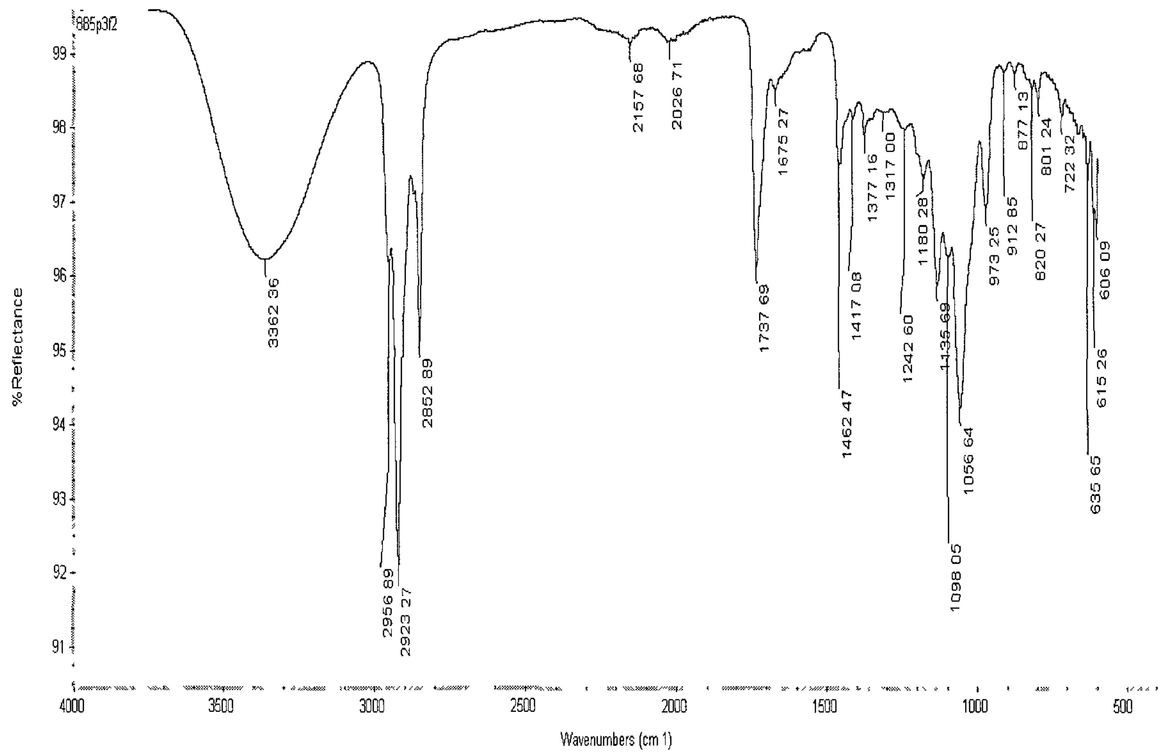


Figure S3. IR Spectrum of GGL 885

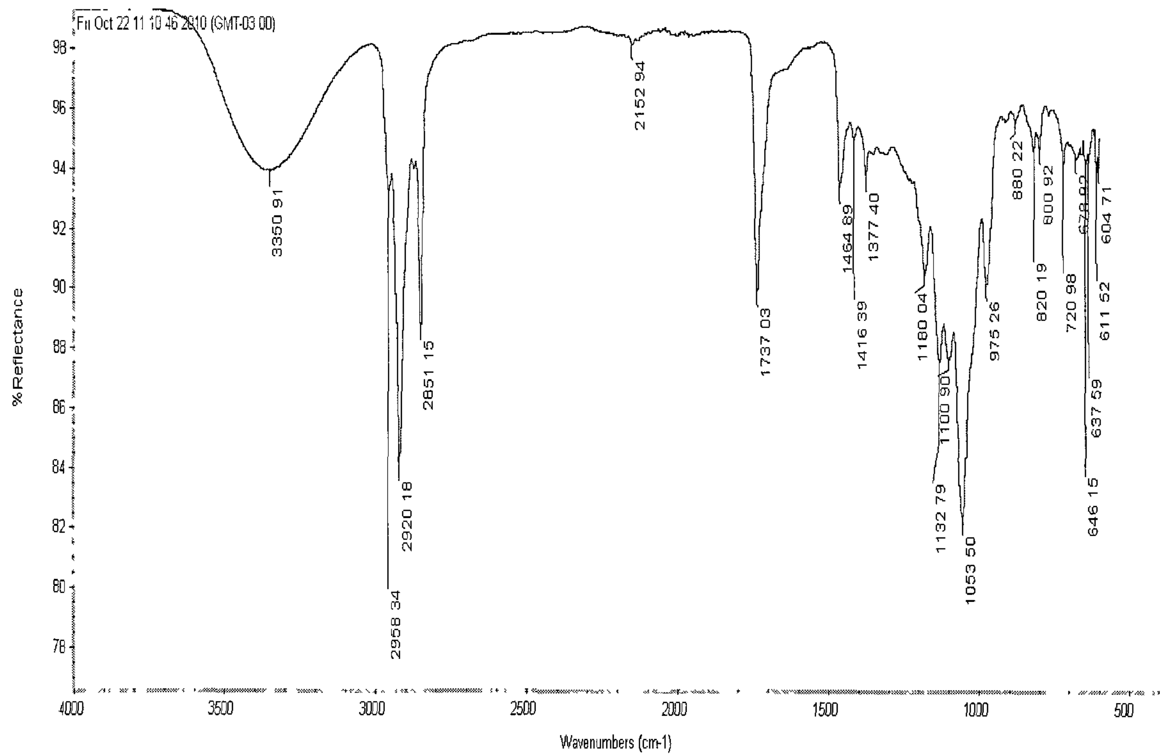


Figure S4. IR Spectrum of GGL 915

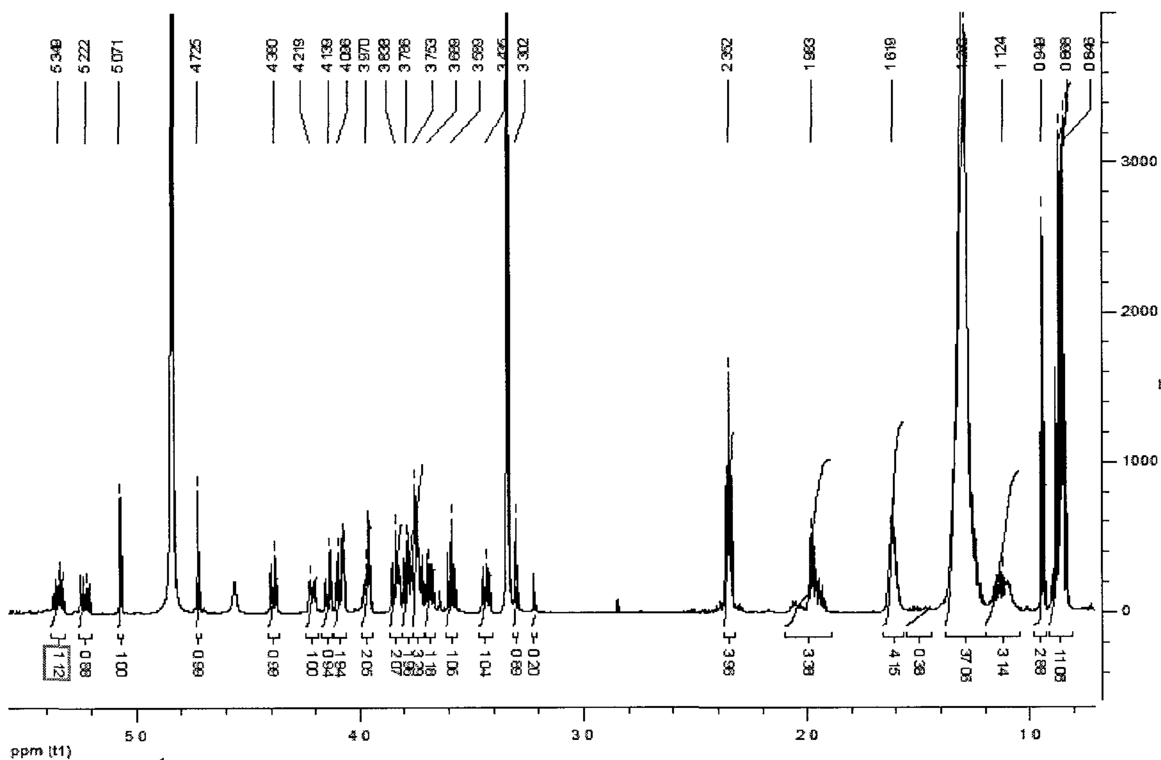


Figure S5. ^{1}H NMR Spectrum of GGL 885 (600 MHz) in CD_3OD

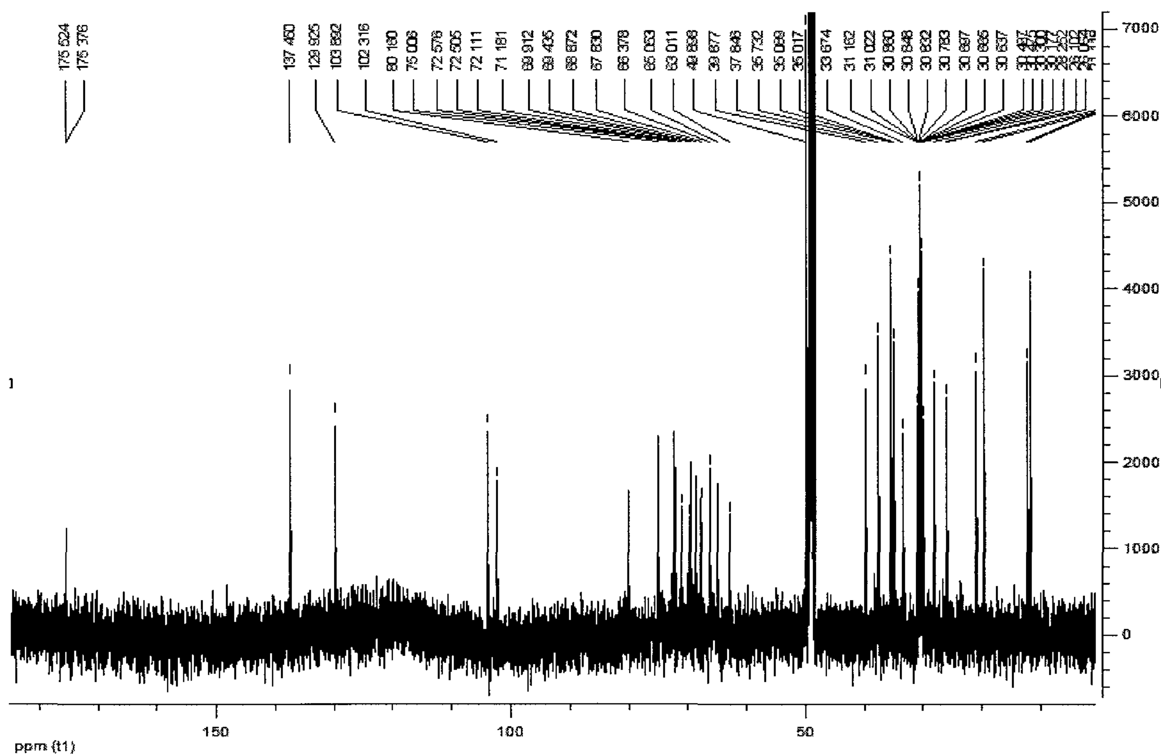


Figure S6. ^{13}C NMR Spectrum of GGL 885 (150 MHz) in CD_3OD

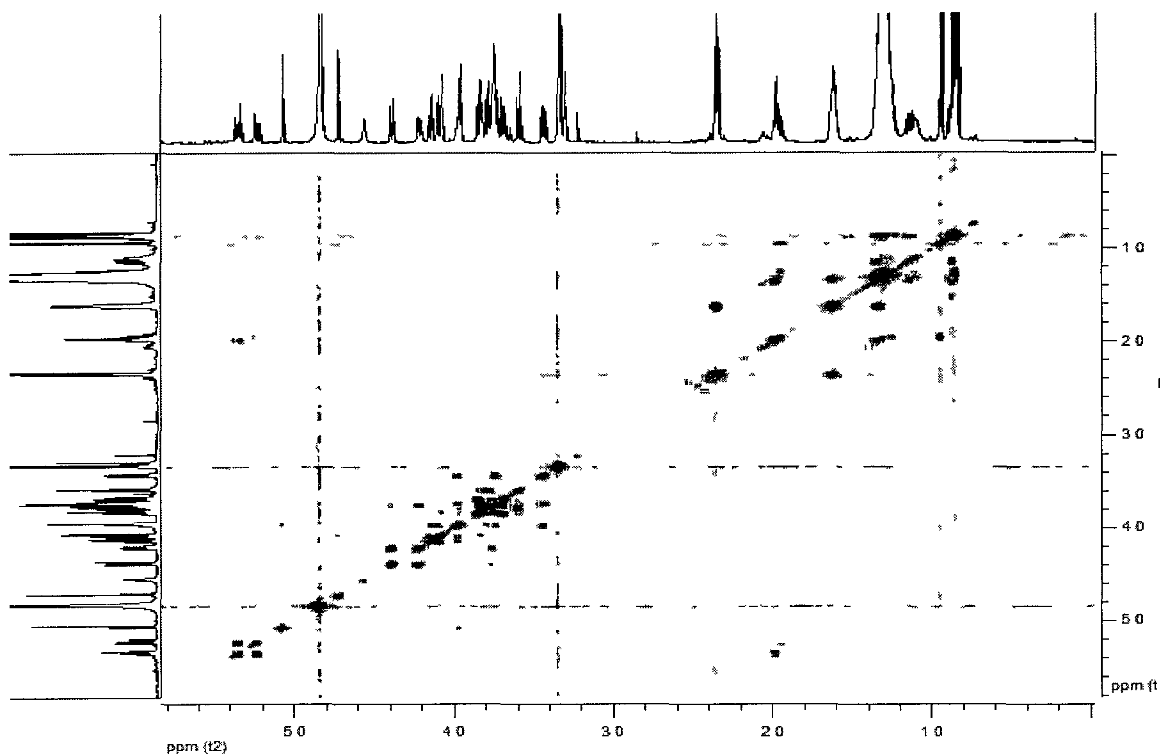


Figure S7. COSY NMR Spectrum of GGL 885 (600 MHz) in CD_3OD

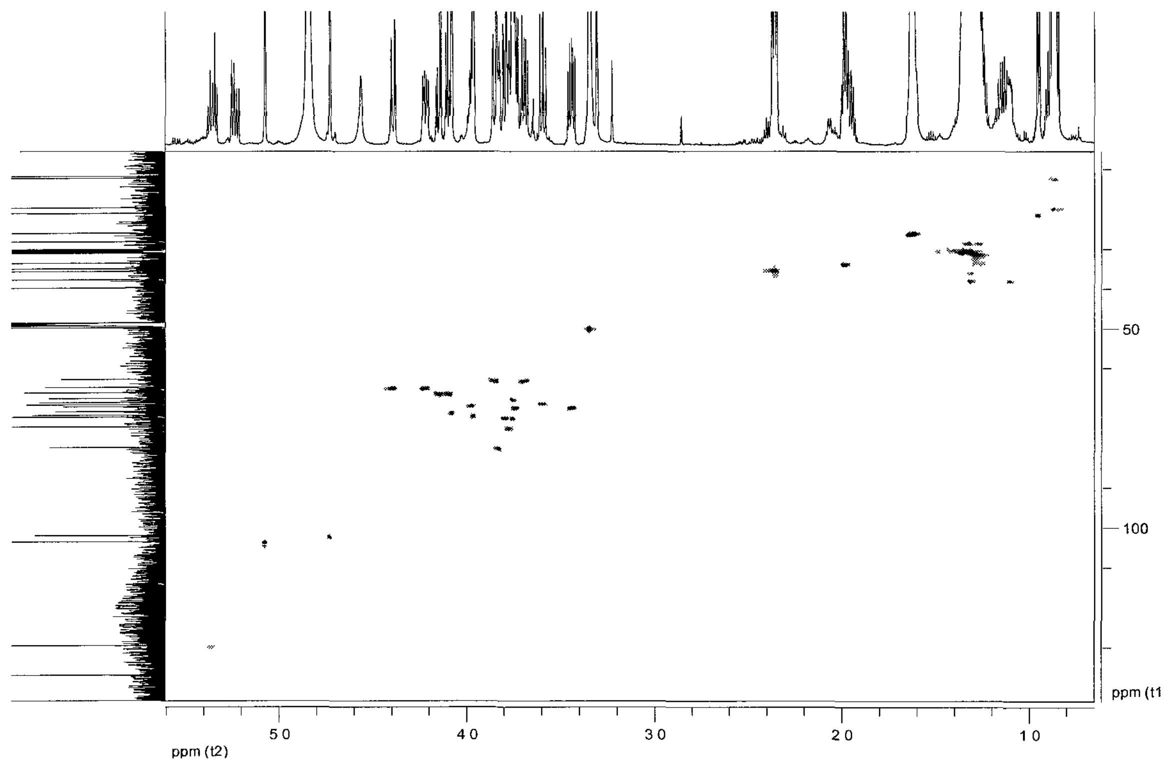


Figure S8. HSQC NMR Spectrum of GGL 885 (600 MHz) in CD_3OD

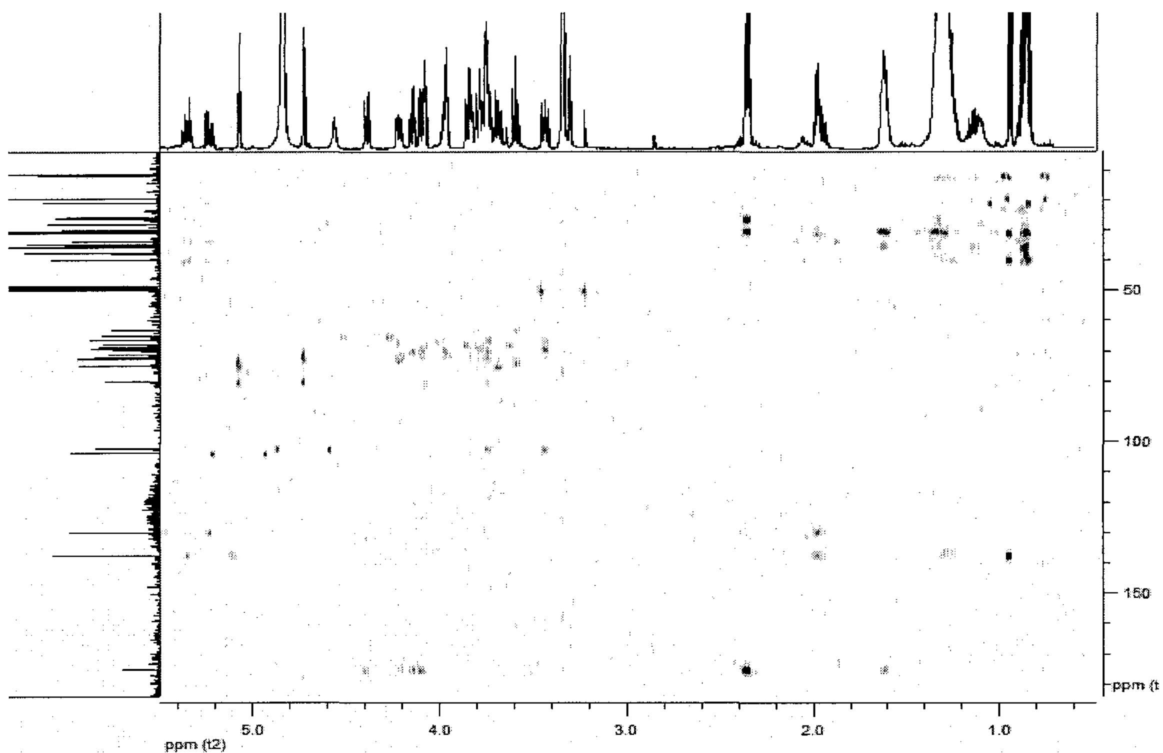


Figure S9. HMBC NMR Spectrum of GGL 885 (600 MHz) in CD_3OD

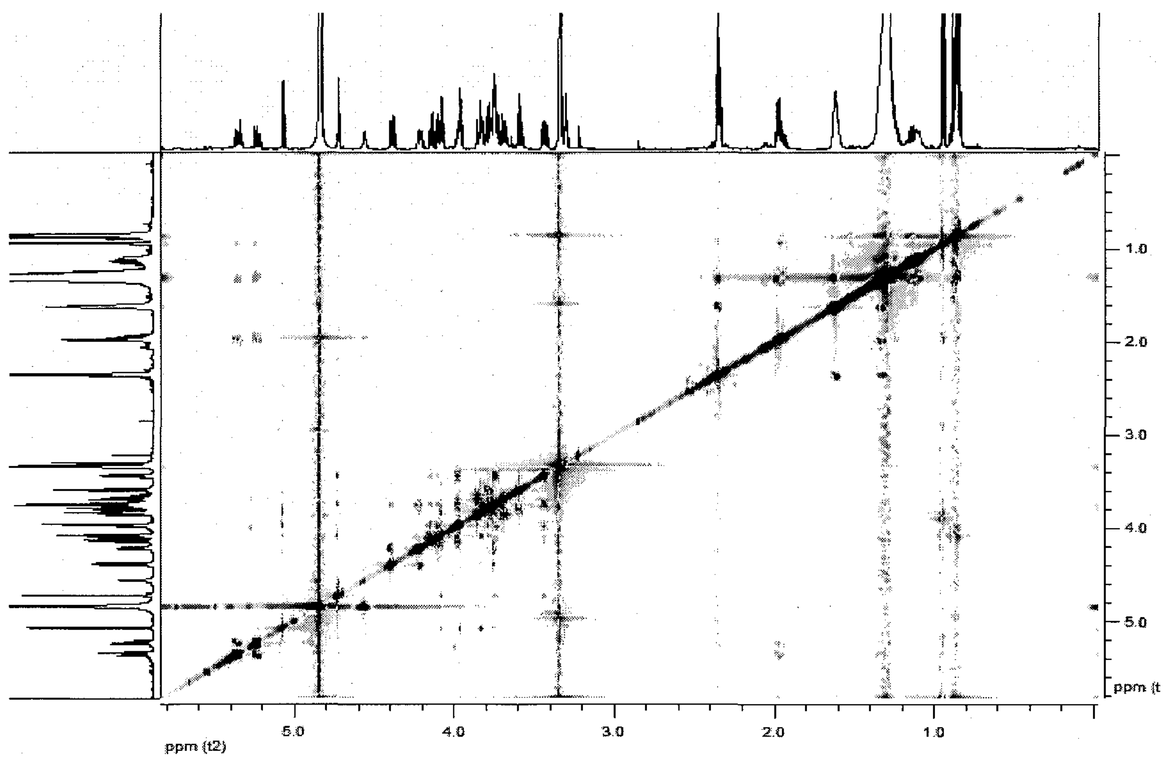


Figure S10. NOESY NMR Spectrum of GGL 885 (600 MHz) in CD_3OD

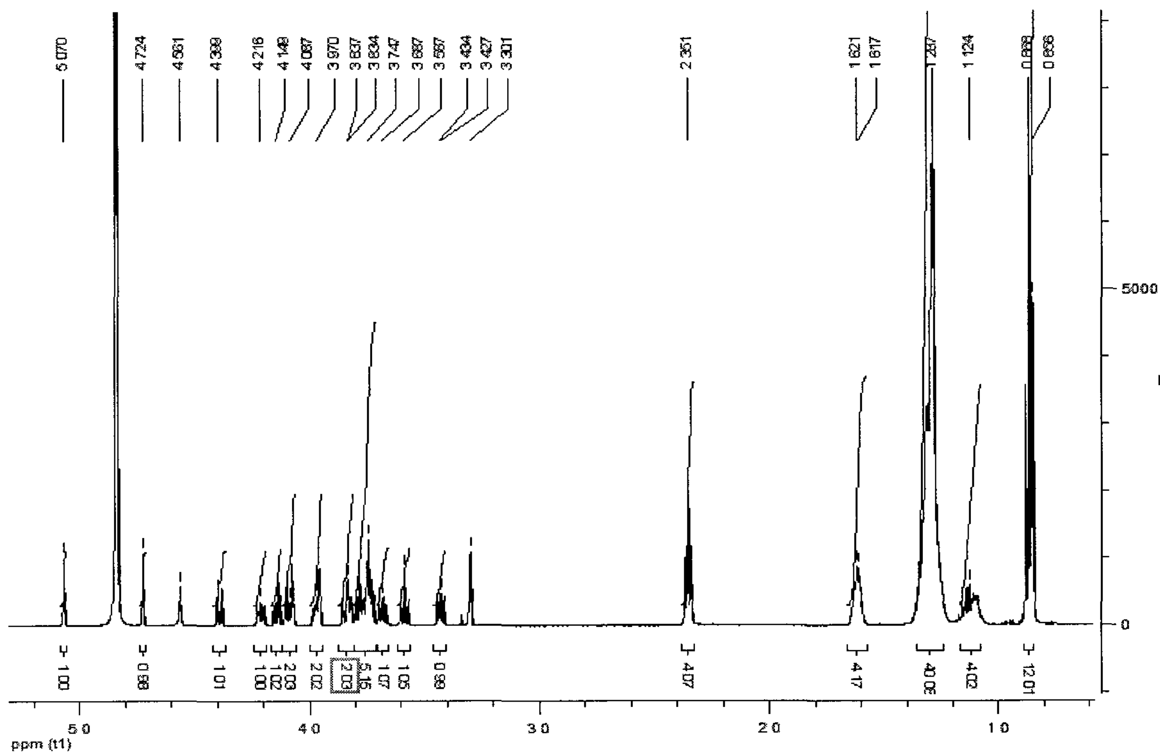


Figure S11. ^{1}H NMR Spectrum of GGL 915 (600 MHz) in CD_3OD

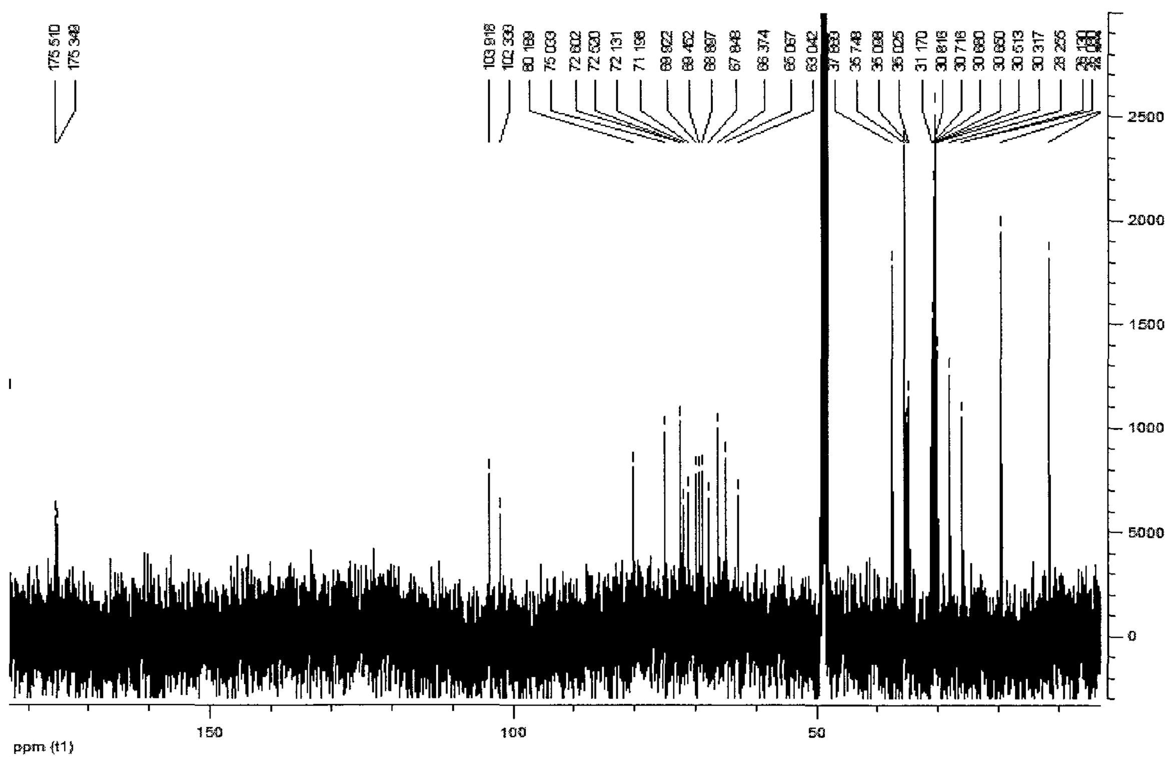


Figure S12. ^{13}C NMR Spectrum of GGL 915 (150 MHz) in CD_3OD

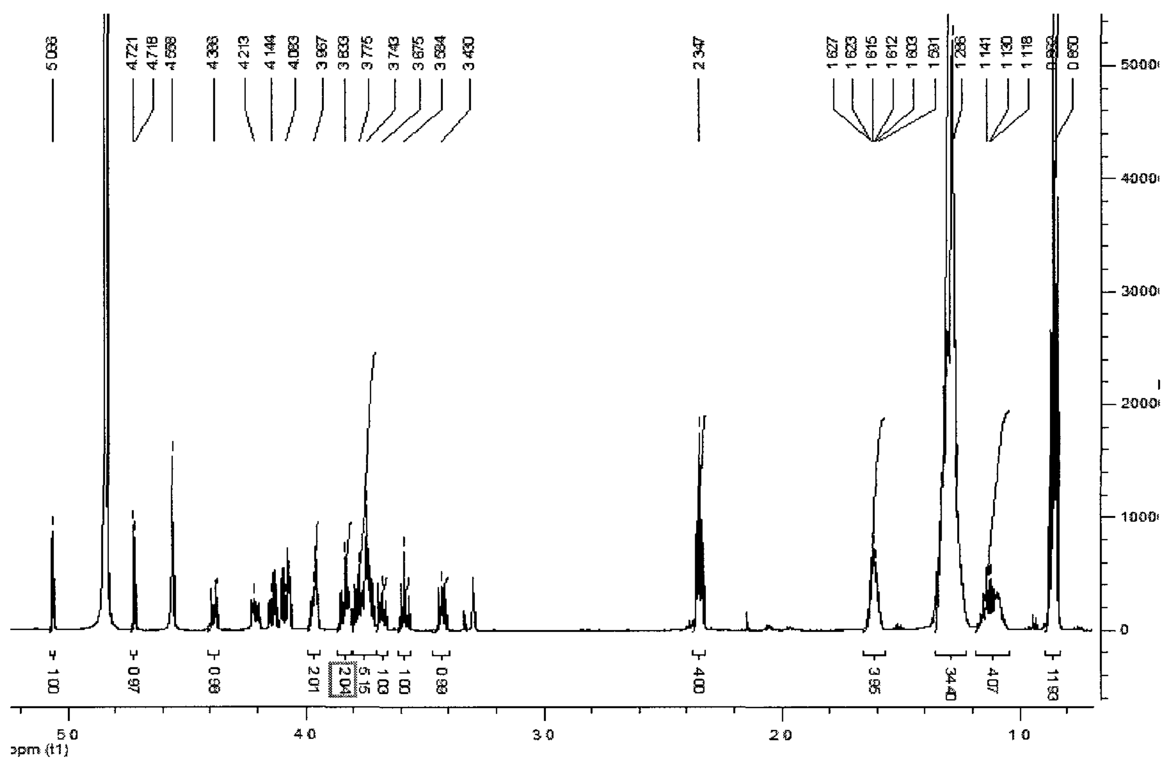


Figure S13. ^{1}H NMR Spectrum of GGL 887 (600 MHz) in CD_3OD

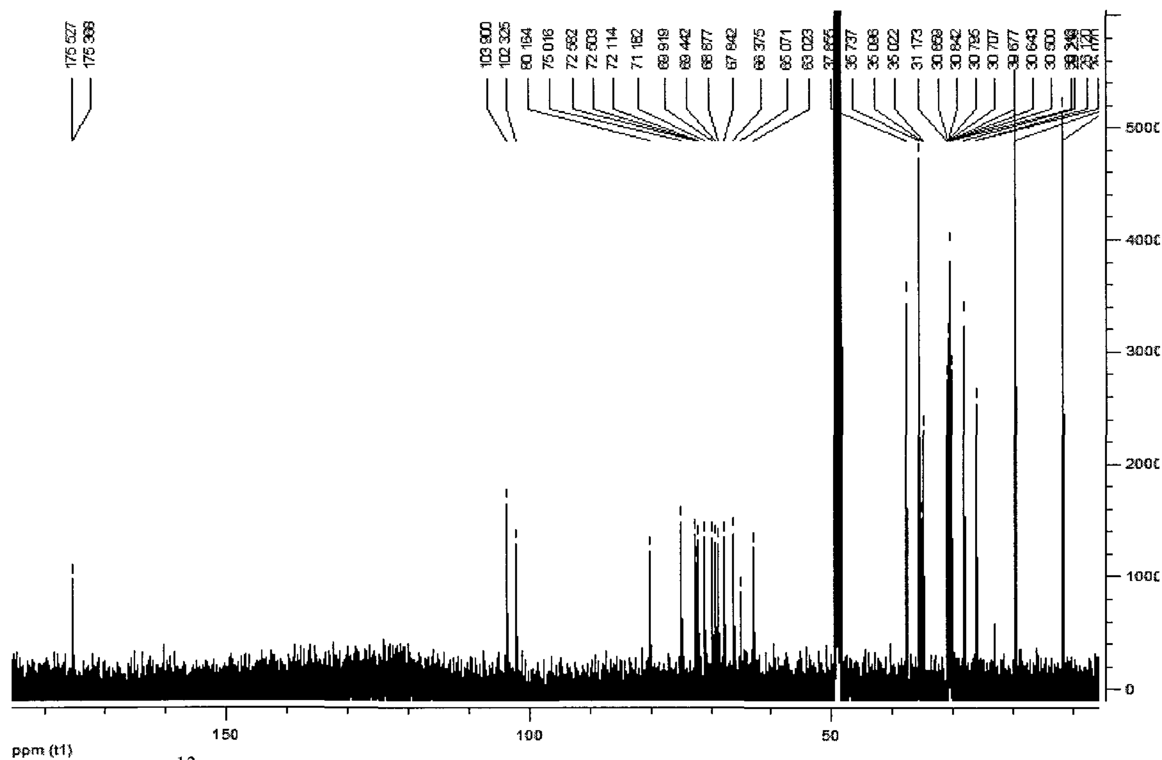


Figure S14. ^{13}C NMR Spectrum of GGL 887 (150 MHz) in CD_3OD

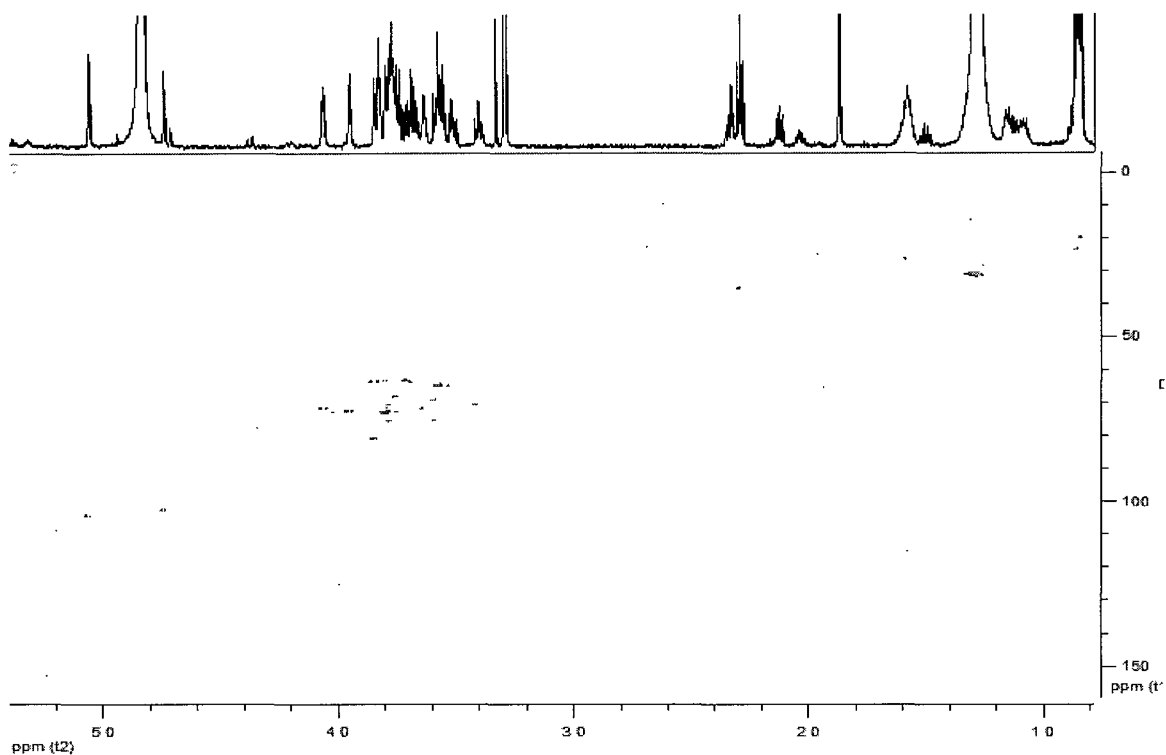


Figure S15. HSQC NMR Spectrum of Hydrolyzed GGL 873 (600 MHz) in CD_3OD

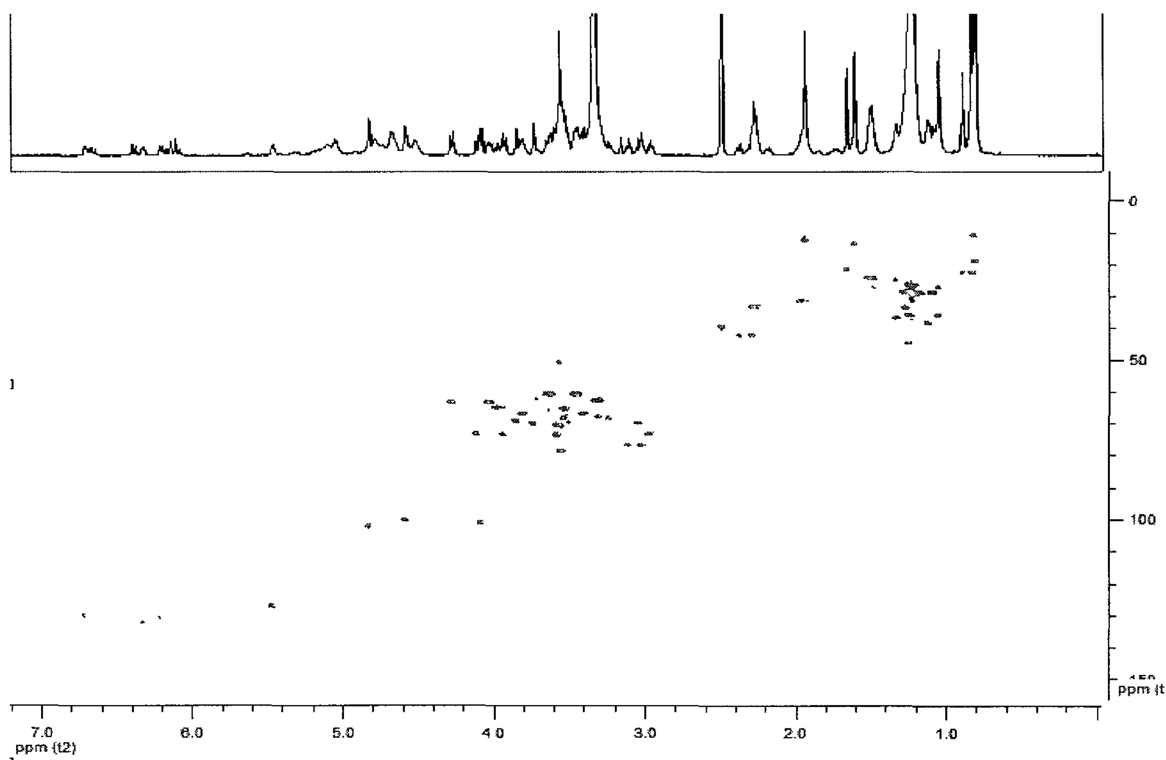


Figure S16. HSQC NMR Spectrum of GGL 873 Mixture (600 MHz) in DMSO-d_6

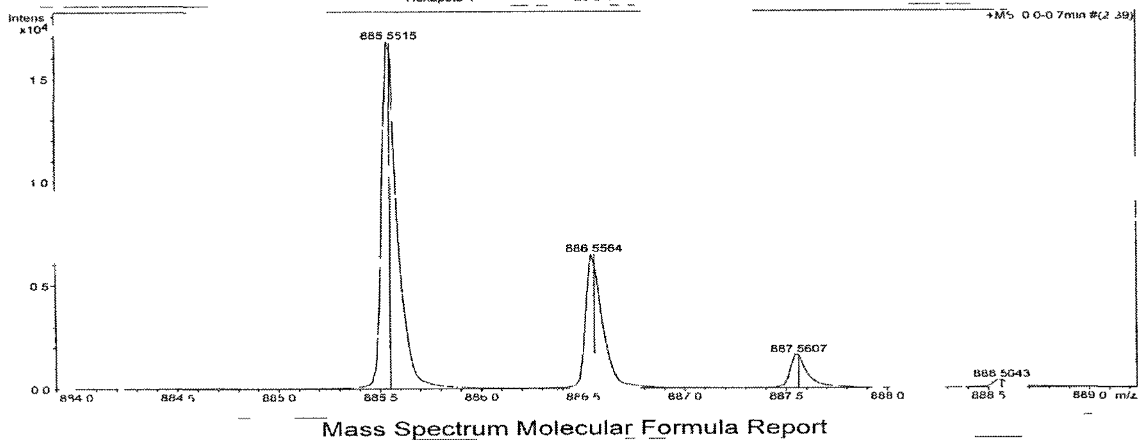
Mass Spectrum Molecular Formula Report

Analysis Info

Analysis Name	D \Data\Xiao\Sept 29 2010000014 d	Acquisition Date	9/29/2010 3 27 06 PM
Method	xiaofengpos m	Operator	Administrator
Sample Name	885 P3F2	Instrument	microTOF 57
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	52 V
Scan Range	n/a	Capillary Exit	110 0 V	Set Pulsar Pull	398 V
Scan Begin	50 m/z	Hexapole RF	150 0 V	Set Pulsar Push	398 V
Scan End	1500 m/z	Skimmer 1	55 0 V	Set Reflector	1300 V
		Hexapole 1	26 0 V	Set Flight Tube	9000 V
				Set Detector TOF	1960 V



Mass Spectrum Molecular Formula Report

Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	rdb	N Rule	e⁻
C 45 H 82 Na 1 O 15	0 07	885 5546	3 52	2 61	4 50	ok	even

Figure S17. HR-MS Spectrum and Data of GGL 885

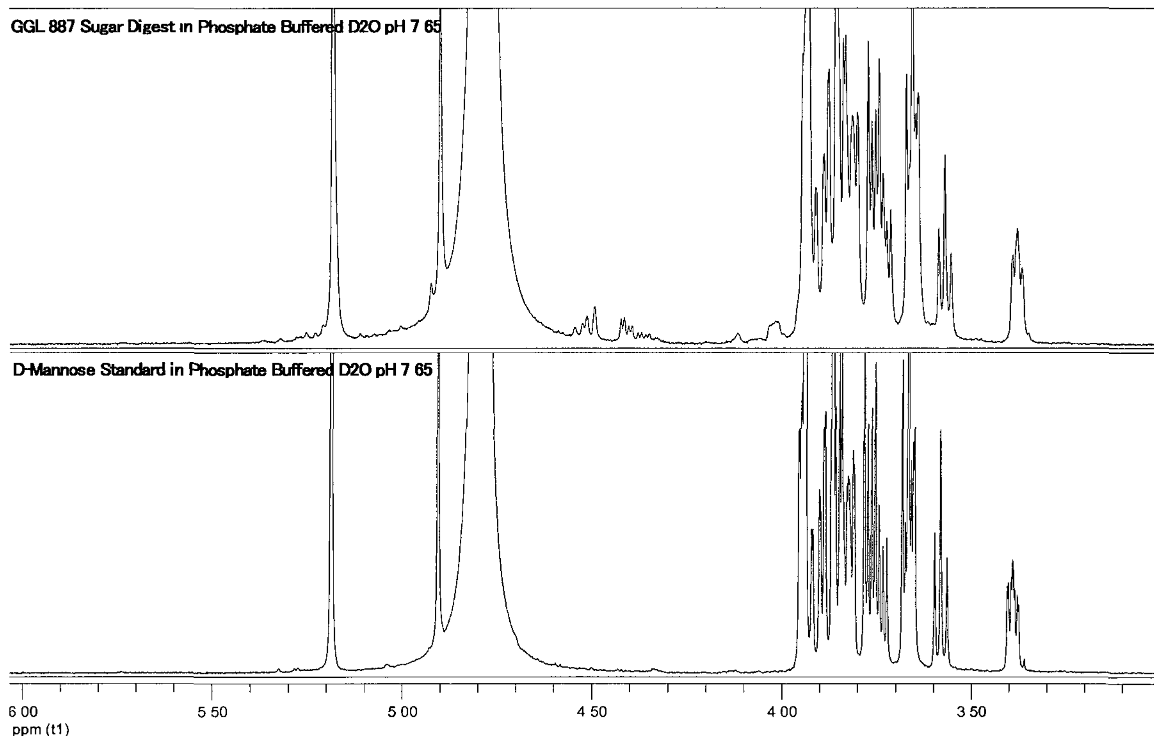


Figure S18. ^{1}H NMR Spectrum of GGL 887 Sugar Digest and D-Mannose Standard (600 MHz) in Phosphate Buffered D_2O at pH 7.65