Secondary Metabolite Regulation And Drug-Lead Discovery From Aquatic Actinomycetes.

BY

SKYLAR NICOLE CARLSON

B.S. Florida State University, 2010

THESIS

Submitted as partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Pharmacognosy in the Graduate College of the

University of Illinois at Chicago, 2015

Chicago, Illinois

Defense Committee:

Brian T. Murphy, PhD	Chair and Advisor, Department of Medicinal Chemistry and Pharmacognosy, Institute for Tuberculosis Research
Joanna Burdette, PhD	Department of Medicinal Chemistry and Pharmacognosy
Jimmy Orjala, PhD	Department of Medicinal Chemistry and Pharmacognosy
Alexander Mankin, PhD	Department of Medicinal Chemistry and Pharmacognosy
Stefan Green, PhD	UIC DNA Services Facility, Department of Biological Sciences and Department of Pathology

This dissertation is dedicated to those whose ladder leaned against the wrong wall and were brave enough to make the leap to start again.

ACKNOWLEDGMENTS

I would like to thank Dr. Brian T. Murphy for allowing me the privilege of being his first graduate student. It has been my pleasure to give everything for the success of his laboratory, be it rummaging through the remnants of other closed laboratories' junk or searching the lab, top to bottom, for the smallest blue plastic stand for a collection trip. Although his favorite phrase is "this is not my first rodeo…" this has been our first rodeo. Thank you for your firm support and high standards. I fully believe that it is these seemingly unachievable standards that have made me the scientist I have become. Your laboratory gave me the opportunity to follow my dreams to pursue natural products chemistry and structure elucidation and your personal vested interest in my success gave me the wings to pursue my next step, wherever that may be.

I would like to acknowledge the members of my preliminary and doctoral defense committees for their time and helpful feedback: Dr. Joanna Burdette, Dr. Stefan Green, Dr. Jimmy Orjala, Dr. Shura Mankin, and Dr. Don Morrison.

My foundations in Florida ensured my success here in Chicago in graduate school. I would like to thank my dear friend and travel buddy Carolyn Hartman, my mormor, my benefactor, my grandmother. Your support has truly made this chapter of my life an adventure and not a struggle. To my parents, who more than anything else they have ever given me, gave me all of their love and truly made me the person I am today. And my dear Mae for her constant joy in the face of life regardless of the obstacles life throws our way.

I would not be studying Pharmacognosy if it were not for the caring and gracious mentorship I received at Florida State University. Dr. Mark Kearley sat with me for hours in his office going over many synthetic routes of macrolide antibiotics. He showed me how to be a mentor and how

to take a vested interest in a student's dreams. I would also like to acknowledge Dr. John Dorsey, for his simple and elegant teaching methods, proving that the simplest execution of a task is often the most powerful. His continued friendship has been a fountain of inspiration and motivation during graduate school.

I would also like to thank Dr. Judy Bolton, who has demonstrated, through her leadership of the department, the power of being a woman and the strength that the fierce application of logic to any situation can clarify even the most difficult of decisions. I would also like to thank Dr. Joanna Burdette for her wisdom and the perspective of a fellow southern lady and always being available to talk. I would like to acknowledge Dr. Melany Puglisi of Chicago State University for all that she has done for the advancement of my career and the opportunity to gain invaluable teaching experience. Her outside perspective has been a breath of fresh air and a dose of reality.

I would also like to acknowledge my new and old friends. To Dr. Chaitanya Aggarwal, for being my rock. To Michelle Vierra and James Paul Marks III, Caw. To my lady girls, Kimberly Nowakowski and Brittany Johnson, for reminding me that Chicago is bigger than UIC or my problems. To Kerem Araci for your continued support during the most exciting year of my life. To my new friends Julia Zelenakova and Anna Balasubramanian for your confidence and support. Lastly, to my cohort of graduate students, we did it. Regardless of what the "it" actually is, we did it.

To my constant companions, my lab mates, those past and present: Urszula Tanouye who made the transition from biochemist to scientist a joy. She demonstrated that you can only "science" if you are happy. To Mark Sadek who reminded me daily that you can always work harder. To Maryam Elfeki, who showed me how to care just enough and to never take myself too seriously.

For Anam Shaikh, who I am incredibly proud of, who always checked on my mental and emotional state and for extensive review of this dissertation. For Dr. Hiyoung Kim who showed me that leadership is not always being loud or in charge. To those lab members who will continue the honorable pursuit of natural products and novel secondary metabolites, Brian Lallier, Michael Mullowney, and Vanessa Nepomuceno. I wish them, and Dr. Brian T. Murphy continued success and excellence.

Last but not least, I would like to thank my collaborators and friends in no particular order: Dr. Nora Vasquez-Laslop, Dr. Tyvette Hilliard, Dr. Tiara Perez, Dr. Michael Federle, James Paul Marks, Dr. Cedric Orelle, Tanja Florin, Dr. Jenny Chang, Dr. Laura Cook, Dr. Chang Hwa Hwang, Dr. David Lankin, Dr. Alec Krunic, Eoghainin Ó hAinmhire, Dan May, Liz Ryan, Divya Vasudevan, Rhea Bovee, Dr. Suzzane Quartuccio, Dr. Shangwen Luo, Dr. Heonjoong Kang, Dr. Hahk Soo Kang, Dr Ben Ramirez, Dr. Gerd Prehna, Dr. Sang Hyun Cho, Dr. Leo Njongmeta, Samatha Glass, Matthew Deraedt, Taylor Highland, Gennaro Paolella, Ying Gao, and Grace Li.

CONTRIBUTIONS OF AUTHORS

Chapter 1 is an original work by Skylar Carlson with editing graciously provided by Dr. Brian T. Murphy. Chapter 2 is a manuscript published in *Marine Drugs* in 2013. The bioassays are the work of Laura Marler in the laboratory of Dr. John Pezzuto. Dr. Sang-Jip Nam provided technical assistance in crystallization of **1** and **2**. Dr. Bernard Santarsiero preformed X-ray experiments on the crystallized **1** and **2**. The isolation and crystallization of **1** and **2**, were completed, and NMR spectroscopic data were acquired at the Scripps Institute of Oceanography, by Dr. Brian T. Murphy. The analysis of spectral data, the characterization of **1** and **2**, and manuscript preparation was completed by Skylar Carlson. Chapter 3 is a manuscript published ASAP on the *Journal of Natural Products* website and an original analysis of the field of secondary metabolism induction completed by Skylar Carlson. All co-cultivation experiments, in-house antimicrobial bioassays, and subsequent follow-up experiments were completed by Skylar Carlson. Sequencing was completed at the UIC DNA Services Facility and at the University of Chicago Cancer Center. Edited by Dr. Brian T. Murphy. Urszula Tanouye provided technical consultation. Dr. Sesselja Omarsdottir hosts Dr. Brian T. Murphy in Iceland during collection-trips as a part of their collaboration. The source sediments of the strains starting with the letter K in Figure 6 were collected during these trips. She also provided editorial review of the completed manuscript. Chapter 4 is an original analysis by Skylar Carlson of the future direction of the natural products field. Appendix I is a summary of selected projects involving some of the collaborations in the laboratory of Brian T. Murphy. The chromatography, NMR spectroscopy, and in-house antimicrobial bioassay were executed by Skylar Carlson. All other biological data in this document is the result of collaborations. Funding was provided by a variety of private and government sources, as described in the acknowledgments section.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iv

LIST OF FIGURES xiii

LIST OF ABBREVIATIONS xvii

SUMMARY xx

Chapter 1. Introduction to thesis research 1

1.1 A historical perspective of natural products 1

1.2 Microbial-derived natural products 1

1.3 The aquatic environment as a source for drug-lead discovery 3

1.4 Aquatic actinomycete-derived natural products 4

1.5 Antibiotic resistance and the development of novel bioassays 5

1.6 The realization of the genetic potential of actinomycetes 6

1.7 Thesis summary 7

1.8 References 9

Chapter 2. Potential chemopreventive activity of a new macrolide antibiotic from a marine-derived *Micromonospora* sp. 14

2.1 Introduction 14

2.2 Results and discussion 16

2.2.1 Structure elucidation 16

2.2.2 Chemopreventive activity of **1** and **2** 20

2.3 Methods 22

2.3.1 General experimental procedures 22

2.3.2 Bacterial isolation and identification 22

2.3.3 Fermentation and extraction 23

2.3.4 Isolation and characterization of juvenimicin C (**1**) and isolation of 5-O-α-L-rhamnosyltylactone (**2**) 23

2.3.5 Quinone reductase 1 (QR1) assay 24

2.3.6 Determination of GSH levels in cell culture 26

2.3.7 Determination of glutathione reductase activity 27

2.3.8 Determination of glutathione peroxidase activity 27

2.4 Conclusions 27

2.5 References 28

Chapter 3. Phylum-specific regulation of resistomycin production in a *Streptomyces* sp. via microbial co-culture 32

3.1 Introduction 32

3.2 Inducible secondary metabolite biosynthetic pathways in Actinobacteria 33

3.3 Targeted methods to induce the production of secondary metabolites 34

3.3.1 Heterologous expression of biosynthetic gene clusters to increase yields of secondary metabolites 34

3.3.2 Adding non-native precursors to culture media to generate “non-natural” natural products 35

3.4 General methods to induce secondary metabolite biosynthesis 36
3.4.1 OSMAC Approach to increase yields of secondary metabolites 36
3.4.2 Employing physical stress to increase yields of secondary metabolites 37
3.4.3 Addition of small molecules to culture media to increase yields of secondary metabolites 38
3.5 Co-culture as a method to alter secondary metabolism 40
3.6 Significance 42
3.7 Results and discussion 42
3.7.1 Co-cultivation of 95 actinomycete strains with Proteobacteria panel 43
3.7.2 Resistomycin induction in co-cultivation experiments is specific to interactions with Proteobacteria 47
3.8 Methods 51
3.8.1 General experimental procedures 51
3.8.2 Identification of strain B033 52
3.8.3 Co-cultivation of actinomycetes with four-strain Proteobacteria panel 52
3.8.4 In vitro antimicrobial growth inhibition assays 53
3.8.5 Chromatographic analysis of co-culture extracts 54
3.8.6 B033 Co-incubation with *Burkholderia vietnamiensis* culture supernatant and cell mass 54
3.8.7 Selection of expanded panel of environmental Proteobacteria 55
3.8.8 B033 Co-cultivated with an expanded panel of Proteobacteria isolates. 55
3.8.9 Monitoring for resistomycin production in B033-environmental isolate co-culture experiments 57

3.8.10 Growth inhibition assays against strains that induce resistomycin production 57

3.9 Conclusions 57

3.10 Future directions 58

3.10.1 Imaging mass spectrometry as a Rosetta stone for bacterial communication 58

3.10.2 One percent induction rate 58

3.11 References 61

Chapter 4. Conclusions 71

Appendix I "Stop. Collaborate and listen." 74

4.1 Introduction 74

4.2 ChIPS process identified chloramphenicol from a secondary metabolite fraction from strain G006 76

4.3 Inhibitor of bacterial translation from strain D085 78

4.4 Kosinostatin identified from Strain D077, a *Micromonospora* sp. 80

4.5 Thiazolyl peptides identified from strain D081 83

4.6 Strain B034 produces resistomycin and bafilomycin analogs 85

4.7 Strain D034 produces granatacin analogs 89

4.8 Summary 89

4.9 References 91

Appendix II Reference Spectra 93

LIST OF TABLES

Table 1 ^{1}H and ^{13}C NMR data (600 MHz, CD_3CN) of juvenimicin C (**1**) 19

Table 2 Chemopreventive activity of juvenimicin C (**1**) and 5-O-α-L-rhamnosyltylactone (**2**). . 21

Table A1 Natural products identified 90

Table A2 Similarity of proteobacterial co-culture panel to strains on Health and Human Services Select Agents List (HHS-SA) 106

Table A3 Ingredients of media types used in nutrient study 112

Table A4 Panel of Proteobacteria used in follow-up cultivation studies..... 113

LIST OF FIGURES

Figure 1 Source of small molecule approved drugs..... 2

Figure 2 Key 2D NMR Correlations of **1** 18

Figure 3 Crystal structure of **1**. 18

Figure 4 Reactions taking place during quantification of QR-1 activity. 25

Figure 5 HPLC chromatograms at 254 nm of the co-culture extract of B033-*B. vietnamiensis* and the extracts from monocultures of B033 and *B. vietnamiensis*..... 45

Figure 6 Phylogenetic diversity of environmental strains in follow-up co-culture panel..... 48

Figure 7 Chemical profiling of co-culture extracts..... 56

Figure 8 Preparation of cell mass and supernatant for co-incubation experiments. 56

Figure 9 The power of one percent induction of secondary metabolism..... 60

Figure A1 Collection sites in Hạ Long Bay, Cat Ba, Vietnam. 75

Figure A2 Identification of indolmycin (**5**) using ^{1}H-NMR in MeOD. 79

Figure A3 Biochromatogram of D077-F4 in the in-house antimicrobial bioassay. 82

Figure A4 HPLC chromatogram of fraction D081-F87 at 350 nm. 84

Figure A5 Biochromatogram of B034-F4 in the MABA and LORA bioassays..... 87

Figure A6 Putative identification of bafilomycin analogs. 88

Figure A7 Strain D034 grown on ISP2 agar..... 89

Figure A8 ^{1}H NMR spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 93

Figure A9 COSY spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 93

Figure A10 HMBC spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 94

Figure A11 NOESY spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 94

Figure A12 Selective 1D-TOCSY spectrum of H2 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 95

Figure A13 Selective 1D-TOCSY spectrum of H3 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 95

Figure A14 Selective 1D-TOCSY spectrum of H8 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 96

Figure A15 Selective 1D-TOCSY spectrum of H15 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 96

Figure A16 Selective 1D-TOCSY spectrum of H18 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 97

Figure A17 Selective 1D-TOCSY spectrum of H1′ (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 97

Figure A18 Selective 1D-TOCSY spectrum of H2′ (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 98

Figure A19 Selective 1D-TOCSY spectrum of H5′ (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3 98

Figure A20 TOCSY spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-*d3* 99

Figure A21 HR-ESI-TOF-MS of juvenimicin C (**1**)..... 99

Figure A22 Circular dichroism spectrum of juvenimicin C (**1**)..... 100

Figure A23 UV spectrum of juvenimicin C (**1**) 100

Figure A24 Crystal structure of juvenimicin C (**1**) 101

Figure A25 ^{1}H-NMR spectrum (500 MHz) of 20-deoxo-23-deoxy-5-O-(6-deoxy-β-D-glucopyranosyl)-tylonolide (**2**) in acetonitrile-d_3..... 101

Figure A26 HR-ESI-TOF-MS of 20-deoxo-23-deoxy-5-O-(6-deoxy-β-D-glucopyranosyl)-tylonolide (**2**)..... 102

Figure A27 Crystal structure of 20-deoxo-23-deoxy-5-O-(6-deoxy-β-D-glucopyranosyl)-tylonolide (**2**)..... 102

Figure A28 ^{1}H NMR spectrum (600 MHz) of chloramphenicol (**4**) in $CDCl_3$..... 103

Figure A29 ^{1}H NMR spectrum (600 MHz) of two indolmycin stereoisomers (**5**) in MeOD..... 103

Figure A30 ^{1}H NMR spectrum (600 MHz) of kosinostatin (**6**) in DMSO-d_6..... 104

Figure A31 ^{1}H NMR spectrum (600 MHz) of a fraction putatively containing a thiazolyl peptide in DMSO-d_6 104

Figure A32 HR-MS of G006-F242 compared to chloramphenicol (**4**) standard..... 105

Figure A33 High-resolution mass spectrometry (IT-TOF) analysis of resistomycin (**3**) 109

Figure A34 Identification of resistomycin in B033-*B. vietnamiensis* co-culture extracts 110

Figure A35 Resistomycin identified in extracts of all Proteobacteria-B033 cultivation experiments 111

Figure A36 Listing of bacterial strains mentioned in this dissertation 115

LIST OF ABBREVIATIONS

A1	A1 media type (Table A3)
A1F	A1 Fresh water media type (Table A3)
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CD	Doubling Concentrations*
CFU	Colony Forming Units
ChIPS	Characterization of Inhibitors of Protein Synthesis
COSY	Correlation Spectroscopy
DAD	Diode Array Detector
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DI	Deionized water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	EthylenediamineTetraacetic acid
ELSD	Evaporative Light Scattering Detector
EMEA	European Medicines Agency
ESI	Electrospray Ionization
EtoAc	Ethyl Acetate
FDA	Food and Drug Administration

* Circular dichroism, which is normally abbreviated CD is not abbreviated in this work to avoid confusion.

GSH	Glutathione
HCI	High Content Imaging
HMBC	Heteronuclear Multiple-Bond Correlation spectroscopy
HPLC	High Performance/Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HRESI-TOF	High resolution electrospray ionization – time of flight
HSQC	Heteronuclear Single Quantum-Coherence Spectroscopy
IR	Induction Ratio[†]
ISP2	International Streptomyces Project, Media 2 (Table A3)
ITR	Institute for Tuberculosis Research
LORA	Low Oxygen Recovery Assay
MA	Massachusetts
MABA	Microplate Alamar Blue Assay
MeOD	Deuterated Methanol
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MOSE	Mouse Surface Epithelium
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
obs	Observed

[†] Infrared, which is normally abbreviated IR is not abbreviated in this work to avoid confusion.

OSMAC	One strain many compounds
OVCAR	Ovarian Cancer
PCA	Principal Component Analysis
QR1	Quinone Reductase-1
Rm	resistomycin
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
SCUBA	Self Contained Underwater Breathing Apparatus
SPE	Solid Phase Extraction
TOCSY	Total Correlation Spectroscopy
TOF	Time of Flight
UIC	University of Illinois at Chicago
UMMPF	University of Illinois at Chicago Mass spectrometry and Proteomics Facility
UV	Ultraviolet
XAD-16N	Proprietary trade name of a polymeric adsorbent resin from Supelco

SUMMARY

Chapter 1 provides an introduction to the field of natural products, drug discovery from the aquatic environment, and the development of Actinobacteria as a resource of drug-leads. Chapter 2 details one such effort that resulted in the isolation and structure elucidation of a novel macrolide marine natural product with potential chemopreventative bioactivity. Appendix I details the known molecules identified in the pursuit of novel natural products. As an alternative to the traditional drug discovery approach and in order to access secondary metabolites that are not normally expressed under laboratory conditions, we attempted to induce the expression of silent biosynthetic gene clusters via microbial co-culture; this is discussed in Chapter 3. The results presented have implications toward improving the drug discovery process by suggesting that it is possible to mine existing strain libraries for new natural products in a phylum specific manner. Lastly, Chapter 4 contains concluding remarks and future directions of the field of natural products. A listing of bacterial strains mentioned in this dissertation is available in Figure A36.

Chapter 1. Introduction to thesis research

1.1 A historical perspective of natural products

Natural products have been used for centuries for the treatment of diseases, with humans relying on the biological activity of mixtures of whole plants and other naturally occurring substances, consumed as teas or applied directly to an affected area. However, it was not until modern times that the molecules responsible for the observed biological activity were isolated and identified using spectroscopic techniques. These studies have led to the pharmaceuticals artemisinin, morphine, paclitaxil, quinine, and the salicylates, among many others.[1-2]

Of all the United States Food and Drug Administration (FDA) approved drugs, natural products account for more than 60% of the small molecules on the market, and 50% of all approved drugs (including non-small molecule drugs such as biologicals and vaccines).[3-4] As demonstrated in Figure 1, even approved synthetic molecules have been strongly influenced by natural product scaffolds and pharmacophores.

1.2 Microbial-derived natural products

Within all natural product approved new chemical entities, 53% are microbial-derived natural products.; of these, bacterial-derived secondary metabolites account for one third.[3] A new chemical entity is defined as a drug that contains an active moiety that has not previously been approved by the FDA in any other application.

The first FDA approved bacterial new chemical entity was streptomycin,[3] a metabolite isolated from a *Streptomyces* sp., which belongs to the phylum Actinobacteria, commonly referred to as actinomycetes. This approval, in part, launched an era of soil derived microbial drug discovery,

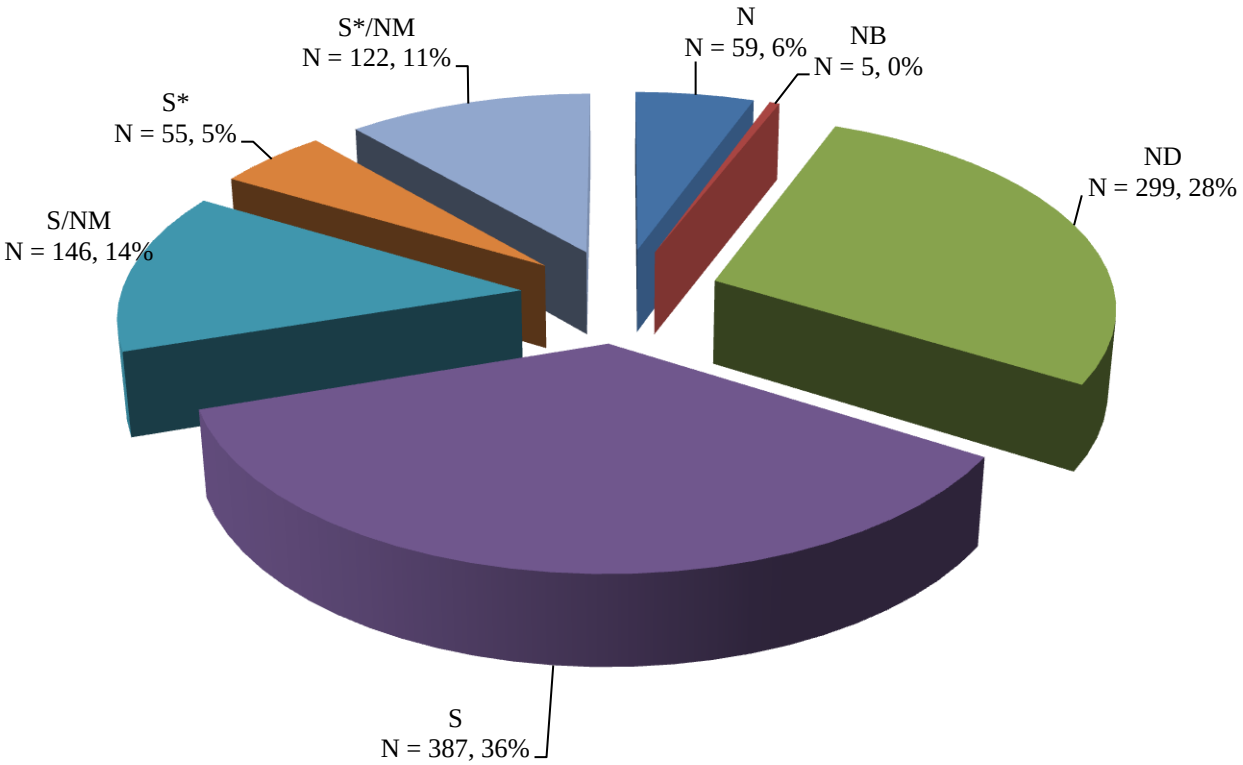


Figure 1 Source of small molecule approved drugs

In their cornerstone review Newman and Cragg[4] quantified the source of all of FDA approved small molecule drugs from 1981 until 2010. As shown in the above figure (adapted from and Newman and Cragg, 2012), natural products account for over 60 percent of approved drugs when synthetic derivatives that have a natural product pharmacophore or inspiration are considered. In this key, the abbreviation for each group is explained followed by the quantity of approved small molecules, then the percentage of the total number approved small molecule drugs is given. N = natural products, small molecules that have been approved without any synthetic modification, 59, 6%. NB = natural product botanical, represents those defined mixtures approved since the FDA published additional guidelines for the dietary supplement industry in 2004, 5, 0%. ND = Natural product derived, usually involves semi-synthetic modification of a natural product, 299, 28%. S = Synthetic, a synthesized molecule typically developed from hits identified in a high-throughput screening or from extensive modification of an existing synthetic bioactive molecule, 387, 36%. S/NM = Synthetic natural product mimic, represents those molecules that originated from a natural product structure and mimic natural products yet have been extensively modified, 146, 14%. S* = Synthetic but contains a natural product pharmacophore, 55, 5%. S*/NM = Synthetic, natural product pharmacophore, natural product mimic, 122, 11%.

which has yielded 120 approved therapeutics.[1] This era was heralded as the end of infectious disease, however this triumph was cut short as bacteria resistant to these antibiotics were isolated after widespread clinical use.[5] To combat this resistance researchers began to explore new environmental sources in order to isolate novel chemical scaffolds to be used against this resistance.

1.3 The aquatic environment as a source for drug-lead discovery

During the golden era of microbial drug discovery the marine environment was ignored, leaving approximately 70% of the earth's surface not investigated. This was due to the incorrect belief that the marine environment was not a viable source of cultivatable bacteria. This belief was reinforced by the discovery of known compounds from sediments collected close to shore. With the isolation of the first marine actinomycete, a *Rhodococcus* sp.,[6] and the subsequent discovery of unprecedented chemical scaffolds from marine sponges and invertebrates by Scheuer and Moore,[1] the marine drug discovery paradigm was launched. As such, there have been three marine natural products FDA or European Medicines Agency (EMEA) approved for a variety of pharmaceutical applications without major modification.[7-8] There are an additional five approved drugs that are derived from natural products isolated from the marine environment.[9]

On average, a milliliter of sea water contains 10^6 bacterial cells and marine sediment contains 10^9 bacterial cells which makes bacteria the most abundant life form in aquatic environments.[10] Additionally, The ocean contains many diverse aquatic microenvironments, that is, niche habitats with unique evolutionary pressures that select for unique adaptations in those species, which are able to inhabit these environments. Bacteria isolated from these aquatic microenvironments boast adaptations such as: halophily, psychrophily, thermophily, chemoautotrophy, and barophily. Additionally the biodiversity represented in the aquatic environment provides symbiotic,

epibiotic, and pathogenic opportunities for microorganisms.[11] Each of these environments selects for unique populations of bacteria and potential secondary metabolite novelty.[10-12] One of the most prolific sources of microbial-derived natural products from the aquatic environment are bacteria within the phylum Actinobacteria, with 778 novel molecules reported in the MarinLit database (covering marine natural products in the 50 years from 1963 to 2013).[13]

1.4 Aquatic actinomycete-derived natural products

Actinomycetes are Gram-positive bacteria that display immense genetic potential to produce natural products known as secondary metabolites (in some cases, up to 9.9% of their genome[14-15]). When purified through biological-assay (bioassay) guided fractionation, these small-molecular weight entities can display potent anticancer, antiviral, antimicrobial, or other biological activities.

Aquatic derived actinomycetes were originally thought to be terrestrial spores that washed into the aquatic environment; however, genomic sequencing technologies have identified several marine-[16] and freshwater-specific[17-21] clades. This indicates that while they share a common ancestor, their individual genetic traits are distinct enough to cause a divergence in their genetic lineages. This evidence establishes some aquatic-derived actinomycetes distinct from their terrestrial counterparts, demonstrating the divergence from terrestrial-derived microbes.[22] Further evidence has suggested there are many novel genera to be discovered.[15] In the case of the marine obligate genus *Salinispora,* several structurally unique compounds have been linked to a specific species.[23] These data suggest that the chemical diversity within the marine environment exists even at the species level. Therefore, following this paradigm the aquatic environment is a viable and productive place to look for bioactive secondary metabolites.

1.5 Antibiotic resistance and the development of novel bioassays

According to a 2014 World Health Organizations report, mankind is headed toward a post-antibiotic era where common infections could once again kill.[24] While bacterial resistance to small molecule inhibitors is an ancient[25] evolutionary advantage, the misuse of our existing arsenal[26] is accelerating the issue of bacterial resistance. Misusing those antibiotics that retain efficacy against human infections by failing to complete an antibiotic regimen or by failing to receive an effective dose of antibiotics to clear an infection, has led to the emergence of pathogens resistant to second and even third line antibiotics. Routinely relying on second or third line antibiotics is an ineffective clinical strategy that relies on the discovery of new antibiotics during a time when there is a disparate investment in antibiotic drug discovery. Increasingly the expensive and high risk initial phase of antimicrobial drug discovery is being left to academia and smaller biotechnology companies,[27] as the pharmaceutical industry is retreating from antimicrobial drug discovery at an unprecedented rate.[28]

In order to further the discovery of new chemical entities with limited financial resources, new sources of natural products and new bioassays must be developed. Since the year 2000, 77% of small molecules approved for use as antibiotics are natural products, all of which are microbial-derived secondary metabolites,[3] validating the focus on screening actinomycete secondary metabolite fraction libraries for antimicrobial drug-lead development.

However, a historically successful source alone will not allow for the identification of new drug leads. New assays must be developed to screen compounds for novel mechanisms of action alongside the continued pursuit of structural novelty from diverse ecosystems. To this end, two successful antimicrobial screening platforms - both relying on basic science techniques - are highlighted here.

Peach et al.[29] describe the development of a screening platform to identify the mechanism of action of antimicrobial compounds based on high content image (HCI) screening, by rapid geometric averaging of bacterial cells after exposure to antimicrobial compounds.[29] Based on the theory that antimicrobial compounds affect the downstream physiology of the microorganism, a blinded set of known antimicrobial compounds were correctly characterized as proof of concept. This method was then used to screen fractions of a marine microbial library. This technique successfully identified the secondary metabolite novobiocin, a DNA gyrase inhibitor used under the trade name Albamycin. While this platform has the potential to find compounds with novel mechanisms of action, the method requires the use of fluorescence reagents and robotic sample automation. While large research intensive institutions, such as UIC or Northwestern University, boast high-throughput screening facilities, smaller academic drug discovery units do not have the same access to these platforms.

Without the use of fluorescent reagents, the laboratory of Dr. Alexander Mankin has described the development of the ChIPS[30] assay to identify inhibitors of protein translation. While this assay is limited to identifying inhibitors of protein translation, it allows for the classification of inhibitors as having a novel or a known mechanism of inhibition. Though a collaboration with Dr. Mankin's laboratory, we were able to screen our fraction library for actinomycete secondary metabolite fractions that inhibit bacterial translation. We were able to identify antibiotics and their mechanism of protein translation inhibition from a crude fraction.

1.6 The realization of the genetic potential of actinomycetes

In addition to the development of new bioassays, the untapped genetic potential of actinomycetes has provided an additional source of novel natural products. Actinomycetes are known to dedicate up to 9.9% of their genome to secondary metabolite production in the form of

biosynthetic gene clusters.[14] However, standard laboratory conditions rarely provide sufficient motivation to express the metabolic product of these encoded biosynthetic gene clusters. Mathematical modeling predicts that there might be as many as 10^4 novel antibiotics yet to be discovered from all *Streptomyces* spp.[31] The induction of the genetic potential of actinomycetes can be accomplished in many ways. In our laboratory, we developed a drug-lead discovery method around the hypothesis that actinomycetes isolated from aquatic environments contain endemic chemical defenses already encoded in their genome. In combination with a member of the most abundant phylum of bacteria, Proteobacteria, we were able to induce the expression of antibiotic production in a *Streptomyces* sp. isolated from Lake Michigan. Furthermore, this method has the potential to mine existing microbial libraries for previously unseen antibiotic potential as discussed further in Chapter 3.

1.7 Thesis summary

In pursuit of novel secondary metabolite drug leads, the laboratory of Dr. Brian T. Murphy at UIC has isolated a library of marine and fresh-water actinomycetes and fungi of approximately 1,500 strains. These strains were isolated from aquatic environmental samples from shallow water by wading or skin diving, and from deeper water by SCUBA or PONAR.

These acronyms are colloquial in aquatic sampling circles but are not abundantly clear. SCUBA stands for self contained underwater breathing apparatus but has entered the English language as an acronym with the increased popularity of SCUBA by vacationers and amateur aquatic explorers. PONAR is a type of sediment grab that was developed in the early 1960's when existing marine and freshwater sampling techniques did not prove satisfactory for the collection of sediments from Lake Michigan. The grab is named after its inventors Charles E. Powers,

Robert A. Ogle, Jr., Vincent E. Noble, John C. Ayers, and Andrew Robertson[32-33] but has since become a standalone name for these sampling devices.

The microbes were isolated from sediment collected from these aquatic environments have been cultivated on a one liter scale and their secondary metabolites extracted. Each extract is then fractionated by polarity via solid phase extraction (SPE) chromatography using a silica gel stationary phase. These fractions are then added to a secondary metabolite fraction library, stored at 10 mg/mL in DMSO. The fraction library now contains over 2,500 fractions from 625 microbes. This fraction library is stored in a 96 well plate format to allow for the generation of daughter plates, which can be screened in high throughput assays for biological activity.

The Murphy laboratory routinely screens the new additions to the fraction library for antimicrobial and anticancer bioactivity, in addition to normal cell cytotoxicity. The antimicrobial biological data we have amassed for our fraction library comes from two sources. The in-house antimicrobial bioassay is used to screen our fraction library for inhibitors of *Escherichia coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*. In collaboration with the Institute for Tuberculosis Research (ITR), our fraction library is also screened for inhibitors of *Mycobacterium tuberculosis* in two *in vitro* inhibition assays, using the microplate alamar blue assay (MABA),[34] and a low-oxygen recovery assay (LORA).[35] Dr. Joanna Burdette's laboratory researches the mechanisms that underlie ovarian cancer progression and maintains several drug-resistant ovarian cancer cell lines and tests our fraction library for anticancer bioactivity. Both the ITR (Vero cell line, normalized monkey kidney) and Dr. Burdette's laboratory (mouse ovarian surface epithelium, MOSE) have normalized cell lines to determine cytotoxicity against normal cells. These bioassays allow for a multifaceted view of the biological activity of the secondary metabolite fractions derived from our aquatic derived microbial library.

When dose response bioassay data is returned from a collaborator, the hits are ranked by potency and only the most potent hits are subjected to the process of dereplication. "Most potent" depends greatly on the assay but is typically defined by the potency of current FDA approved inhibitors relative to the test concentration and fraction purity. The dereplication process starts with chemical profiling via liquid chromatography-mass spectrometry (LCMS). The molecular weight and ultra-violet (UV) profile are compared to in-house and electronic databases. If there is no match and the compounds are thought to be putatively new, several rounds of chromatography are undertaken in order to isolate a single biologically active secondary metabolite. This process is a derivative of the traditional natural product discovery paradigm. Chapter 2 details one such effort that resulted in the isolation and structure elucidation of a novel macrolide marine natural product with potential chemopreventive bioactivity. However, dereplication is not always a straight forward process. Appendix I details our important collaborations here at UIC, the bioassay data generated by our collaborators, and the putative identification of several structurally diverse natural products.

1.8 References

1. Fenical, W. Marine Pharmaceuticals: Past, Present, and Future. *Oceanography* **2006**, *19*, 110-119.
2. Dias, D. A.; Urban, S.; Roessner, U. A historical overview of natural products in drug discovery. *Metabolites* **2012**, *2*, 303-336.
3. Patridge, E.; Gareiss, P.; Kinch, M.; Hoyer, D. An analysis of FDA-approved drugs: natural products and their derivatives. *Drug Disc. Today* **2015**, 1283-1287.
4. Newman, D. J.; Cragg, G. M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **2012**, *75*, 311-335.

5. Lewis, K. Antibiotics: Recover the lost art of drug discovery. *Nature* **2012**, *485*, 439-440.

6. Helmke, E.; Weyland, H. *Rhodococcus marinonascens* sp. nov., an actinomycete from the sea. *Int. J. Syst. Bacteriol.* **1984**, *34*, 127-138.

7. 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 in Pharmacological Sciences* **2010**, *31*, 255-265.

8. Jeon, Y. J.; Samarakoon, K. W.; Elvitigala, A. S., 41. Marine-derived pharmaceuticals and future prospects. Kim, S. K., Ed. 2015.

9. Martins, A.; Vieira, H.; Gaspar, H.; Santos, a. S. Marketed marine natural products in the pharmaceutical and cosmeceutical Industries: tips for success. *Mar. Drugs* **2014**, *12*, 1066-1101.

10. Rusch, D. B.; Halpern, A. L.; Sutton, G.; Heidelberg, K. B.; Williamson, S.; Yooseph, S.; Wu, D.; Eisen, J. A.; Hoffman, J. M.; Remington, K.; Beeson, K.; Tran, B.; Smith, H.; Baden-Tillson, H.; Stewart, C.; Thorpe, J.; Freeman, J.; Andrews-Pfannkoch, C.; Venter, J. E.; Li, K.; Kravitz, S.; Heidelberg, J. F.; Utterback, T.; Rogers, Y.-H.; Falcon, L. I.; Souza, V.; Bonilla-Rosso, G.; Eguiarte, L. E.; Karl, D. M.; Sathyendranath, S.; Platt, T.; Bermingham, E.; Gallardo, V.; Tamayo-Castillo, G.; Ferrari, M. R.; Strausberg, R. L.; Nealson, K.; Friedman, R.; Frazier, M.; Venter, J. C. The Sorcerer II global ocean sampling expedition: northwest Atlantic through eastern tropical Pacific. *PLoS Biol.* **2007**, *5*, e77.

11. Murphy, B. T. M., K.; Fenical, W., Marine Microrganisms. Eolss Publishers: Oxford, UK, 2011.

12. Biers, E. J.; Sun, S.; Howard, E. C. Prokaryotic genomes and diversity in surface ocean waters: interrogating the global ocean sampling metagenome. *Appl. Environ. Microbiol.* **2009**, *75*, 2221-2229.

13. Blunt, J. W.; Copp, B. R.; Keyzers, R. A.; Munro, M. H. G.; Prinsep, M. R. Marine natural products. *Nat. Prod. Rep.* **2015**, *32*, 116-211.

14. Udwary, D. W.; Gontang, E. A.; Jones, A. C.; Jones, C. S.; Schultz, A. W.; Winter, J. M.; Yang, J. Y.; Beauchemin, N.; Capson, T. L.; Clark, B. R.; Esquenazi, E.; Eustaquio, A. S.; Freel, K.; Gerwick, L.; Gerwick, W. H.; Gonzalez, D.; Liu, W. T.; Malloy, K. L.; Maloney, K. N.; Nett, M.; Nunnery, J. K.; Penn, K.; Prieto-Davo, A.; Simmons, T. L.; Weitz, S.; Wilson, M. C.; Tisa, L. S.; Dorrestein, P. C.; Moore, B. S. Significant natural product biosynthetic potential of actinorhizal symbionts of the genus *Frankia*, as revealed by comparative genomic and proteomic analyses. *Appl. Environ. Microbiol.* **2011**, *77*, 3617-3625.

15. Udwary, D. W.; Zeigler, L.; Asolkar, R. N.; Singan, V.; Lapidus, A.; Fenical, W.; Jensen, P. R.; Moore, B. S. Genome sequencing reveals complex secondary metabolome in the marine actinomycete Salinispora tropica. *Proc. Natl. Acad. Sci.* **2007**, *104*, 10376-10381.

16. Fenical, W.; Jensen, P. R. Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat. Chem. Biol.* **2006**, *2*, 666-673.

17. Hahn, M. W. Description of seven candidate species affiliated with the phylum Actinobacteria, representing planktonic freshwater bacteria. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 112-117.

18. Newton, R. J.; Jones, S. E.; Eiler, A.; McMahon, K. D.; Bertilsson, S. A guide to the natural history of freshwater lake bacteria. *Microbiol. Mol. Biol. Rev.* **2011**, *75*, 14-49.

19. Newton, R. J.; Jones, S. E.; Helmus, M. R.; McMahon, K. D. Phylogenetic ecology of the freshwater Actinobacteria acI lineage. *Appl. Environ. Microbiol.* **2007**, *73*, 7169-7176.

20. Warnecke, F.; Amann, R.; Pernthaler, J. Actinobacterial 16S rRNA genes from freshwater habitats cluster in four distinct lineages. *Environ. Microbiol.* **2004**, *6*, 242-253.

21. Allgaier, M.; Grossart, H. P. Diversity and seasonal dynamics of Actinobacteria populations in four lakes in northeastern Germany. *Appl. Environ. Microbiol.* **2006**, *72*, 3489-3497.

22. Jensen, P. R.; Mincer, T. J.; Williams, P. G.; Fenical, W. Marine actinomycete diversity and natural product discovery. *Anton. Leeuw. Int.* **2005**, *87*, 43-48.

23. Jensen, P. R.; Williams, P. G.; Oh, D. C.; Zeigler, L.; Fenical, W. Species-specific secondary metabolite production in marine actinomycetes of the genus *Salinispora*. *Appl. Environ. Microbiol.* **2007**, *73*, 1146-1152.

24. Organization, W. H. *Antimicrobial resistance global report on surveillance*; Geneva, Switzerland, 2014; p 256.

25. D'Costa, V. M.; King, C. E.; Kalan, L.; Morar, M.; Sung, W. W. L.; Schwarz, C.; Froese, D.; Zazula, G.; Calmels, F.; Debruyne, R.; Golding4, G. B.; Poinar, H. N.; Wright, G. D. Antibiotic resistance is ancient. *Nature* **2011**, *477*, 457-461.

26. Wright, G. D. Something old, something new: revisiting natural products in antibiotic drug discovery. *Can. J. Microbiol.* **2014**, *60*, 147-154.

27. Khanna, I. Drug discovery in pharmaceutical industry: productivity challenges and trends. *Drug Disc. Today* **2012**, *17*, 1088-1102.

28. Kinch, M. S.; Patridge, E.; Plummer, M.; Hoyer, D. An analysis of FDA-approved drugs for infectious disease: antibacterial agents. *Drug Disc. Today* **2014**, *19*, 1283-1287.

29. Peach, K. C.; Bray, W. M.; Winslow, D.; Linington, P. F.; Linington, R. G. Mechanism of action-based classification of antibiotics using high-content bacterial image analysis. *Mol. Biosyst.* **2013**, *9*, 1837-1848.

30. Orelle, C.; Carlson, S.; Kaushal, B.; Almutairi, M. M.; Liu, H.; Ochabowicz, A.; Quan, S.; Pham, V. C.; Squires, C. L.; Murphy, B. T.; Mankin, A. S. Tools for characterizing bacterial protein synthesis inhibitors. *Antimicrob. Agents Chemother.* **2013**, *57*, 5994-6004.

31. Watve, M.; Tickoo, R.; Jog, M.; Bhole, B. How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.* **2001**, *176*, 386-390.

32. Instructors manual: bottom sampling. http://www.gvsu.edu/wri/education/instructors-manual-bottom-sampling-31.htm (accessed March 12, 2015).

33. TePas, K. Lake Guardian Shipboard and Shoreline Science Workshop: Report Out From Day 2! http://blog.epa.gov/blog/2011/07/lake-guardian-shipboard-and-shoreline-science-workshop-%C2%A0report-out-from-day-2/ (accessed March 12, 2015).

34. Collins, L.; Franzblau, S. G. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob. Agents Chemother.* **1997**, *41*, 1004-1009.

35. Cho, S. H.; Warit, S.; Wan, B.; Hwang, C. H.; Pauli, G. F.; Franzblau, S. G. Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **2007**, *51*, 1280-1385.

Chapter 2. Potential chemopreventive activity of a new macrolide antibiotic from a marine-derived *Micromonospora* sp.‡

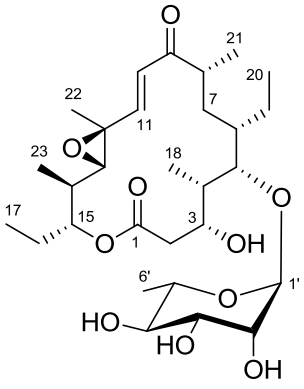
2.1 Introduction

Cancer initiation is the result of subcellular damage, two small molecule initiators are electrophilic metabolites of exogenous carcinogens and endogenous reactive oxygen species (ROS).[1] Quinone reductase 1 (QR1) is a phase II enzyme responsible for two-electron reduction and detoxification of such metabolites. Induction of this enzyme has been shown to be a biomarker for chemoprevention.[2] Accordingly, secondary metabolites capable of inducing this enzyme can be used to slow the process of carcinogenesis.

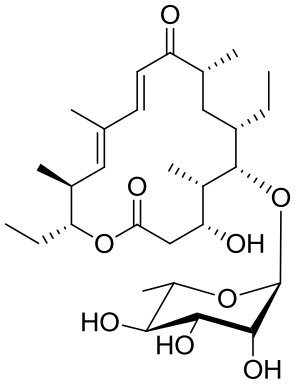
Several studies report QR1-inducing compounds of both semi-synthetic and natural product origins. This suite of compounds exhibits QR1 doubling concentrations (CD) in the mM to nM range. Some common QR1-inducing structural classes include stilbenes and tetrahydro-β-carbolines. In regard to the latter, semi-synthetic efforts significantly improved QR1 CD values, reaching values as low as 0.2 μM through addition of alkylated N-urea derivatives to a core piperidine ring system;[3] this was an improvement in bioactivity observed from naturally occurring derivatives (e.g., perlolyrin, CD = 1.7 μM).[4] Similarly, the QR1 inducing activity of the stilbene resveratrol (CD = 21 μM) was enhanced to submicromolar levels through generation of thiazole linked analogues (CD values ranging from 0.087 to 0.98 μM).[5] This bioactivity was comparable to that of a potent inducer 4'-bromoflavone (CD = 0.1 μM), a simple, available, and inexpensive chemopreventive agent that induces phase II metabolism.[6]

A few other classes of secondary metabolites have been shown to induce QR1 activity such as triterpenes,[7] flavonoids,[8-11] labdane diterpenes,[12] and other small molecular weight phenolic compounds,[4, 13] though the majority of these structures exhibit moderate to weak CD values when compared to their stilbene, flavonoid, and β-carboline counterparts.

As part of a program to identify molecules that induce the expression of QR1, screening of an actinomycete secondary metabolite fraction library led to the selection of strain CNJ-878 for further investigation on the basis of bioactivity in QR1 enzyme assays. In the current study, we present the first report of a macrolide antibiotic, juvenimicin C (**1**), that induces QR1 and other phase II detoxifying enzymes with moderate potency, while **2** failed to exhibit the same bioactivity. The isolation of a macrolide glycoside containing an epoxide at position C-11 and C-12 (**1**) and a second molecule containing the reduced double bond at the same position (**2**), provided an opportunity to study the structure activity relationship of this epoxide functional group. Details of the structure elucidation and biological activities are described herein.



1



2

2.2 Results and discussion

2.2.1 Structure elucidation

After several rounds of chromatography, juvenimicin C (**1**) was obtained as white powder. The molecular formula of **1** was assigned as $C_{29}H_{48}O_{10}$ on the basis of combined NMR and MS experiments. This formula demanded six degrees of unsaturation. Analysis of HMBC and HSQC NMR experiments suggested the presence of an α,β-unsaturated ketone carbonyl (δ_C 202.4, C-9), two olefinic carbons (δ_C 124.8, C-10; 150.5, C-11), two epoxide carbons (δ_C 60.7, C-12; 68.8, C-13), and an ester functional group (δ_C 174.8, C-1). There was evidence of eight sp^3 oxygenated carbons (δ_C 67.4, C-3; 84.7, C-5; 77.7, C-15; 72.0, C-2′; 72.3, C-3′; 73.3, C-4′; 69.8, C-5′), one of which was identified as an anomeric carbon (δ_C 104.0, C-1′) (Table 1). Given that the molecular formula afforded six degrees of unsaturation and the molecule contained one sugar, an α,β-unsaturated ketone, an epoxide, and an ester group, the remaining degree was satisfied by the macrolide ring system. Key HMBC, COSY, and TOCSY correlations are given in Figure 2. Interpretation of COSY data defined six spin systems which were then connected to each other using HMBC correlations.

An HMBC correlation from H_2-2 to C-4 positioned fragment C-2−C-3 next to the spin system of C-18, C-4, C-5, C-6, C-7, C-8, and C-21. A COSY correlation was observed from H_3-20 to H_2-19, though no correlation was observed from H_2-19 to H-6; thus an HMBC correlation from H_3-20 to C-6 helped solidify the position of the ethyl substituent. An HMBC correlation from H_3-21 to C-9 suggested the α,β-unsaturated ketone was adjacent to the aforementioned spin system. A J-3 correlation from H-11 to C-9 further supported the position of the α,β-unsaturated carbonyl moiety, the chromophore of which was observed in the UV spectrum of **1**. An HMBC correlation from H-11 to C-13 linked the epoxide to the α,β-unsaturated system, while COSY connectivities

placed a five-carbon unit (H_3-23, H-14, H-15, H_2-16, and H_3-17) adjacent to C-13. Finally, an HMBC correlation connected the oxygenated methine H-15 to the ester carbonyl at C-1 to complete the flat macrolide skeleton.

Additional signals were present in the ^{1}H NMR spectrum that could not be attributed to the macrolide aglycone. Subtracting those atoms accounted for by the macrolide, the remaining fragment had a formula of $C_6H_{11}O_5$. Resonances typical of an anomeric carbon (δ_C 104.0 and δ_H 4.62) were observed in both ^{1}H NMR and HMBC experiments suggesting the macrolide was glycosylated. Five of the eight observed oxygenated carbons were connected via COSY and TOCSY experiments to afford the fragment C-1′−C-6′. The macrolide was glycosylated at C-5, as evidenced by an HMBC correlation from the anomeric proton H-1′ to C-5.

To facilitate stereochemical determination, **1** was crystallized from methanol using a slow evaporation technique, and its relative configuration was determined by X-ray crystallographic analysis (Figure 3). We propose **1** to be the C3(R), C4(S), C5(S), C6(S), C8(R), C12(S), C13(S), C14(S), C15(R), C1′ (R), C2′(R), C3′(R), C4′(R), C5′(S) enantiomer, given its stereochemical similarity to **2**. Compound **2** was isolated during the purification process of **1** and identified on the basis of HRMS, NMR, and crystallographic analysis to be 5-O-α-L-rhamnosyltylactone (**2**)[14] (Figure A25, Figure A26, and Figure A27). X-ray analysis afforded the determination of absolute stereochemistry of **2**.

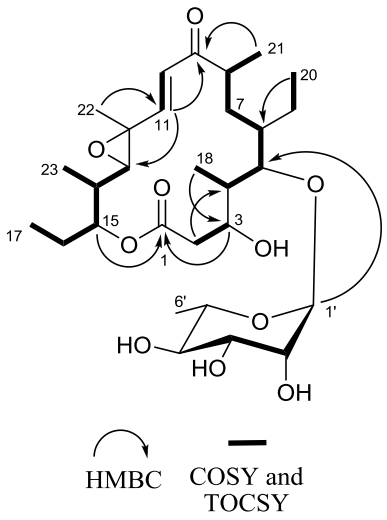


Figure 2 Key 2D NMR Correlations of **1**
The spin systems identified via homonuclear experiments, COSY and TOCSY, are indicated by bold lines. The heteronuclear correlations that connected these spin systems are shown by arrows.

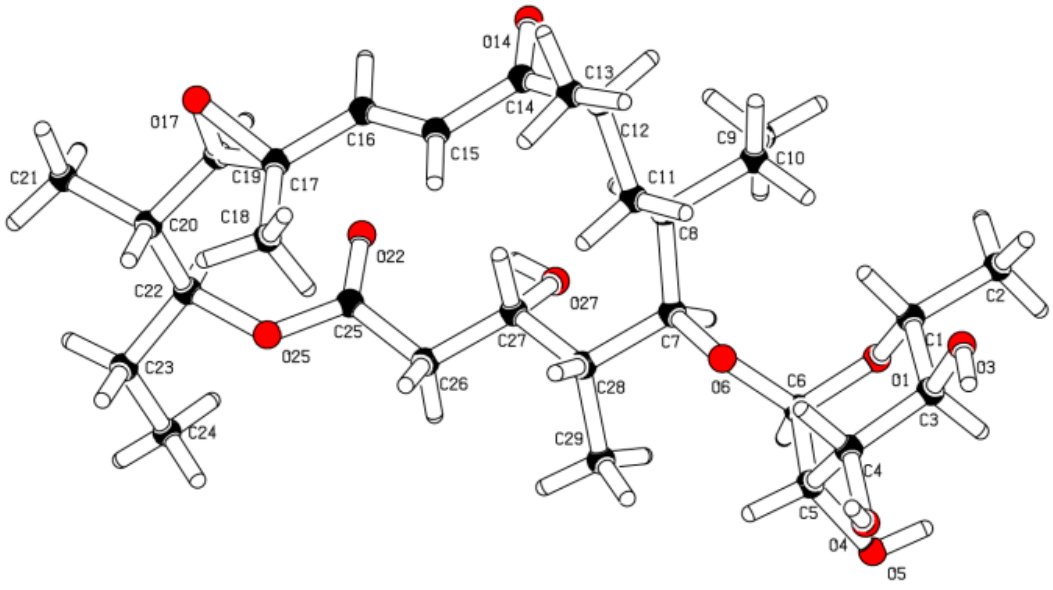


Figure 3 Crystal structure of **1**.
This image and the corresponding crystal lattice data allowed for the determination of the relative configuration of **1**.

Table 1 ^{1}H and ^{13}C NMR data (600 MHz, CD_3CN) of juvenimicin C (**1**)

	1	
Pos	^{13}C	δ ^{1}H mult. (J, Hz)
1	174.8	
2	40.4	2.59 dd (9.0, 10.2)
		2.19 d (9.0)
3	67.4	3.69 d (10.2)
4	41.6	1.75 m[a]
5	84.7	3.58 d (9.6)
6	39.6	1.14 m
7	33.3	1.76 m
		1.38 m
8	46.0	2.62 m
9	202.4	
10	124.8	6.65 d (15.9)
11	150.5	6.34 d (15.9)
12	60.7	
13	68.8	2.80 d (9.6)
14	38.4	1.75 m[a]
15	77.7	4.83 dt (10.0, 2.4)
16	25.1	1.80 m
		1.49 m
17	9.3	0.88 d (7.2)
18	10.1	0.95 d (6.6)
19	22.5	1.50 m
		1.36 m
20	12.5	0.87 d (7.2)
21	17.5	1.16 d (6.6)
22	15.3	1.43 s
23	14.5	1.08 d (6.6)
1'	104.0	4.62 br s
2'	72.0	3.89 br s
3'	72.3	3.47 d (7.2)
4'	73.3	3.29 m
5'	69.8	3.63 m
6'	17.5	1.18 d (6.0)
3-OH		3.22 br s

[a] signals are overlapping

*2.2.2 Chemopreventive activity of **1** and **2***

Assessment of QR1 induction is used as a generic biomarker since it is known to positively correlate with the induction of other phase II enzymes responsible for metabolic detoxification. Juvenimicin C (**1**) was found to induce QR1 activity with an induction ratio (IR) of 4.3. A more quantitative parameter, amenable for comparison of active substances, is the concentration required to double activity (CD). For compound **1**, the CD was found to be 10.1 μM. Comparison with 5-O-α-L-rhamnosyltylactone (**2**), with an IR of only 1.4, indicates the epoxide group is vital for activity. The most likely explanation for this elevated response mediated by **1** is nucleophilic addition, as is the case with α,β-unsaturated ketones. To further investigate the response of compound **1**, the effect on the levels of other detoxifying enzymes and glutathione were determined. Relative to the control, **1** increased the activity of glutathione (GSH) reductase (12.2 μmol NADPH oxidized/mg protein/min) and glutathione peroxidase (138 μmol NADPH oxidized/mg protein/min), and increased glutathione levels (CD = 27.7 μM). While these responses are lower than the positive control, 4'-bromoflavone,[6] **1** displays activity similar to that of 2,4-dibromo-phenazine (QR1 CD = 5.9 μM, GSH CD = 20.1 μM, 12.9 μmol NADPH oxidized/mg protein/min in a glutathione reductase assay),[15] a compound which served as a scaffold for the development of inducers active in the nanomolar concentration range. Taken together, these activities demonstrate the chemopreventive potential of **1** and establish it as a target for further semi-synthetic optimization.

Table 2 Chemopreventive activity of juvenimicin C (**1**) and 5-O-α-L-rhamnosyltylactone (**2**).

Sample	QR1 IR	QR1 (CD)μM	Glutathione (CD) μM	Glutathione reductase*	Glutathione peroxidase*
Control	1.0	na	na	9.6	63.7
1	4.3	10.1	27.7	12.2	138
2	1.4	nd	nd	nd	nd
4'-BF	8.4	0.1	5.67	18.1	154

na, not applicable; nd, not determined; (**2**) did not exhibit significant QR1 induction, thus further experiments were nd. *Reported as μmol NADPH oxidized/mg protein/min. 4'-BF (4'-bromoflavone) was used as a positive control.

2.3 Methods

2.3.1 General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were measured on a Shimadzu Pharma Spec UV-1700 spectrophotometer. Circular dichroism spectra were acquired using a Jasco J-710 spectropolarimeter. NMR spectra were obtained on a Bruker 600 MHz DRX-600 equipped with a 1.7 mm cryoprobe and Avance III console. Chemical shifts (δ) are given in ppm and coupling constants (*J*) are reported in Hz. ^{1}H and ^{13}C NMR resonances of juvenimicin C (**1**) are reported in Table 1. High resolution mass spectra were obtained on an Agilent ESI-TOF spectrometer at the Scripps Center for Mass Spectrometry. Liquid chromatography mass spectrometry (LCMS) data were obtained using a Hewlett-Packard series 1100 system equipped with a reversed-phase C_{18} column (Phenomenex Luna, 100 x 4.6 mm, 5 µm) at a flow rate of 0.7 mL min^{-1}. High-performance liquid chromatography (HPLC) separations were performed using a Waters 600E system controller and pumps with a Model 480 spectrophotometer. Separation was achieved using a Phenomenex Luna semi-preparative C_{18} column (250 x 10 mm, 5 µm) with a flow rate of 2 mL min^{-1}.

2.3.2 Bacterial isolation and identification

Strain CNJ-878, a gift from the Scripps Institute of Oceanography and Professor William Fenical, was isolated from sediment collected off the coast of Palau using SCUBA at a depth of 25 m. Strain CNJ-878 (GenBank accession number DQ448714) shared 98.9% 16S rRNA gene sequence identity with the most closely related type strain *Micromonospora yangpuensis* (GenBank accession number GU002071)[16-17] suggesting it may represent a new species. It shared high levels of sequence identity with other marine derived strains including two

Micromonospora spp. (CNQ-335_SD01, EU214915; CNS-633_SD06, EU214967), and 99.9% identity to three strains belonging to the proposed genus "*Solwaraspora*" (UMM543, AY552769; UMM566, AY552764; UMM 483, AY552761).[18]

2.3.3 Fermentation and extraction

Strain CNJ-878 was cultured in 39 x 1 L portions in Fernbach flasks containing high nutrient medium (filtered ocean water, 10 g starch, 4 g yeast extract, 2 g peptone, 1 g calcium carbonate, 100 mg potassium bromide, and 40 mg iron sulfate) for 7 d at 25°C while shaking at 230 rpm.

Sterilized Amberlite XAD-16N resin (20 g L^{-1}) was added to each flask to absorb the extracellular metabolites. The culture medium and resin were shaken for 6 h, filtered using cheesecloth to remove the resin, and washed with deionized water to remove salts. The resin, cell mass, and cheesecloth were extracted with acetone overnight, concentrated under vacuum, and partitioned between water and ethyl acetate. The organic layer was dried under vacuum to afford 2.5 g of extract.

*2.3.4 Isolation and characterization of juvenimicin C (**1**) and isolation of 5-O-α-L-rhamnosyltylactone (**2**)*

The crude extract was fractionated using silica gel flash column chromatography eluting with a methanol-dichloromethane (DCM) step gradient to afford seven fractions. Fraction 4 (DCM-methanol 95:5), contained the bioactive constituents, thus it was separated using C_{18} flash column chromatography eluting with 50, 80, and 100% aqueous acetonitrile. The 50% acetonitrile fraction was further separated using RP-C_{18} HPLC (2 mL min^{-1} isocratic flow, 70% aqueous methanol) to afford 10 fractions. Fraction 2 (t_R 11.3 min, 163 mg) was separated using RP-C_{18} semi-preparative HPLC using an isocratic flow of 70% aqueous methanol to afford semi-

pure **1** (t_R 11.3 min, 6.6 mg) and **2** (t_R 12.50 min, 16.0 mg). Each fraction was subsequently purified using C_{18} reversed-phase HPLC eluting with 68% aqueous methanol to yield juvenimicin C (**1**, 4.1 mg, 0.16% yield) and 5-O-α-L-rhamnosyltylactone (**2**, 13.4 mg, 0.54% yield).

Juvenimicin C (**1**): White amorphous powder (4.1 mg). $[\alpha]^{25}_{D}$ -16 (c 0.03, MeOH), UV (MeOH) λ_{max} (log ε) 239 (517). ^{1}H NMR (600 MHz, CD_3CN) and ^{13}C NMR (600 MHz, CD_3CN), see Table 1. HRESI-TOF MS *m/z* 579.3160 $[M+Na]^+$ (calcd. for $C_{29}H_{48}O_{10}Na$: 579.3139). Supplementary crystallographic data for **1** were deposited under accession number CCDC 915943 and can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

5-O-α-L-Rhamnosyltylactone (**2**): White amorphous powder (13.4 mg). UV (MeOH) λ_{max} 240 nm. ^{1}H NMR (600 MHz, CD_3CN), see Figure A25; HRESI-TOF MS *m/z* 563.3203 $[M+Na]^+$ (calcd. for $C_{29}H_{48}O_9Na$: 563.3191).

2.3.5 Quinone reductase 1 (QR1) assay

This assay was modified from a previously described protocol.[19] Cultured Hepa 1c1c7 mouse hepatoma cells were plated at a density of 2 x 10^4 cells mL^{-1} in 96-well plates and incubated for 24 h. The medium was then changed, and test compounds, dissolved in 10% dimethyl sulfoxide (DMSO), were introduced and serially diluted to a concentration range of 0.15-20 μg mL^{-1}. The cells were incubated for an additional 48 h. Quinone reductase activity was measured by the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan as demonstrated in Figure 4. A preliminary measure of QR activity is IR, that is the specific enzyme activity of treated cells

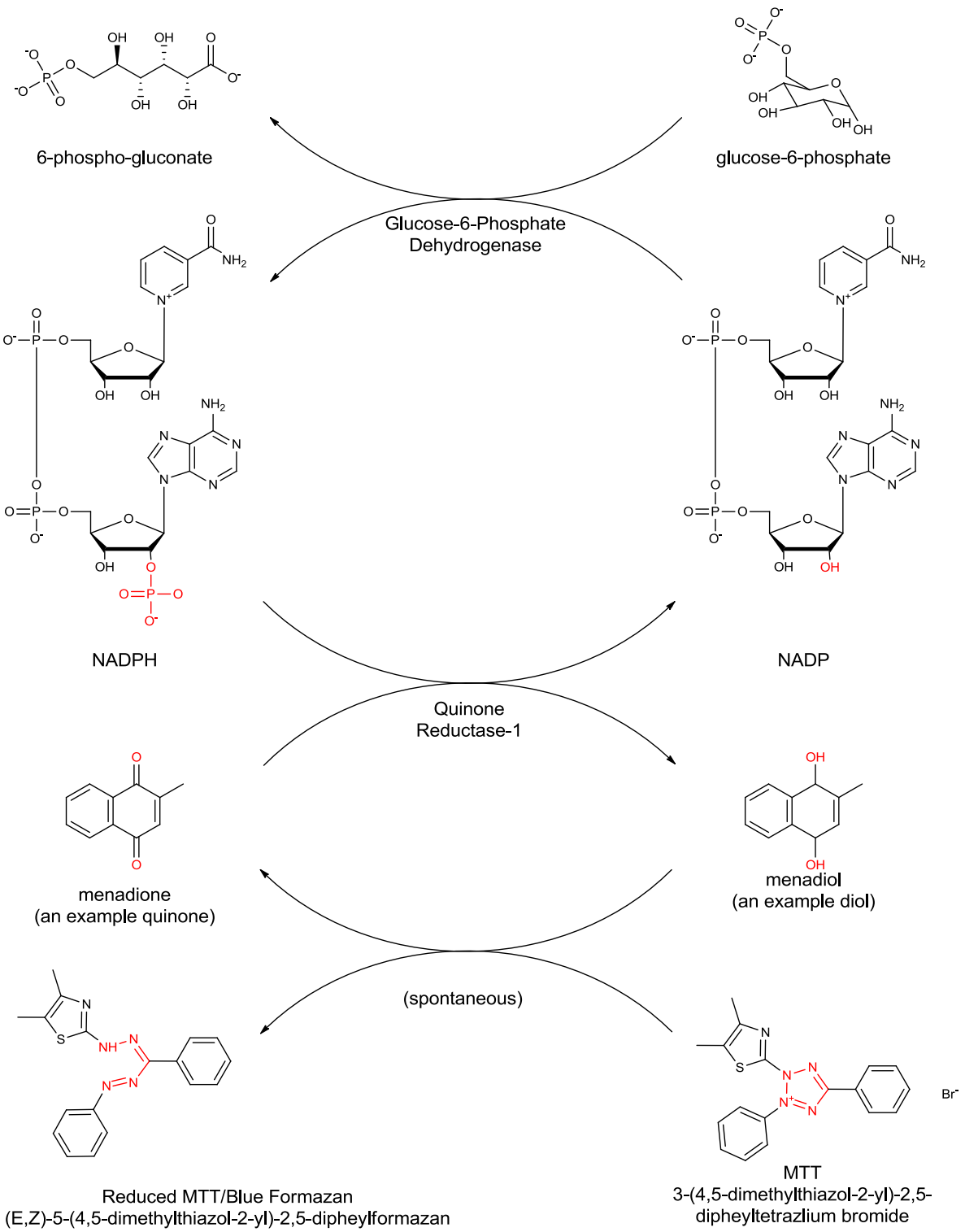


Figure 4 Reactions taking place during quantification of QR-1 activity.
Figure adapted from Prochaska and Santamaria, 1987.[21]

compared with DMSO-treated control.[20] Normalization to protein levels was accomplished using crystal violet staining of duplicate plates, and subsequent measurement at 595 nm.[21]

Enzyme activity was expressed as a CD value, the concentration of test material needed to double the specific activity of quinone reductase (reported in micromolar). The known QR1 inducer 4'-bromoflavone (CD = 0.1 μM) was used as a positive control.[6]

2.3.6 Determination of GSH levels in cell culture

Glutathione was measured via oxidation of 5,5-dithiobis-(2-nitrobenzoic acid) (Ellman reagent) and reduction by NADPH in the presence of glutathione reductase, as previously described.[22] Briefly, Hepa 1c1c7 cells were seeded in 96-well plates (200 μL/well) at a concentration of 2 x 10^4 cells mL^{-1}. Following a 24 h incubation, five serial dilutions of test compounds in 0.5% DMSO (final concentration) and fresh medium were added in duplicate. Plates were further incubated for 48 h, washed three times with PBS (pH 7.4), and frozen at -80°C. Cells were lysed by three consecutive freeze-thaw cycles, followed by the addition of 40 μL of 125 μM sodium phosphate buffer (pH 7.5) containing 6.3 mM EDTA (solution A). A reaction mixture was prepared consisting of 20 μL of 6 mM 5,5-dithiobis-(2-nitobenzoic acid) in solution A, 10 μL of glutathione reductase solution (50 units in 10 mL solution A), and 140 μL of NADPH-generating system (solution B). Solution B contained 2.5 mL of 0.5 M Tris-HCl (pH 7.4), 330 μL of 150 mM glucose 6-phosphate, 100 units of glucose 6-phosphate dehydrogenase, and 30 μL of 50 mM $NADP^+$ in a total volume of 50 mL distilled water. Freshly prepared reaction mixture (170 μL)

was added to each well and plates were shaken at room temperature for five minutes. After five minutes of further incubation, the formation of 2-nitro-5-thiobenzoic acid was measured at 405

nm. Protein content was measured using a bicinchoninic acid protein assay kit with BSA as a standard.

2.3.7 Determination of glutathione reductase activity

To measure the activity of glutathione reductase, cell lysates were added to a mixture containing 1.78 mM EDTA in 178 mM potassium phosphate buffer, 1 mM glutathione disulfide (GSSG), and 0.1 mM NADPH in 10 mM Tris-HCl in a final volume of 1 mL. The linear decrease in absorbance of NADPH was measured at 340 nm for 2 min. Activity was normalized per mg protein.[23]

2.3.8 Determination of glutathione peroxidase activity

Glutathione peroxidase activity was measured by combining tissue supernatant and 31.5 mM sodium phosphate buffer, 1 mM GSH, 0.2 mM β-NADPH, 11.25 mM sodium azide, and 10 units glutathione reductase in a final volume of 1.0 ml. After allowing the incubation mixture to equilibrate for a few minutes, 0.238 mM hydrogen peroxide was added. The linear decrease in absorbance of NADPH was measured at 340 nm for 1 min following a lag time of 30 s. Activity was normalized per mg protein.[23]

2.4 Conclusions

We identified a new macrolide glycoside, juvenimicin C (**1**), from a marine-derived *Micromonospora* sp. Compound **1** enhanced QR1 enzyme activity and glutathione levels by two-fold with CD values of 10.1 and 27.7 μM, respectively. In addition, glutathione reductase and glutathione peroxidase activities were elevated. A γ,δ-unsaturated analog (**2**) was not active, indicating the structural importance of the epoxide associated with compound **1** that was not seen

in the not significantly bioactive metabolite **2**. This is the first reported member of the macrolide class of antibiotics found to mediate these responses.

2.5 References

1. Talalay, P.; Fahey, J. W.; Holtzclaw, W. D.; Prestera, T.; Zhang, Y. Chemoprotection against cancer by phase 2 enzyme induction. *Toxicol. Lett.* **1995**, *82-83*, 173-179.
2. Dinkova-Kostova, A. T.; Talalay, P. Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen. *Free Radic. Biol. Med.* **2000**, *29*, 231-240.
3. Shen, L.; Park, E. J.; Kondratyuk, T. P.; Guendisch, D.; Marler, L.; Pezzuto, J. M.; Wright, A. D.; Sun, D. Design, synthesis, and biological evaluation of callophycin A and analogues as potential chemopreventive and anticancer agents. *Bioorg. Med. Chem.* **2011**, *19*, 6182-6195.
4. Li, Y.; Zhao, M.; Parkin, K. L. β-Carboline derivatives and diphenols from soy sauce are *in vitro* quinone reductase (QR) inducers. *J. Agric. Food Chem.* **2011**, *59*, 2332-2340.
5. Mayhoub, A. S.; Marler, L.; Kondratyuk, T. P.; Park, E. J.; Pezzuto, J. M.; Cushman, M. Optimization of thiazole analogues of resveratrol for induction of NAD(P)H:quinone reductase 1 (QR1). *Bioorg. Med. Chem.* **2012**, *20*, 7030-7039.
6. Song, L. L.; Kosmeder, J. W., 2nd; Lee, S. K.; Gerhauser, C.; Lantvit, D.; Moon, R. C.; Moriarty, R. M.; Pezzuto, J. M. Cancer chemopreventive activity mediated by 4'-bromoflavone, a potent inducer of phase II detoxification enzymes. *Cancer Res.* **1999**, *59*, 578-585.
7. Cheng, L.; Shen, L. M.; Zhang, M.; Li, N.; Li, X.; Ma, Z. J.; Qu, H. B. Eleven new triterpenes from *Eurycorymbus cavaleriei*. *Helv. Chim. Acta* **2010**, *93*, 2263-2275.

8. Fahey, J. W.; Stephenson, K. K. Pinostrobin from honey and Thai ginger (*Boesenbergia pandurata)*: a potent flavonoid inducer of mammalian phase 2 chemoprotective and antioxidant enzymes. *J. Agric. Food Chem.* **2002**, *50*, 7472-7476.

9. Wollenweber, E.; Stevens, J. F.; Klimo, K.; Knauft, J.; Frank, N.; Gerhauser, C. Cancer chemopreventive in vitro activities of isoflavones isolated from *Iris germanica*. *Planta Med.* **2003**, *69*, 15-20.

10. Carcache-Blanco, E. J.; Kang, Y. H.; Park, E. J.; Su, B. N.; Kardono, L. B. S.; Riswan, S.; Fong, H. H. S.; Pezzuto, J. M.; Kinghorn, A. D. Constituents of the stem bark of *Pongamia pinnata* with the potential to induce quinone reductase. *J. Nat. Prod.* **2003**, *66*, 1197-1202.

11. Yin, H. Q.; Lee, B. W.; Kim, Y. C.; Sohn, D. H.; Lee, B. H. Induction of the anticarcinogenic marker enzyme, quinone reductase, by *Dalbergiae Lignum*. *Arch. Pharm. Res.* **2004**, *27*, 919-922.

12. Yuan, Y.; Ji, L.; Luo, L.; Lu, J.; Ma, X.; Ma, Z.; Chen, Z. Quinone reductase (QR) inducers from *Andrographis paniculata* and identification of molecular target of andrographolide. *Fitoterapia* **2012**, *83*, 1506-1513.

13. Li, F.; Wang, Y.; Parkin, K. L.; Nitteranon, V.; Liang, J.; Yang, W.; Li, Y.; Zhang, G.; Hu, Q. Isolation of quinone reductase (QR) inducing agents from ginger rhizome and their in vitro anti-inflammatory activity. *Food Res. Int.* **2011**, *44*, 1597-1603.

14. Schell, U.; Haydock, S. F.; Kaja, A. L.; Carletti, I.; Lill, R. E.; Read, E.; Sheehan, L. S.; Low, L.; Fernandez, M. J.; Grolle, F.; McArthur, H. A.; Sheridan, R. M.; Leadlay, P. F.; Wilkinson, B.; Gaisser, S. Engineered biosynthesis of hybrid macrolide polyketides

containing D-angolosamine and D-mycaminose moieties. *Organic & Biomolecular Chemistry* **2008**, *6*, 3315-3327.

15. Marler, L.; Conda-Sheridan, M.; Cinelli, M. A.; Morrell, A. E.; Cushman, M.; Chen, L.; Huang, K.; Van Breemen, R.; Pezzuto, J. M. Cancer chemopreventive potential of aromathecins and phenazines, novel natural product derivatives. *Anticancer Res.* **2010**, *30*, 4873-4882.

16. Zhang, L.; Xi, L.; Ruan, J.; Huang, Y. *Micromonospora yangpuensis* sp. nov., isolated from a sponge. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 272-278.

17. Kim, O. S.; Cho, Y. J.; Lee, K.; Yoon, S. H.; Kim, M.; Na, H.; Park, S. C.; Jeon, Y. S.; Lee, J. H.; Yi, H.; Won, S.; Chun, J. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 716-721.

18. Magarvey, N. A.; Keller, J. M.; Bernan, V.; Dworkin, M.; Sherman, D. H. Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. *Appl. Environ. Microbiol.* **2004**, *70*, 7520-7529.

19. Zhang, Y.; Talalay, P.; Cho, C. G.; Posner, G. H. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci.* **1992**, *89*, 2399-23403.

20. Nam, S.-J.; Gaudencio, S. P.; Kauffman, C. A.; Jensen, P. R.; Kondratyuk, T. P.; Marler, L. E.; Pezzuto, J. M.; Fenical, W. Fijiolides A and B, Inhibitors of TNF-α-Induced NFκB Activation, from a Marine-Derived Sediment Bacterium of the Genus Nocardiopsis. *J. Nat. Prod.* **2010**, *73*, 1080-1086.

21. Prochaska, H. J.; Santamaria, A. B. Direct measurement of NAD(P)H: quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. *Anal. Biochem.* **1988**, *169*, 328-336.

22. Gerhäuser, C.; You, M.; Liu, J.; Moriarty, R. M.; Hawthorne, M.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive potential of sulforamate, a novel analogue of sulforaphane that induces phase 2 drug-metabolizing Enzymes. *Cancer Res.* **1997**, *57*, 272-278.

23. Carlson, G. P.; Turner, M.; Mantick, N. A. Effects of styrene and styrene oxide on glutathione-related antioxidant enzymes. *Toxicology* **2006**, *227*, 217-226.

Chapter 3. Phylum-specific regulation of resistomycin production in a *Streptomyces* sp. via microbial co-culture[§]

3.1 Introduction

Actinomycete genomes contain an immense potential to produce secondary metabolites, however standard laboratory culture experiments rarely provide the conditions under which associated biosynthetic pathways are expressed. Despite years of research attempting to access these pathways, and aside from a few well-studied bacterial quorum sensing systems, little is known about the specificity of secondary metabolite regulation in bacteria. In order to induce silent biosynthetic gene clusters in bacteria, researchers have attempted a number of experimental designs that are outlined in the introduction to this chapter. We chose to develop a co-cultivation approach to induce the expression of biosynthetic gene clusters based on the documented successes of co-cultivation experiments in the literature. However, to demonstrate that antibiotic biosynthetic gene clusters could be selectively induced by the presence of specific taxa of bacteria in co-culture, we chose aquatic-derived actinomycete strains from our library that were not known to produce antibiotics under our laboratory culture conditions. We then co-cultivated each of them with a panel of four Proteobacteria. This panel induced the production of resistomycin from a Lake Michigan derived actinomycete. The induction of this antimicrobial secondary metabolite was then used to trace the specificity of this phenomenon to the phylum level of bacteria-bacteria interaction. The antibiotic induction rate observed in this study can be used to predict future efforts to mine existing actinomycete microbial libraries for novel drug

---

[§] Reproduced manuscript with permission from Carlson, S.; Tanouye, U; Omarsdottir, S; and Murphy, B. T. Phylum-specific regulation of resistomycin production in a *Streptomyces* sp. via microbial co-culture. *J. Nat. Prod.* **2014** Article ASAP DOI: 10.1021/np500767u. Copyright 2014 American Chemical Society.

leads. This chapter summarizes the field of secondary metabolite induction and the co-cultivation experiments undertaken in our laboratory. The conclusions and future directions are presented at the end of the chapter.

3.2 Inducible secondary metabolite biosynthetic pathways in Actinobacteria

Actinomycete genomes have been found to be up to 10.2 Mb[1] with 1600 to 9000 protein encoding genes.[2] They are known to dedicate a significantly large portion, in some cases up to ten percent, of their genomes to secondary metabolite biosynthesis.[2] In some organisms, this can represent over 20 biosynthetic gene clusters.[2] For example, in the genus *Streptomyces*, 25-400 kb of DNA[3] could be devoted to secondary metabolite biosynthetic gene clusters that range in size from 5 kb to over 100 kb.[1] Despite the size of this structural repository, in most cases only a few molecules associated with these biosynthetic gene clusters have been experimentally observed.[4]

Biosynthetic gene clusters that are expressed at low levels under standard laboratory culture conditions, resulting in trace to currently undetectable amounts of the metabolic product, are referred to as silent biosynthetic gene clusters. Alternatively, the term cryptic biosynthetic gene clusters refer to those clusters that have been identified via whole genome sequencing but a specific secondary metabolite has not yet been assigned to the gene cluster either by predictive modeling or by isolating and identifying the resultant secondary metabolite.[5]

Numerous ways to elicit the secondary metabolites produced by these biosynthetic gene clusters have been described in the literature, such as heterologous expression and co-cultivation. For the purposes of this dissertation, attempts to induce silent and cryptic secondary metabolite gene clusters in bacteria will be described as either (A) targeted methods to increase the expression of a specific biosynthetic pathway, resulting in the increased recovery of a single secondary

metabolite or are (B) non-targeted, general methods to increase secondary metabolism in a non-specific manner.[6]

3.3 Targeted methods to induce the production of secondary metabolites

With the advancement of genome sequencing technologies, cryptic biosynthetic gene clusters are being identified faster than secondary metabolites can be ascribed to them. Genome mining, or the process of identifying these biosynthetic gene clusters and accessing the metabolic product of these genes, utilizes bioinformatics, synthetic chemistry, and molecular biology, in addition to the standard natural product isolation and structure elucidation methods.[7] Two experimental approaches to utilize genome mining to induce the production of secondary metabolites are heterologous expression of biosynthetic gene clusters and the feeding of non-native precursors in order to initiate biosynthesis from these biosynthetic gene clusters.

3.3.1 Heterologous expression of biosynthetic gene clusters to increase yields of secondary metabolites

Once identified, the biosynthetic gene clusters are cloned into and expressed by heterologous hosts. Two common hosts are *E. coli* and *Streptomyces coelicolor*.[8] The requirements of the chosen host are often quite difficult when transferring biosynthetic gene clusters between genera, as each host must have the appropriate precursors present, a common problem for using *E. coli* as a heterologous host. Other problems could arise at any step of the process as the large enzymes must be functionally expressed, the enzyme complex must then be correctly decorated with post translational modifications, and the heterologous host must be resistant to the toxicity of the completed natural product.[7-9]

This method holds promise for secondary metabolite discovery and the discovery of new chemical scaffolds. While heterologous expression allows the expression of specific cryptic pathways, it is a time consuming molecular biology technique. This is disadvantageous because it requires extensive knowledge of the biosynthetic gene cluster responsible for the production of the secondary metabolite and is therefore not a high throughout method to induce secondary metabolism.

3.3.2 Adding non-native precursors to culture media to generate "non-natural" natural products

The substrate specificity of biosynthetic enzymes or the lack there of, leads to the branching of secondary metabolite biosynthetic pathways that results in the generation of many diverse secondary metabolites.[10-12] Capitalizing on this promiscuity,[13] culture media can be supplemented with non-native intermediates in predetermined biosynthetic pathways. These supplements can be as simple as increasing the concentration of specific primary metabolites that are known to be incorporated in a small molecule or as complex as synthesizing precursors with uniquely synthetic non-natural functional groups.[14] In order to study the biosynthesis or to solve complex natural product structures with many quaternary carbons, primary metabolic products can be added to culture media that are enriched with ^{13}C in order to trace the primary metabolite building block incorporation into secondary metabolites. Sometimes the addition of the required primary metabolic product is sufficient to initiate the production of a secondary metabolite. Building on this technique, it is possible to supplement culture media with synthetic precursors that possess functional groups created in a laboratory and not seen in nature. These studies have yielded "unnatural" natural products in both polyketide[15-16] and non-ribosomal peptide synthetases.[17] While this method does not initially appear to be a targeted way to produce

secondary metabolites, it requires knowing which compounds a strain produces. Unfortunately, this method does not allow for the induction of cryptic or silent biosynthetic gene clusters, nor is this method a high-throughput way to induce secondary metabolism.

3.4 General methods to induce secondary metabolite biosynthesis

Previous studies that attempt to access secondary metabolites in a general fashion have been well summarized.[18-19] The inducers of global secondary metabolism include but are not limited to: altering the culture media, employing physical stress, searching for small-molecule modulators, and co-cultivation. A brief summary of each method is presented herein with the aim of highlighting the novelty of our study.

3.4.1 OSMAC Approach to increase yields of secondary metabolites

The simplest technique to alter secondary metabolism in bacteria is to cultivate a single bacterial strain using different culture conditions, which is referred to as the one strain many compounds (OSMAC) approach.[20] This method requires little pre-existing knowledge of secondary metabolite biosynthetic pathways. For example, changing the osmotic balance (increasing the concentration of NaCl, KCl, or sucrose) of the culture media of the α-proteobacterium *Sinorhizobium meliloti* induced the expression of the diketopiperizine N-acetylglutamiylglutamine.[21] Similarly, cultivation of *Streptomyces venezuelae* in the presence of 3% DMSO caused an increase in the production of chloramphenicol by 300%.[22] The exact mechanism of induction remains unknown, however it is possible that the bacteria were provided with an additional carbon source or that during cultivation the bacteria were stressed by the presence of DMSO. (DMSO is a commonplace laboratory cryopreservative but is toxic to cells at higher concentrations.) These alterations in the regulatory machinery of the responsible

biosynthetic gene cluster remain largely unknown. Other culture conditions such as phosphate concentration,[23] dissolved oxygen, hydrostatic pressure, altering pH,[20] and changing culture vessel shape[24] have also been shown to induce secondary metabolism not seen under standard cultivation conditions. The OSMAC approach requires no knowledge of existing biosynthetic pathways and can be made relatively high throughput. However, altering the culture media still may not provide sufficient motivation to elicit the production of secondary metabolites otherwise not seen under standard laboratory culture conditions.

3.4.2 Employing physical stress to increase yields of secondary metabolites

Actinomycetes form spores and aerial mycelia when exposed to nutrient deprivation. In order to execute such a complex biochemical feat, a signal cascade is initiated that sometimes leads to antibiotic production.[25] Rigali et al.[25] describe the production of actinorhodin or undecylprodigiosin caused by the accumulation of N-acetylglucosamine following nutrient deprivation, likely due to the initiation of cell wall breakdown.

A strain of *Streptomyces venezuelae* was induced to produce the novel antibiotic jadomycin B during nutrient deprivation when exposed to heat, phage, and ethanol shock prior to cultivation.[26-27] The strain originally produced chloramphenicol but due to the stress, the same biosynthetic machinery switches to producing jadomycin derivatives.[25]

Physically stressing microorganisms prior to or during cultivation has yielded natural products not seen under standard laboratory conditions. These studies demonstrate the inextricable link between primary and secondary metabolism.

3.4.3 Addition of small molecules to culture media to increase yields of secondary metabolites

Another straightforward approach to inducing secondary metabolism in bacteria is to search for small molecules that can be added to culture media that would generally up regulate secondary metabolism. While no single chemical entity has been identified that can globally upregulate secondary metabolite production, efforts have been made to identify small molecule inducers of specific biosynthetic pathways in an effort to identify such a small molecule.

For example, Seyedsayamodst[28] established a platform to screen small molecule libraries for modulators of one cryptic and one silent biosynthetic gene cluster in the β-proteobacteria *Burkholderia thailandensis*. This high throughput screen identified nine inducers of secondary metabolism, all of which were antibiotics at higher concentrations. The antibiotic trimethoprim, an inhibitor of dihydrofolate reductase (an essential enzyme for the de novo synthesis of purines and amino acids), induced five biosynthetic pathways in *B. thailandensis*, including two cryptic clusters whose metabolic product remains to be identified. The findings presented demonstrated that small molecule inducers of secondary metabolism are possibly antibiotics acting as signaling molecules at sub-inhibitory concentrations.[28] Similarly, Craney et al.[29] found that small molecule inhibitors of the fatty acid biosynthesis upregulated secondary metabolite biosynthesis in *S. coelicolor*.[29] The methods presented here are high-throughput ways to identify inducers of secondary metabolism but due to the small scale of high throughput experiments, additional cultivation experiments and optimization are required to identify the induced antibiotic. These data support antimicrobial secondary metabolites used out of their ecological context to treat human disease are possibly acting as quorum-sensing molecules in the environment.

As further evidence, lactic acid bacteria produce bacteriocins, small antimicrobial peptides with narrow-spectrum activity, in response to specific Gram-positive competitors. This narrow antibacterial spectrum is hypothesized to affect specific bacteria from the same environment.[30] This species-specific response is similarly likely due to quorum-sensing.

During co-cultivation, metabolites exchanged between the bacteria during cultivation may be behaving as quorum-sensing molecules.[31] Bassler et al.[32] demonstrated cross-species control of *Vibrio harveyi* bioluminescence, demonstrating that supernatants from several non-bioluminescent species could induce bioluminescence in other *V. harveyi* species.[32] Separately, it was shown that antibiotic production is under the control of quorum sensing in a *Serratia* sp. (γ-Proteobacteria). The production of the alkaloid pigment prodigiosin was an indicator of the induction of secondary metabolite biosynthetic gene clusters. Additionally, two independent quorum-sensing systems were identified; one system that was species-specific, and another system that could be induced by multiple species. This demonstrates that co-cultivation can induce secondary metabolism in a species specific manner by the exchange of small molecules.

The quorum-sensing language of *V. harveyi* is the exchange of diffusible N-acyl homoserine lactones. These signals have also been directly linked to the production of violacein, a bactericidal secondary metabolite composed of a 5-hydroxyindole, an oxindole, and a 2-pyrrolidone,[33-34] in *Chromobacterium violaceum*.[35-36] These groups both demonstrated that the small molecular weight N-acyl homoserine lactones transcriptionally regulate secondary metabolite biosynthesis via quorum-sensing.

Aside from a few well characterized systems discussed here, little is known about the specificity of secondary metabolite regulation in bacteria, such as the conditions under which a bacterium

produces a secondary metabolite and the extent to which it does so in recognition of a competing species in the immediate environment.

3.5 Co-culture as a method to alter secondary metabolism

Co-cultivation between microbial species is the simultaneous cultivation of two or more species in the same culture flask. This method has been extensively reviewed[19, 37-38] and is responsible for several novel natural products such as the emericellamides,[37, 39-40] libertellenones,[41-42] pestalone,[43] marinamide,[44] and N-formyl alkaloids,[45] among many others.

Culturing two microbes in the same flask is rarely exploited to study cross-species or cross-kingdom signaling; however, the utility of cultivating two microbes is both an undeniably simple and successful way to induce secondary metabolism. Our study aimed to induce the production of species-specific antibiotics in actinomycetes against Proteobacteria competitors. Members of the phylum Proteobacteria were chosen as competitors because Proteobacteria are the most abundant bacteria in the aquatic environment, the source for all of our actinomycetes. This emphasizes the ecological relevance of our study, as aquatic derived actinomycetes are likely to have defenses encoded against these endemic strains. As such, our study is different from most cross-species bacteria co-cultivation experiments. Current bacteria-bacteria co-cultivation literature reflects the use of cross-species combinations to induce antimicrobial activity, increase the yield of a known metabolite, or the happenstance isolation a novel antibiotic.

Mearns-Spragg et al.[46] co-cultivated (or co-incubated with cell-free supernatants) unidentified marine bacteria with various terrestrial pathogens. Six marine bacteria that did not produce antibiotics in monoculture, were grown in the presence of two γ-proteobacterial pathogens (*E. coli* and *P. aeruginosa*) contained within 8000 Da dialysis tubes. Three of the six marine bacteria

exhibited antimicrobial activity in the disc diffusion assay after competition with the γ-Proteobacteria.[46-47] Similar to ours, this study demonstrated that antimicrobial activity (where none was previously observed) could be induced by the presence of Proteobacteria in co-cultivation. Extracts of the co-culture supernatant were assessed for antimicrobial activity yet no antibiotics were identified.

While searching for biosurfactants and quorum-sensing inhibitors, Dusane et al.[48] demonstrated that co-cultivation of marine epibiotic bacteria with the terrestrial pathogen *P. aeruginosa* (a γ-Proteobacteria) induced the production of compounds that displayed antimicrobial activity.[48] The induction of secondary metabolism was not specific to γ-Proteobacteria as the induced antimicrobial activity was also observed when co-cultivated with *Bacillus pumilius*.

Onaka et al.[49] demonstrated the production of novel antibiotic alchivemycin A by a strain of *Streptomyces lividans* only when the strain was co-cultivated with living *Tsukamurella pulmonis* (a mycolic acid containing bacterium, belonging to the Corynebacterineae). The authors went on to note that the induction was specific to bacteria that contained mycolic acid in their cell wall. However, alchivemycin A was only produced when there was direct contact between the two bacterial species during co-culture, as adding mycolic acid to the culture media was not sufficient to induce the production of this antibiotic. Studies utilizing heat-killed cell wall and cell-free supernatant were also unable to induce the production of alchivemycin.[49-50] In more recent studies the same laboratory demonstrated the induction of an indolocarbazole from the bacteria *Streptomyces cinnamoneus* and the fungus *Tsukamurella pulmonis* using the same methodology.[51] These studies demonstrated that interactions during co-cultivation experiments are often more complex than the exchange of a single molecule and may require live cells to induce the production of secondary metabolites.

In an attempt to increase the amount of a metabolite produced, Trischman et al.[52] saw an 320% increase over monoculture in the amount of the diketopiperazine containing pheylalanine-proline, cyclo(Phe-Pro), produced by a *Bacillus* sp. when challenged with a second *Bacillus* sp.[52]

Slattery et al.[53] investigated the increased production of istamycin (originally isolated from 'sea mud') by the marine bacterium *Streptomyces tenjimariensis* when co-cultivated with a panel of 53 other marine bacteria.[53] Twelve of these bacteria increased the production of istamycin, the majority (eight of the twelve) of which were Gram-negative. While these marine bacteria were not identified by 16S rRNA gene sequencing, this study demonstrates the strength of cultivating microbes from the same source. Co-cultivation of similarly sourced marine bacteria can be used to upregulate endemic chemical defenses.

3.6 Significance

With few exceptions, the majority of these studies focused on the induction of specified gene clusters, happenstance discovery of structural novelty, or increasing metabolite production. The drug lead discovery system presented here utilizes a phylum of bacteria typically found in aquatic environment to induce endemic antimicrobial defense systems in aquatic actinomycetes; the degree of induction specificity observed in this study is highly novel.

3.7 Results and discussion

We have isolated over 1,500 actinomycete strains from unique aquatic environments around the world including Vietnam, Iceland, the Great Lakes, and several marine sites in the USA. From the fermentation broth of each of these strains we have created a secondary metabolite fraction library that we use to screen in high-throughput biological assays that target a variety of bacterial strains. In unpublished previous studies, this fraction library was screened against a panel of

bacteria strains *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), and *Mycobacterium tuberculosis* (H37Rv) for antimicrobial activity.

For the current co-cultivation study, we selected 95 aquatic-derived actinomycete strains whose secondary metabolite fractions did not display significant antimicrobial activity (minimum inhibitory concentration [MIC] greater than 16 μg/mL in dose response assays) against the aforementioned four strains. This allowed us to infer that any antimicrobial activity observed from extracts of co-culture experiments was likely due to interaction with the competing bacterial species. Finally, our four-strain Proteobacteria panel was selected on the basis of 1) phylogenetic similarity to known bioterrorism agents and 2) natural occurrence in the aquatic environment (justification for the selection of these four strains is listed in Table A2). These two reasons allowed us to not only investigate potential ecological interactions of our aquatic-derived actinomycetes, but also to explore a unique platform toward the discovery of antimicrobial agents. The strains chosen for the Proteobacteria panel were: *Burkholderia vietnamiensis* (ATCC BAA-248), *Brucella neotomae* (ATCC 23459), *Yersinia pestis* (A1122), and *Xanthomonas axonopodis* (ATCC 8718).

3.7.1 Co-cultivation of 95 actinomycete strains with Proteobacteria panel

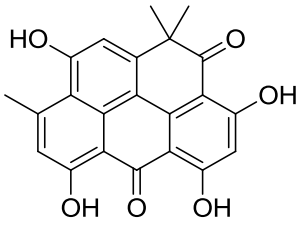
Each of the 95 actinomycetes was cultivated under five conditions (as described in detail under General Experimental Procedures): in combination with one of the four Proteobacteria strains and one monoculture to serve as a control. After a set incubation period, the broths of the resulting 475 co-cultivation experiments were extracted, evaporated to dryness, and subjected to biological and chemical profiling (via growth inhibition assays and HPLC-DAD (diode array

detector)-MS analysis) in order to detect induced antibiotics. The co-cultivation extracts were screened for biological activity against eight bacterial strains (*B. neotomae* ATCC 23459, *B. vietnamiensis* ATCC BAA-248, *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *M. tuberculosis* H37Rv, *P. aeruginosa* ATCC 27853, *X. axonopodis* ATCC 8718, and *Y. pestis* A1122) using *in vitro* growth inhibition assays. Antibacterial activity observed in the co-culture extract(s) and the lack thereof in the corresponding actinomycete monoculture extract indicated that an antibiotic was produced as a result of microbial competition. Of the 475 extracts, we identified a single strain that displayed significant antimicrobial activity against *E. faecalis* (<32 μg/mL) in the co-culture and not in the monoculture.

The co-culture extract of strain B033-*B. vietnamiensis* exhibited an MIC of 16 μg/mL against *E. faecalis*, whereas the B033 monoculture extract did not exhibit significant inhibitory activity in any other bioassay. As a result, the corresponding extracts were analyzed using HPLC (Figure 5) and HRESIMS (Figure A33).

One chromatographic peak at t_R = 17.9 min (Figure 5) was detected in the co-culture extract but not in the monoculture extracts of strains B033 and *B. vietnamiensis*. We suspected the compound was resistomycin based on its HRMS data and characteristic UV spectrum. This was confirmed after comparison of HRMS, UV, and t_R data to a purchased standard (Figure A33). Resistomycin (**3**) is a compound that is reported to possess potent cytotoxicity and is a weak, broad spectrum antibiotic[54-55] that inhibits DNA-dependent RNA polymerase.[56-58]

In order to verify that the observed induction was a reproducible phenomenon, in follow-up experiments strain B033 was co-cultivated in triplicate with each of the Proteobacteria strains in



3

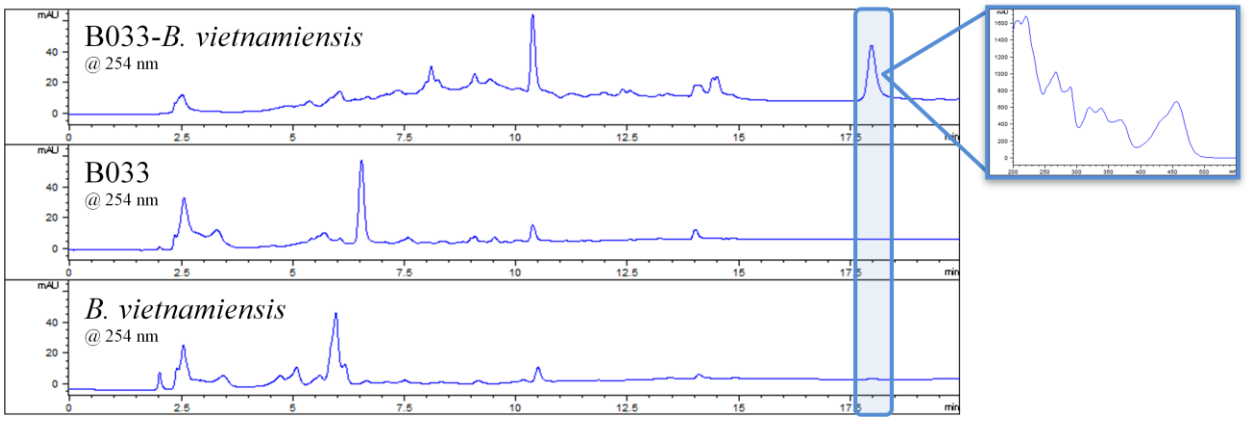


Figure 5 HPLC chromatograms at 254 nm of the co-culture extract of B033-*B. vietnamiensis* and the extracts from monocultures of B033 and *B. vietnamiensis*.
The highlighted box indicates the chromatographic peak of the induced antibiotic resistomycin (**3**); the UV profile of resistomycin is shown to the right.

the panel. All co-cultures induced the production of resistomycin, as observed via HPLC-DAD analysis (Figure A34). Given that resistomycin was detected in the co-culture extracts of B033 and all four Proteobacteria in the panel, it is reasonable to conclude that the molecule is derived from B033 and not each of the competitor strains. Additionally, resistomycin has been reported from other *Streptomyces* species.[59-62]

Since we identified the producer of the antibiotic, we then set out to determine whether antibiotic production was specific to microbial co-cultivation or whether it was a general response to stress, we assessed resistomycin production under a variety of monoculture conditions. First, we cultivated B033 under five different media types (Table A3) and did not observe resistomycin production, suggesting that altering carbon and nitrogen sources has no effect on the production of the molecule. Previous reports suggested that a *Streptomyces* sp. increased its production of resistomycin upon the addition of excess starch,[63] though we did not observe this effect on B033. We then grew B033 in minimal freshwater-based A1 media (one tenth the strength of our nutrient rich A1 media), and both filter sterilized Lake Michigan water and DI water in order to represent a nutrient limited environment. Resistomycin was not detected in the culture broths, thus nutrient limitation is likely not the cause for the observed antibiotic induction. To complement this, we cultivated B033 in freshwater-based A1 media for up to 14 days and monitored the broth for the presence of resistomycin on a daily basis; we were unable to detect the metabolite in any of the daily aliquots. Additionally, cultivating the strain in a saline environment did not affect production of the small molecule. The only parameter that affected resistomycin production in monoculture was increasing the cultivation temperature, as we observed the antibiotic when B033 was cultivated in freshwater-based A1 media at 37 °C (though not at 20 °C or 25 °C).

3.7.2 Resistomycin induction in co-cultivation experiments is specific to interactions with Proteobacteria

To determine whether resistomycin production could be induced in a phylum-specific manner, we co-cultivated strain B033 with a panel of 51 bacteria (44 environmental isolates and 7 ATCC type strains; experiments carried out in freshwater-based A1 media at 21 °C). These isolates are representative of the major phyla found in aquatic environments (Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes). Each had a unique morphological appearance and phylogenetic identity based on the analysis of their corresponding 16S ribosomal RNA gene; this analysis was performed in order to prevent strain duplication that would risk skewing the calculation of percent resistomycin induction observed within each phylum (Table A4).

Of 51 total B033 pairings, resistomycin was detected in the extract of 15 co-cultures. Specifically, within the B033-Proteobacteria co-culture group (20 of 51), resistomycin was detected in 13 of the samples (65%). Conversely, resistomycin was detected once in each of the following phylum groupings: B033-Actinobacteria (9.1%; N = 11) and B033-Firmicutes (5.9%; N = 17), (Figure 6). Additionally, B033 was co-cultivated with two strains from the phylum Bacteroidetes and resistomycin was not detected in either of these culture extracts (data not shown). Further co-cultivation experiments are needed with isolates of this phylum in order to determine whether this trend would follow that observed with Actinobacteria and Firmicutes.

Our data suggest that the production of resistomycin in strain B033 strongly favors the particular phylum of bacteria present in its immediate environment, a discovery that has implications

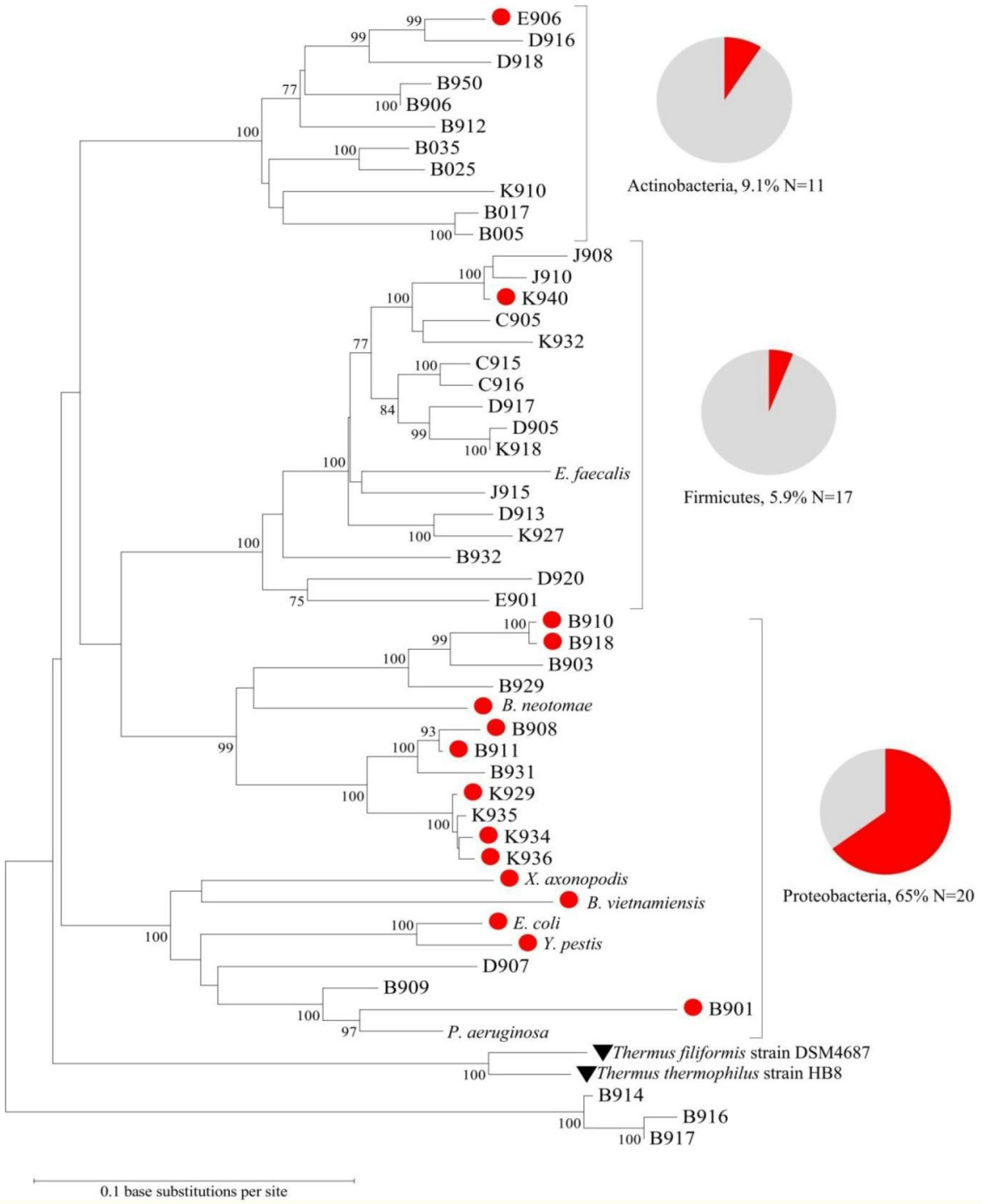


Figure 6 Phylogenetic diversity of environmental strains in follow-up co-culture panel. The phylogenetic tree was constructed within the software package MEGA using the Neighbor-Joining method, and implementing a maximum composite likelihood nucleotide substitution model.[64-65] Nodes supported by bootstrap analyses (>70%) are indicated.[66] The red circles indicate the strains that induced resistomycin production when co-cultivated with strain B033. The black triangles are reference strains and were not directly part of this study. The percent of strains within each grouping that induced resistomycin production is shown to the right of the phylogenetic tree.

toward understanding when and how bacteria commit to producing antibiotics. We attempted to assess whether antibiotic induction was a result of a) small molecule production from the competing species in co-culture, which would implicate quorum sensing as an induction mechanism, or b) the physical presence of a particular cell type, which would implicate direct cell to cell contact as an induction mechanism.[49] Both sets of experiments were inconclusive and further method optimization is currently underway in our laboratory in order to address this question. We hypothesize that there may be some large complex (greater than 2 μm) that is adheres to the filter cup yet is resistant to centrifugation that could be responsible for the observed induction. Furthermore, an alternative filter method could be used to separate the cell mass in order to attain this component. For instance, we co-incubated B033 with the spent culture broth of each strain that induced resistomycin production. Though results were inconclusive and not reproducible, the presence or concentration of any extracellular signaling molecules is difficult to quantify and may not have provided sufficient conditions for upregulation of the antibiotic's biosynthetic genes. Additionally, when co-incubating B033 with the autoclaved cell mass of inducing strains, the added amount of, or physical changes to the cellular structure are likely other important factors that require more detailed tailoring. Thus, our attempts to induce resistomycin production by incubating strain B033 in a cell-free or heat-killed system were not sufficient to determine the induction mechanism.

Next, we tested the ability of resistomycin to inhibit each of the competing strains that were responsible for its induction using in vitro growth inhibition assays. The inducing Actinobacteria strain (E906) and Firmicutes strain (K940) were inhibited by resistomycin with MIC values of 0.43 μg/mL and 0.25 μg/mL, respectively. However, of the 13 Proteobacteria strains that induced its production, resistomycin exhibited inhibitory activity against none of them (single dose

testing at 50 μg/mL), suggesting that it may not be an effective means of warding off competing Proteobacteria in the environment. The reason for resistomycin production is unclear, given that B033 produces it in the presence of Proteobacteria and it is not an effective antibiotic against the Gram-negative bacteria that we tested. It is possible that resistomycin has other ecological roles, beyond that of warding off some Gram-positive bacteria. Previous reports have shown that certain antibiotics act as signaling molecules at sub-inhibitory concentrations, so it is possible that resistomycin mediates other processes related to Proteobacteria in the environment. Sub-inhibitory concentrations of antibiotics have been shown to affect genes that regulate virulence, biofilm formation, motility, and even cell to cell communication; these studies have been well summarized.[67-71] Though the ecological processes driving this induction remain unclear, these results hold greater significance toward optimizing the drug discovery process, namely "squeezing the sponge dry" to access a greater number of secondary metabolites from existing microbial strain libraries.

The observation that a bacterium has the capacity to recognize competitors or communicate at the phylum level and upregulate genes that encode for secondary metabolite biosynthesis supports the hypothesis that microbial co-cultivation may be used to discover antibiotics that selectively target particular microbial taxa. By elucidating the intricacies of environmental cues that trigger antibiotic production, it will be possible to re-mine previously studied microbial strain libraries and increase the discovery rate of antibiotic leads by applying the identified inducing mechanism to strains previously discarded as not producing antibiotics. This philosophy need not be confined to bacteria, as antibiotic production is triggered through cross-kingdom microbial interactions as well. Given that the evolution of drug-resistant pathogens has

forged far ahead of the discovery of new antibiotic scaffolds, this would stand as a needed improvement to the traditional drug discovery paradigm.

However, the current set of experiments was simply a pilot study to assess the specificity of antibiotic production in bacteria using ecologically compatible mimics of known bacterial pathogens. As reported, this experimental model is not suitable for mining large strain libraries for antibiotics. Cultivating tens or even thousands of bacteria in numerous combinations on a milliliter scale is far too labor intensive and extraordinarily impractical. To truly explore the chemical space of existing strain libraries, these co-cultivation experiments must occur in high-throughput, should be coupled to sensitive biological and chemical detection methods, and streamlined into an automated data-mining algorithm to identify potential leads. (These future directions are expounded upon in section 3.10)

3.8 Methods

3.8.1 General experimental procedures

Resistomycin standard was purchased from BioViotica Naturstoffe GmbH (Liestal, Switzerland). All environmental strains (actinomycete and other) were previously isolated from aquatic-derived environmental samples from various locations. The remaining strains were purchased from the ATCC. Unless explicitly stated otherwise, all cultures were fermented at 20 ^{0}C in 60 mL of A1, freshwater derived strains were cultured in freshwater-derived A1 and marine derived stains were cultured in A1 (30 g L^{-1} Instant Ocean ® Purchased from Pet Co. Animal Supplies, Inc.).

3.8.2 Identification of strain B033

Strain B033 was isolated from sediment collected via PONAR from a depth of 57 m from Lake Michigan (43°13'27.63"N, 87°34'10.62"W). Strain B033 (GenBank KM589856) shared 99% 16S rRNA gene sequence identity with the most closely related type strain *Streptomyces griseoflavus* (GenBank NR_042291).

3.8.3 Co-cultivation of actinomycetes with four-strain Proteobacteria panel

All Proteobacteria strains were stored at 10^{10} CFU mL^{-1} (as calculated using the spread plate method) and grown in 60 mL of nutrient rich A1 broth in 250 mL Erlenmeyer flasks (10 g starch, 4 g yeast extract, 2 g peptone and one of the following: 50% Lake Michigan water passed through a 0.2 μm filter, 50% DI water, or 30 g L^{-1} Instant Ocean ® [Purchased from Pet Co. Animal Supplies, Inc.] in DI water).

The flasks were placed on an Innova 5000 gyratory shaker (220 rpm) at 20 °C, and the co-cultures were aerated for 4 to 14 days, monitoring each culture individually for advancement to log phase growth. Each aquatic-derived actinomycete strain (previously grown to late log phase in A1 media and stored as a 500 μL aliquot of 18% glycerol in cryovials) was cultivated in four flasks with one of four Proteobacteria [*B. vietnamiensis* (ATCC BAA-248), *B. neotomae* (ATCC 23459), *Y. pestis* (A1122), and *X. axonopodis* (ATCC 8718)]. A fifth flask in each set was an actinomycete monoculture. The inoculation volume was determined by first inoculating each Proteobacteria strain from cryopreserved stocks into A1 broth and observing growth over a 14-day period. The inoculation volume of Proteobacteria added to each co-cultivation experiment was optimized to allow for adequate actinomycete growth (because most actinomycete strains are relatively slow growing, this was to avoid the rapid accumulation of Proteobacteria in the

flask). Generally speaking, actinomycetes were separated into three categories: slow, medium, and fast growers. The determined inoculation volume of Proteobacteria (ranging from 50 µL to 200 µL of cryopreserved stock stored at 10^{10} CFU mL^{-1}) was transferred into each corresponding actinomycete culture on the first day of the experiment.

Each co-culture was individually monitored for actinomycete growth. Cultures exhibited a wide range of growth rates, requiring as little as 4 days or as many as 14 days to reach late log phase. After a minimum of four days of growth, each culture was extracted at late log phase by adding two grams of XAD-16N polymeric resin (encased in customized, homemade fine mesh bags). The flasks and resin were shaken for 5 h at 220 rpm. The resin bags were then removed, rinsed with DI water, extracted with acetone, and the eluent was concentrated *in vacuo* to obtain secreted secondary metabolite extracts. The extracts were resuspended in MeOH, centrifuged to remove excess salts, and the supernatant was transferred to pre-weighed vials. The average weight of each extract (per 60 mL culture) was 30 mg. Each extract was weighed and dissolved in DMSO at a final concentration of 50 mg mL^{-1}. Aliquots of the resulting extracts were then transferred to deep 96-well plates and stored at 4 °C.

3.8.4 In vitro antimicrobial growth inhibition assays

To assess whether antibiotics were produced during co-cultivation, an *in vitro* antimicrobial assay was used to assess the ability of co-culture extracts to inhibit the growth of the four Proteobacteria strains (*B. vietnamiensis* ATCC BAA-248, *B. neotomae* ATCC 23459, *Y. pestis* A1122, and *X. axonopodis* ATCC 8718), and a select panel of strains (*P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, and *M. tuberculosis* H37Rv). Initially, the extracts were tested for their ability to inhibit the growth of each pathogen at a single dose (100

μg/mL). A 50 μL aliquot of bacterial stock culture was inoculated in 10 mL of tryptic soy broth (TSB) and incubated (37 °C) overnight on a shaker. All strains grew under these conditions except for *X. axonopodis*, which was grown at 30 °C. The optical density (OD) of the test bacterial strain was then measured at 625 nm, and the concentration of cells was adjusted to approximately 5 x 10^5 CFU per 100 μL in each well, as verified by the spread plate method. Test extracts were then added to each well of a flat bottom 96-well plate for a final volume of 100 μL per well, and incubated overnight. Extracts exhibiting greater than 95% inhibition (as determined by the turbidity of a 200 μL culture at a single dose by measuring absorbance at 625 nm) after initial single dose screening were subjected to a second round of dose response assays, which allowed for the generation of MIC values.

3.8.5 Chromatographic analysis of co-culture extracts

Chromatographic analysis was performed on an Agilent 1100 HPLC employing DAD detection. High resolution mass spectra were obtained on a Shimadzu ESI-IT-TOF spectrometer at the UIC Metabolomics, Mass Spectrometry and Proteomics Facility. Extracts that displayed antimicrobial activity at MIC values <32 μg/mL were analyzed using reversed-phase C18 (RP-C18) HPLC, equipped with a photodiode array detector (PDA). The first 10 min. of the run was a gradient from 10% aqueous MeOH to 100% MeOH, followed by a 100% MeOH wash for 10 min. A flow rate of 0.5 mL min^{-1} was employed. The column utilized during this experiment was a Phenomenex Luna C18 (2), 100 x 4.6 mm, 5 μm pore size.

3.8.6 B033 Co-incubation with Burkholderia vietnamiensis culture supernatant and cell mass

B033 was co-incubated with the culture supernatant from *B. vietnamiensis* cultures. *B. vietnamiensis* was grown in 60 mL of freshwater-based A1 medium for 2, 5, and 10 days. After

this period, the culture media was centrifuged and filtered (0.2 μm filter) to yield the culture supernatant and a cell pellet. Supernatant (30 mL) and autoclaved cell pellet were added on the first day of a growing B033 culture (also 30 mL; total volume equals 60 mL) in freshwater-based A1 medium. The actinomycete was grown to log phase, extracted as previously described, and the resulting extracts were analyzed using the aforementioned HPLC conditions (Figure 8).

3.8.7 Selection of expanded panel of environmental Proteobacteria

Additional environmental strains were purified from existing bacterial diversity agar plates from sediment collected in Lake Huron, Lake Michigan, Lake Tampier, Iceland, the Florida Keys, and the Atlantic Ocean off of the coast of Rockport, Plymouth, and Winthrop, Massachusetts. Isolates were grown in 60 mL of freshwater-based A1 media and cryopreserved at 10^{10} CFU mL^{-1} in 18% glycerol until use in the study. Genomic DNA was isolated, and the 16S ribosomal RNA gene was amplified using primers FC27 and RC1492. The resulting sequencing product from each isolate has been uploaded to GenBank (Table A4)

3.8.8 B033 Co-cultivated with an expanded panel of Proteobacteria isolates.

Actinomycete strain B033 (cryopreserved at 10^{10} CFU mL^{-1}) was cultivated in 60 mL of freshwater-based A1 media in a 250 mL Erlenmeyer flask. Between 50 and 100 μL (based on previous estimates) of Proteobacteria stock were added to the actinomycete culture on the first day of the experiment. The flasks were placed on an Innova 5000 gyratory shaker (220 rpm) at 20 °C, and the co-cultures were aerated for 14 days. These experiments were performed in duplicate.

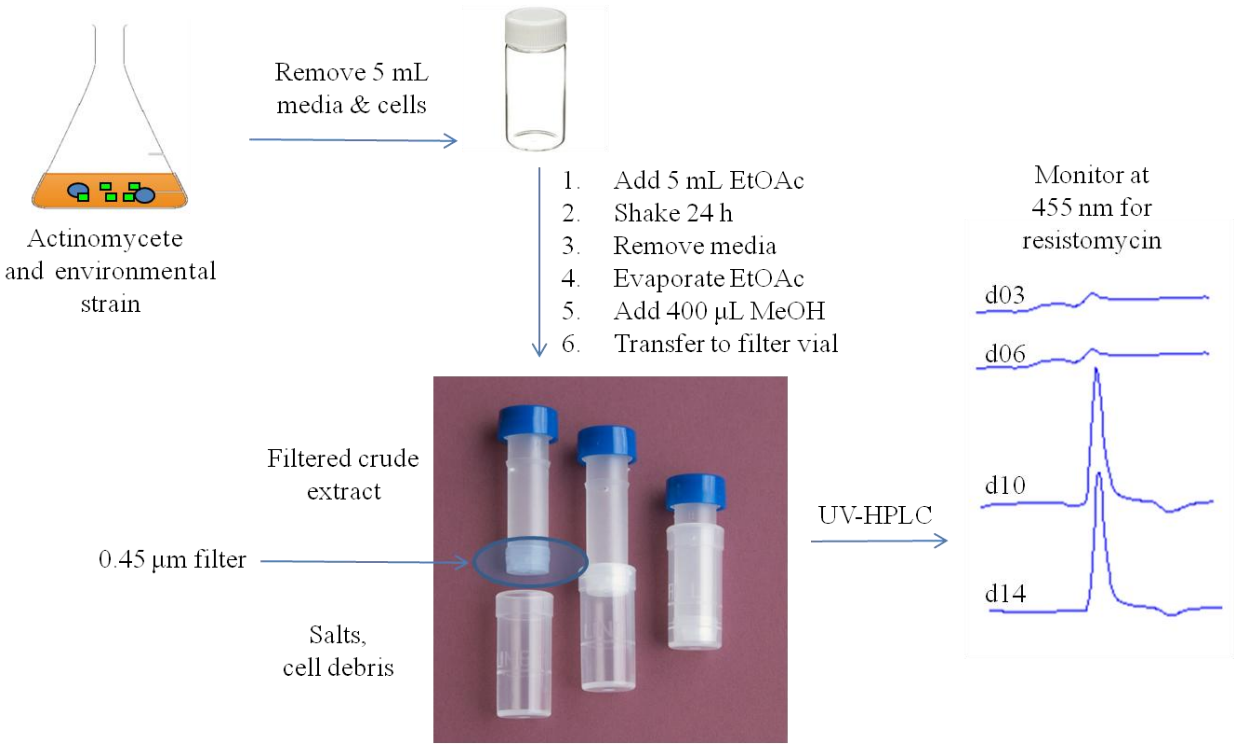


Figure 7 Chemical profiling of co-culture extracts

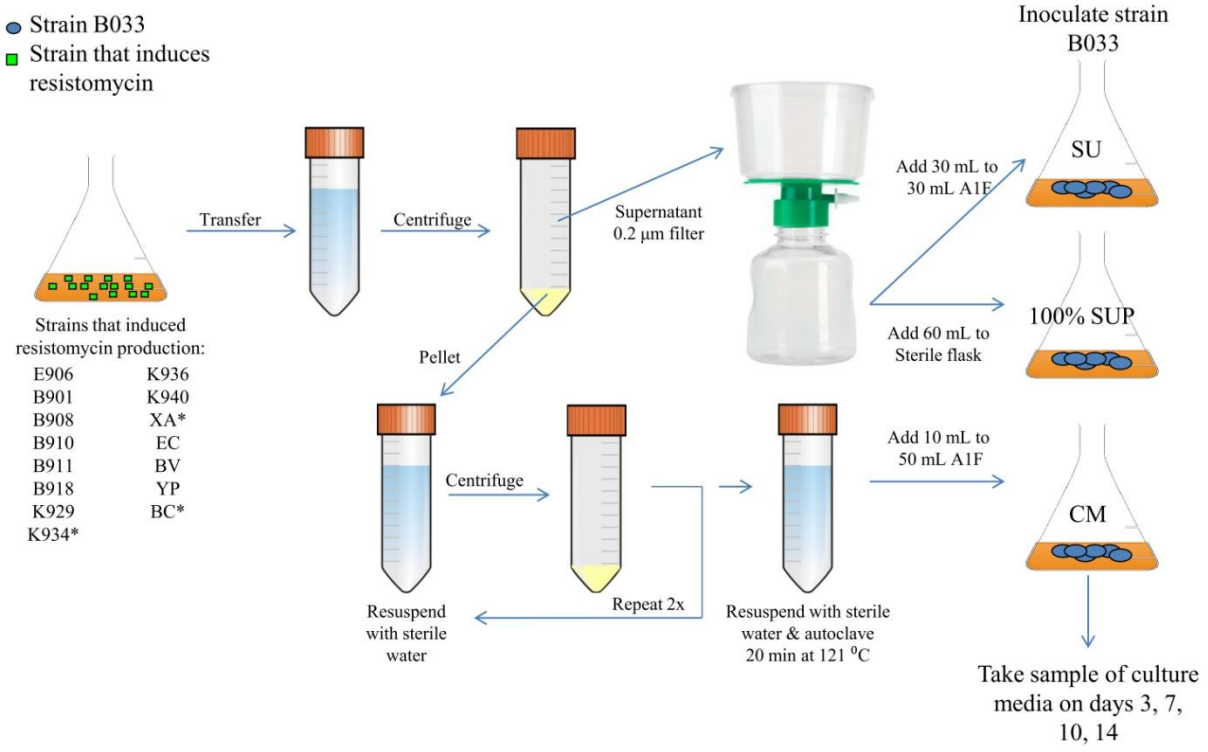


Figure 8 Preparation of cell mass and supernatant for co-incubation experiments.

3.8.9 Monitoring for resistomycin production in B033-environmental isolate co-culture experiments

Aliquots (5 mL) of growing co-cultures were removed and replaced with 5 mL of autoclaved freshwater-based A1 medium to maintain a constant culture volume. Each 5 mL aliquot of media was mixed on a gyratory shaker with an equal volume of EtOAc. After 12 h of shaking, the EtOAc layer was removed and concentrated *in vacuo*. The extract was then dissolved in MeOH, filtered, and analyzed using HPLC as previously described (Figure 7).

3.8.10 Growth inhibition assays against strains that induce resistomycin production

Resistomycin was tested for antimicrobial activity against the environmental isolates that induced its expression during co-cultivation. A 50 μL aliquot of bacterial stock culture was inoculated in 60 mL of freshwater-based A1 media and aerated overnight on a shaker (at 20 °C). The optical density (OD) of the test bacterial strain was then measured at 625 nm, and the concentration of cells was adjusted to approximately 5 x 10^5 CFU per 100 μL. The culture was then aliquoted into each well of a 96-well flat bottom plate. Resistomycin (BioViotica, Switzerland) was then added to test wells, mixed, and the plate was incubated overnight. Strains that were inhibited by resistomycin, greater than 95% inhibition (at a test concentration of 50 μg/mL) after initial single dose screening were subjected to a second round of dose response assays, which allowed for the generation of MIC values.

3.9 Conclusions

Our study suggests that Proteobacteria induce the production of resistomycin in a *Streptomyces* sp. at significantly higher rates than bacterial strains from Firmicutes and Actinobacteria, suggesting that the regulation of secondary metabolism in bacteria is in some cases dependent on

the species present in the environment. These results support the notion that small molecule production in bacteria is not always promiscuous. The results also suggest that future drug lead discovery efforts should take into consideration environmental factors that regulate secondary metabolite gene expression, and use these factors to selectively mine for antibiotics in high throughput on a phylum-specific level.

3.10 Future directions

3.10.1 Imaging mass spectrometry as a Rosetta stone for bacterial communication

The laboratory of Dr. Pieter C. Dorrestein at the University of California, San Diego, has studied competition on solid agar as a Rosetta stone to decipher the chemical communication utilized between bacteria and other microbes in more complex systems via imaging mass spectrometry.[72-73] This complementary method could be applied to our system in order to determine what chemical cues are exchanged that initiate resistomycin production, provided resistomycin is produced by B033 on solid agar.

3.10.2 One percent induction rate

In our study we observed that one actinomycete strain that was not previously shown to have antimicrobial activity, was able to produce an antibiotic during co-cultivation. Of the ~ 100 strains co-cultivated, this translates to a one percent induction rate. If this observation is applied to a large strain library of 100,000 microbes, similar to those acquired by pharmaceutical companies, this could result in the induction of 1,000 new active strains that were not seen previously (Figure 9). This could speed up the discovery of antimicrobial drug lead discovery significantly, in order to combat continually emerging resistance to clinical antibiotic therapies.

In order to screen large microbial libraries, like those created by pharmaceutical companies, the current method must be miniaturized. This process is currently being optimized in the laboratory of Dr. Tim Bugni at the University of Wisconsin Madison (unpublished work, personal communication and poster presented at 2013 annual meeting of the American Society of Pharmacognosy, St. Louis, MO). Their use of mass spectrometry as a detection method allows for rapid screening of co-cultivation experiments and prioritization of induced metabolites using principal component analysis (PCA). The use of statistical software has the potential to automate this process and further increase the high-throughput nature of our study. Finally, combining miniaturization and mass spectrometry, changes in the metabolic fingerprint could be detected even if the induced metabolites are not biologically active.

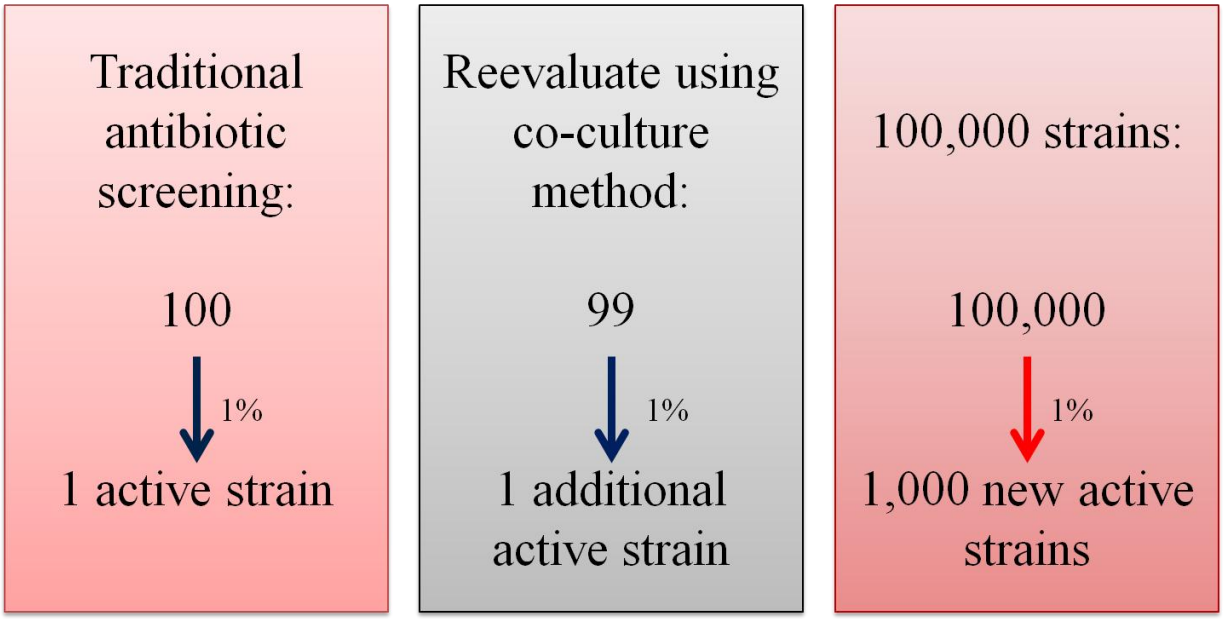


Figure 9 The power of one percent induction of secondary metabolism.
The potential of our co-cultivation method to identify 1,000 strains that produce antimicrobial compounds when co-cultivated with Proteobacteria.

3.11 References

1. Bibb, M. J. Regulation of secondary metabolism in streptomycetes. *Curr. Opin. Microbiol.* **2005**, *8*, 208-215.
2. Udwary, D. W.; Zeigler, L.; Asolkar, R. N.; Singan, V.; Lapidus, A.; Fenical, W.; Jensen, P. R.; Moore, B. S. Genome sequencing reveals complex secondary metabolome in the marine actinomycete Salinispora tropica. *Proc. Natl. Acad. Sci.* **2007**, *104*, 10376-10381.
3. Walsh, C. T.; Fischbach, M. A. Natural Products Version 2.0: Connecting Genes to Molecules. *J. Am. Chem. Soc.* **2010**, *132*, 2469-2493.
4. Chiang, Y. M.; Chang, S. L.; Oakley, B. R.; Wang, C. C. Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms. *Curr. Opin. Chem. Biol.* **2011**, *15*, 137-143.
5. Wasil, Z.; Pahirulzaman, K. A. K.; Buttsa, C.; Simpson, T. J.; Lazarus, C. M.; Cox, R. J. One pathway, many compounds: heterologous expression of a fungal biosynthetic pathway reveals its intrinsic potential for diversity. *Chem. Sci.* **2013**, *4*, 3845-3856.
6. Craney, A.; Ahmed, S.; Nodwell, J. Toward a new science of secondary metabolism. *J. Antibiot.* **2013**, *66*, 387-400.
7. Gomez-Escribano, J. P.; Bibb, M. J. Heterologous expression of natural product biosynthetic gene clusters in *Streptomyces coelicolor*: from genome mining to manipulation of biosynthetic pathways. *J. Ind. Microbiol. Biotechnol.* **2014**, *41*, 425-431.
8. Pfeifer, B. A.; Khosla, C. Biosynthesis of polyketides in heterologous hosts. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 106-118.
9. Challis, G. L. Mining microbial genomes for new natural products and biosynthetic pathways. *Microbiology* **2008**, *154*, 1555-1569.

10. Kuzuyama, T.; Noel, J. P.; Richard, S. B. Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products. *Nature* **2005**, *435*, 983-987.

11. Guo, J.; Huang, F.; Huang, C.; Duan, X.; Jian, X.; Leeper, F.; Deng, Z.; Leadlay, P. F.; Sun, Y. Specificity and Promiscuity at the Branch Point in Gentamicin Biosynthesis. *Chem. Biol.* **2014**, *21*, 608-618.

12. Verdel-Aranda, K.; López-Cortina, S. T.; Hodgson, D. A.; Barona-Gómez, F. Molecular annotation of ketol-acid reductoisomerases from *Streptomyces* reveals a novel amino acid biosynthesis interlock mediated by enzyme promiscuity. *Microb. Biotech.* **2014**, *8*, 239–252.

13. Grond, S. P., I.; Zeeck, A. Novel alpha-L-rhamnopyranosides from a single strain of *Streptomyces* by supplement-induced biosynthetic steps. *Eur. J. Org. Chem.* **2002**, *2002*, 3237-3242.

14. Sun, H.; Liu, Z.; Zhao, H.; Ang, E. L. Recent advances in combinatorial biosynthesis for drug discovery. *Drug Des. Devel. Ther.* **2015**, *9*, 823-833.

15. Koryakina, I.; McArthur, J.; Draelos, M.; Williams, G. Promiscuity of a modular polyketide synthase towards natural and non-natural extender units. *Org Biomol Chem* **2013**, *11*, 4449-4458.

16. Fischer, R. Promiscuity breeds diversity: the role of polyketide synthase in natural product biosynthesis. *Chem. Biol.* **2014**, *21*, 701-702.

17. Xie, Y.; Cai, Q.; Ren, H.; Wang, L.; Xu, H.; Hong, B.; Wu, L.; Chen, R. NRPS substrate promiscuity leads to more potent antitubercular sansanmycin analogues. *J. Nat. Prod.* **2014**, *7*, 1744-1748.

18. Cichewicz, R. H.; Henrikson, J. C.; Wang, X.; Branscum, K. M., Strategies for accessing microbial secondary metabolites from silent biosynthetic pathways. In *Manual of Industrial Microbiology and Biotechnology (3rd Edition)*, Baltz, R. H.; Demain, A. L.; Davies, J. E., Eds. ASM Press: Washington, D.C., 2010; pp 78-95.

19. Scherlach, K.; Hertweck, C. Triggering cryptic natural product biosynthesis in microorganisms. *Org. Biomol. Chem.* **2009**, *7*, 1753-1760.

20. Bode, H. B.; Bethe, B.; Hofs, R.; Zeeck, A. Big effects from small changes: possible ways to explore nature's chemical diversity. *ChemBioChem* **2002**, *3*, 619-627.

21. Sagot, B.; Gaysinski, M.; Mehiri, M.; Guigonis, J. M.; Rudulier, D. L.; Alloing, G. Osmotically induced synthesis of the dipeptide N-acetylglutaminylglutamine amide is mediated by a new pathway conserved among bacteria. *Proc. Natl. Acad. Sci.* **2010**, *107*, 12652-12657.

22. Chen, G.; Wang, G.; Li, X.; Waters, B.; Davies, J. Enhanced production of microbial metabolites in the presence of dimethyl sulfoxide. *J. Antibiot.* **2000**, 1145-1153.

23. Masuma, R.; Tanaka, Y.; Tanaka, H.; Omura, S. Production of nanaomycin and other antibiotics by phosphate-depressed fermentation using phosphate-trapping agents. *J. Antibiot.* **1986**, *39*, 1557-1564.

24. Pettit, R. K. Small-molecule elicitation of microbial secondary metabolites. *Microb. Biotech.* **2011**, *4*, 471-478.

25. Rigali, S.; Titgemeyer, F.; Barends, S.; Mulder, S.; Thomae, A. W.; Hopwood, D. A.; Wezel, G. P. v. Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. *EMBO Rep* **2008**, *9*, 670-675.

26. Doull, J. L.; Ayer, S. W.; Singh, A. K.; Thibault, P. Production of a novel polyketide antibiotic, jadomycin B, by *Streptomyces venezuelae* following heat shock. *J. Antibiot.* **1993**, *46*, 869-871.

27. Doull, J. L.; Singh, A. K.; Hoarel, M.; Ayer, S. W. Conditions for the production of jadomycin B by *Streptomyces venezuelae* ISP5230: effects of heat shock, ethanol treatment and phage infection *J. Ind. Microbiol. Biotechnol.* **1994**, *13*, 120-125.

28. Seyedsayamdost, M. R. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. *Proc. Natl. Acad. Sci.* **2014**, *111*, 7266-7271.

29. Craney, A.; Ozimok, C.; Pimentel-Elardo, S.; Capretta, A.; Nodwell, J. Chemical perturbation of secondary metabolism demonstrates important links to primary metabolism. *Chem. Biol.* **2012**, *19*, 1020-1027.

30. Eijsink, V. G.; L, A.; Diep, D. B.; Havarstein, L. S.; Holo, H.; Nes, I. F. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Anton. Leeuw. Int.* **2002**, 639-654.

31. LaSarre, B.; Federle, M. J. Exploiting quorum sensing to confuse bacterial pathogens. *Microb Mol Biol Rev* **2013**, *77*, 73-111.

32. Bassler, B. L.; Greenberg, E. P.; Stevens, A. M. Cross-species induction of luminescence in the quorum-sensing bacterium Vibrio harveyi. *J. Bacteriol.* **1997**, *179*, 4043-4045.

33. Lopes, S. C. P.; Blanco, Y. C.; Justo, G. Z.; Nogueira, P. A.; Rodrigues, F. L. S.; Goelnitz, U.; Wunderlich, G.; Facchini, G.; Brocchi, M.; Duran, N.; Costa, F. T. M. Violacein extracted from *Chromobacterium violaceum* inhibits plasmodium growth *in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* **2998**, *53*, 2149-2152.

34. Hoshino, T. Violacein and related tryptophan metabolites produced by *Chromobacterium violaceum*: biosynthetic mechanism and pathway for construction of violacein core. *Appl. Microbiol. Biotechnol.* **2011**, *91*, 1463-1475.

35. McClean, K. H.; Winson, M. K.; Fish, L.; Taylor, A.; Chhabra, S. R.; Camara, M.; Daykin, M.; Lamb, J. H.; Swift, S.; Bycroft, B. W.; Stewart, G. S. A. B.; Williams, P. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiol.* **1997**, *143*, 3703-3711.

36. Thomson, N. R.; Crow, M. A.; McGowan, S. J.; Cox, A.; Salmond, G. P. C. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* sp. is under quorum sensing control. *Mol. Microbiol.* **2000**, *36*, 539-556.

37. Bertrand, S.; Bohni, N.; Schnee, S.; Schumpp, O.; Gindro, K.; Wolfender, J. L. Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery. *Biotechnol. Adv.* **2014**, *32*, 1180-1204.

38. Marmann, A.; Aly, A. H.; Lin, W. H.; Wang, B. G.; Proksch, P. Co-cultivation-a powerful emerging tool for enhancing the chemical diversity of microorganisms. *Mar. Drugs* **2014**, *12*, 1043-1065.

39. Pettit, R. K. Mixed fermentation for natural product drug discovery. *Appl. Microbiol. Biotechnol.* **2009**, *83*, 19-25.

40. Marmann, A.; Aly, A.; Lin, W.; Wang, B.; Proksch, P. Co-Cultivation—A Powerful Emerging Tool for Enhancing the Chemical Diversity of Microorganisms. *Marine Drugs* **2014**, *12*, 1043-1065.

41. Park, H. B.; Kim, Y. J.; Park, J. S.; Yang, H. O.; Lee, K. R.; Kwon, H. C. Glionitrin B, a cancer invasion inhibitory diketopiperazine produced by microbial coculture. *J. Nat. Prod.* **2011**, *74*, 2309-2312.

42. Park, H. B.; Kwon, H. C.; Lee, C. H.; Yang, H. O. Glionitrin A, an antibiotic-antitumor metabolite derived from competitive interaction between abandoned mine microbes. *J. Nat. Prod.* **2009**, *72*, 248-252.

43. Cueto, M.; Jensen, P. R.; Kauffman, C.; Fenical, W.; Lobkovsky, E.; Clardy, J. Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. *J. Nat. Prod.* **2001**, *64*, 1444-1446.

44. Zhu, F.; Lin, Y. Marinamide, a novel alkaloid and its methyl ester produced by the application of mixed fermentation technique to two mangrove endophytic fungi from the South China Sea. *Chin. Sci. Bull.* **2006**, *51*, 1426-1430.

45. Zuck, K.; Shipley, S.; Newman, D. J. Induced production of N-forml alkaloids from *Aspergillus fumigatus* by co-culture with *Streptomyces peucetius*. *J. Nat. Prod.* **2011**, *74*, 1653-1657.

46. Mearns-Spragg, A.; Bregu, M.; Boyd, K. G.; Burgess, J. G. Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Lett. Appl. Microbiol.* **1998**, *27*, 142-146.

47. Burgess, J. G.; Jordan, E. M.; Bregu, M.; Mearns-Spragg, A.; Boyd, K. G. Microbial antagonism: a neglected avenue of natural products research. *J. Biotechnol.* **1999**, *70*, 27-32.

48. Dusane, D. H.; Matkar, P.; Venugopalan, B. P.; Kumar, A. R.; Zinjarde, S. S. Cross-species induction of antimicrobial compounds, biosurfactants and quorum-sensing inhibitors in tropical marine epibiotic bacteria by pathogens and biofouling microorganisms. *Curr. Microbiol.* **2011**, *62*, 974-980.

49. Onaka, H.; Mori, Y.; Igarashi, Y.; Furumai, T. Mycolic acid-containing bacteria induce natural-product biosynthesis in *Streptomyces* species. *Appl. Environ. Microbiol.* **2011**, *77*, 400-406.

50. Igarashi, Y.; Kim, Y.; In, Y.; Ishida, T.; Kan, Y.; Fujita, T.; Iwashita, T.; Tabata, H.; Hiroyasu Onaka; Furumai, T. Alchivemycin A, a bioactive polycyclic polyketide with an unprecedented skeleton from *Streptomyces* sp. *Org. Lett.* **2010**, *12*, 3402-3405.

51. Hoshino, S.; Zhang, L.; Awakawa, T.; Wakimoto, T.; Onaka, H.; Abe, I. Arcyriaflavin E, a new cytotoxic indolocarbazole alkaloid isolated by combined-culture of mycolic acid-containing bacteria and *Streptomyces cinnamoneus* NBRC 13823. *J. Antibiot.* **2014**, 1-3.

52. Trischman, J. A.; Oeffner, R. E.; Luna, M. G. d.; Kazaoka, M. Competitive induction and enhancement of indole and a diketopiperazine in marine bacteria. *Mar. Biotechnol.* **2004**, *6*, 215-220.

53. Slattery, M.; Rajbhandari, I.; Wesson, K. Competition-mediated antibiotic induction in the marine bacterium *Streptomyces tenjimariensis*. *Microb. Ecol.* **2001**, *41*, 90-96.

54. Vijayabharathi, R.; Bruheim, P.; Andreassen, T.; Raja, D.; Devi, P.; Sathyabama, S.; Priyadarisini, V. Assessment of resistomycin, as an anticancer compound isolated and characterized from *Streptomyces aurantiacus* AAA5. *J. Microbiol.* **2011**, *49*, 920-926.

55. Adinarayana, G.; Venkateshan, M.; Bapiraju, V.; Sujatha, P.; Premkumar, J.; Ellaiah, P.; Zeeck, A. Cytotoxic compounds from the marine actinobacterium *Streptomyces corchorusii* AUBN1/71. *J. Bioorg. Chem.* **2006**, *32*, 295-300.

56. Arora, S. Molecular structure of heliomycin, an inhibitor of RNA synthesis. *J. Antibiot.* **1985**, *38*, 113-115.

57. Vijayabharathi, R.; Sathyadevi, P.; Krishnamoorthy, P.; Senthilraja, D.; Brunthadevi, P.; Sathyabama, S.; Priyadarisini, V. Interaction studies of resistomycin from *Streptomyces aurantiacus* AAA5 with calf thymus DNA and bovine serum albumin. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* **2012**, *89*, 294-300.

58. Haupt, I.; Wahnert, U.; Pitra, C.; Lober, G.; Luck, G.; Eckardt, K. Effects of the antibiotic resistomycin on the synthesis of macromolecules. *Z. Allg. Mikrobiol.* **1975**, *15*, 411-421.

59. Kock, I.; Maskey, R. P.; Biabani, M. A. F.; Helmke, E.; Laatsch, H. 1-Hydroxy-1-norresistomycin and resistoflavin methyl ether: new antibiotics from marine-derived Streptomycetes. *J. Antibiot.* **2005**, *58*, 530-534.

60. Jakobi, K.; Hertweck, C. A gene cluster encoding resistomycin biosynthesis in *Streptomyces resistomycificus*; exploring polyketide cyclization beyond linear and angucyclic patterns. *J. Am. Chem. Soc.* **2004**, *126*, 2298-2299.

61. Hofle, G.; Wolf, H. Isolierung, I3C-NMR-spektren und biogenese von resistomycin und resistoflavin aus *Streptornyces griseoflavus* B 71 (Actinomycetales). *Liebigs. Ann. Chem.* **1983**, *1983*, 835-843.

62. Shiono, Y.; Shiono, N.; Seo, S.; Oka, S.; Yamazaki, Y. Effects of polyphenolic anthrone derivatives, resistomycin and hypericin, on apoptosis in human megakaryoblastic leukemia CMK-7 cell line. *Z. Naturforsch.* **2002**, *57c*, 923-929.

63. Vijayabharathi, R.; Brunthadevi, P.; Sathyabama, S.; Bruheim, P.; Priyadarisini, V. B. Optimization of resistomycin production purified from *Streptomyces aurantiacus* AAA5 using response surface methodology. *J. Biochem. Tech.* **2012**, *3*, 402-408.

64. Saitou, N.; Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406-425.

65. Tamura, K.; M, N.; Kumar, S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci.* **2004**, *101*, 11030-11035.

66. Felsenstein, J. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution* **1985**, *39*, 783-791.

67. Romero, D.; Traxler, M. F.; López, D.; Kolter, R. Antibiotics as signal molecules. *Chem. Rev.* **2011**, *111*, 5492-5505.

68. Yang, Y. L.; Xu, Y.; Straight, P.; Dorrestein, P. C. Translating metabolic exchange with imaging mass spectrometry. *Nat. Chem. Biol.* **2009**, *5*, 885-887.

69. Linares, J. F.; Gustafsson, I.; Baquero, F.; Martinez, J. L. Antibiotics as intermicrobial signaling agents instead of weapons. *Proc. Natl. Acad. Sci.* **2006**, *103*, 19484-19489.

70. Andersson, D. I.; Hughes, D. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* **2014**, *12*, 465-478.

71. Fang, J.; Dorrestein, P. C. Emerging mass spectrometry techniques for the direct analysis of microbial colonies. *Curr. Opin. Microbiol.* **2014**, *19*, 120-129.

72. Watrous, J. D.; Phelan, V. V.; Hsu, C. C.; Moree, W. J.; Duggan, B. M.; Alexandrov, T.; Dorrestein, P. C. Microbial metabolic exchange in 3D. *ISME J* **2013**, *7*, 770-780.

73. Watrous, J. D.; Dorrestein, P. C. Imaging mass spectrometry in microbiology. *Nat. Rev. Microbiol.* **2011**, *9*, 683-694.

Chapter 4. Conclusions

My doctoral thesis detailed work on the discovery of potential drug-leads and the induction of secondary metabolite biosynthetic gene clusters in bacteria. A broad overview of the field and the utility of aquatic-derived actinomycetes for drug discovery were presented as a foundation for my studies. Next, the discovery of a novel macrolide with potential chemopreventive activity similar to the FDA approved macrolide antibiotics erythromycin and telithromycin was presented. This study demonstrates that the use of these antibiotics might be working to extend our lives beyond warding off bacterial infection. However, the discovery of novel molecules requires wading through thousands of known molecules. The identification of novel secondary metabolites is only possible through collaborative efforts between natural products drug discovery laboratories and laboratories that have developed biological assays.

In the second study presented, we aimed to induce the full potential of actinomycetes to produce secondary metabolites that are rarely seen under standard laboratory culture conditions. In an effort to induce actinomycete secondary metabolite biosynthetic gene clusters, we co-cultivated aquatic derived Proteobacteria with actinomycetes not known to display antimicrobial activity. Through the experiments presented we demonstrated the potential of this co-cultivation to induce small molecule production in one percent of the strains surveyed. I project that, if miniaturized, the combination of Proteobacteria with a large microbe library (that would have otherwise been discarded as not producing antibiotics) may yield as many as one thousand new lead strains.

As time passes and technology develops, several trends rise in popularity and fade as quickly as they appeared. For example, computer aided drug design, meant to revolutionize drug-discovery, has yet to provide the originally proposed return on investment. Similarly, combinatorial chemistry has not yielded the expected returns. It is apparent that looking to nature for structural

novelty is relatively more sustainable, as other fields are continually seeking natural products as a source for structural novelty. Synthetic chemists continue racing each other to synthesize newly published natural products. High-throughput screening has also experienced an increased demand for natural product hallmarks such as chiral centers and an increasing number of heteroatoms.[1] Complementary to drug-discovery, the characterization of naturally derived cytotoxic compounds has illuminated areas of cell biology that were previously poorly understood, expanding our knowledge about the mode of action of natural products in living systems. As such, I have two speculations regarding the future of the field of natural products drug lead discovery.

The first speculation is that we will see an increase in the number of laboratories studying the regulation of secondary metabolite production. While there have been several studies on the induction of the secondary metabolism in model organisms, there have been no "translational studies." That is, applying the lessons learned from these studies to strains isolated from the environment in an attempt to induce the production of biosynthetic gene clusters not seen during cultivation under standard laboratory culture conditions. The application of the lessons learned in the Seyedsayamdost et al. (antibiotic secondary metabolites can induce biosynthetic gene clusters),[2] and Slattery et al.[3] (culturing the actinomycete first and adding a competitor a few days later increases the yield of secondary metabolites) studies to strains isolated from the environment in a drug-discovery setting, could yield a vast number of new active strains.

Secondly, it is my speculation that as we continue to advance genomic sequencing and enzyme purification technologies, entirely cell free systems designed to produce natural products without heterologous expression will be available for order in a "fee-for-service" format. Further, as the research of Hopwood et al. suggested in 1985,[4] we will see the genetic engineering of the

constituent modules of these biosynthetic gene clusters in a “plug-and-play” fashion, where units are swapped with ease in an entirely cell free *in vitro* system. While development is undoubtedly occurring to these ends, I expect to see the expansion and commercialization of these techniques.

References

1. Newman, D. J.; Cragg, G. M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **2012**, *75*, 311-335.
2. Seyedsayamdost, M. R. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. *Proc. Natl. Acad. Sci.* **2014**, *111*, 7266-7271.
3. Slattery, M.; Rajbhandari, I.; Wesson, K. Competition-mediated antibiotic induction in the marine bacterium *Streptomyces tenjimariensis*. *Microb. Ecol.* **2001**, *41*, 90-96.
4. Hopwood, D. A.; Malpartida, F.; Kieser, H. M.; Ikeda, H.; Duncan, J.; Fujii, I.; Rudd, B. A. M.; Floss, H. G.; Omura, S. Production of 'hybrid' antibitoics by geneic engineering. *Nature* **1985**, *314*, 642-644.

Appendix I "Stop. Collaborate and listen."*

A.1 Introduction

During the course of a natural products study it is common to identify known compounds. Ideally, this will happen prior to any major investment of time or resources. A critical aspect of maintaining an efficient drug-lead discovery process is identifying known secondary metabolites from a lead strain quickly. When bioassay data is returned from a collaborator, fractions containing known bioactive molecules can be de-prioritized in favor of those that are likely to produce novel molecules. This chapter highlights the collaborations that led to the screening of our fraction library for biological activity and the putative identification of the known molecules responsible for the observed bioactivity; this process is known as "dereplication."

* Ice, Vanilla. Ice, Ice Baby *(original track)*. By Earthquake, Freddie Mercury, Brian May, Roger Taylor, John Deacon, and David Bowie. Vanilla Ice. Vanilla Ice, 1990. MP3.

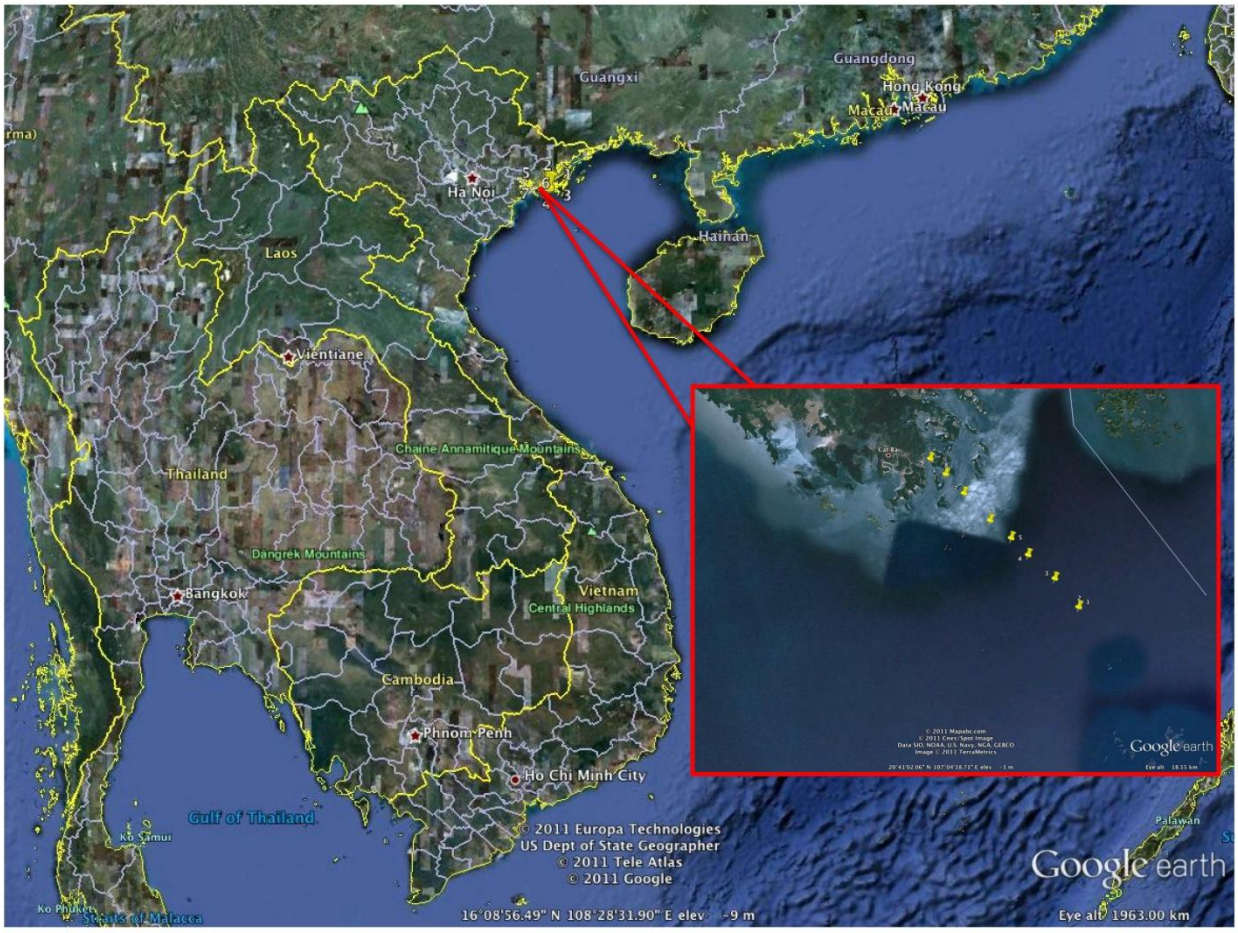


Figure A1 Collection sites in Hạ Long Bay, Cat Ba, Vietnam.
Samples were collected along via PONAR in the transect indicated by yellow pushpins. The depths sampled ranged from 5-22 m.

A.2 ChIPS process identified chloramphenicol[†] from a secondary metabolite fraction from strain G006

Through a collaboration with the Vietnamese Academy of Science and Technology (VAST), sediments were collected via PONAR from Hạ Long Bay along a transect with depths ranging from 5 m to 22 m, as shown in Figure A1. From a sediment sample collected at 22 m, strain G006 was isolated. This strain was identified as *Micromonospora matsumotoense* by comparison of the sequence of the 16S ribosomal rRNA gene sequence, to the sequence of type strains in the NCBI GenBank database. As a part of a program to discover small molecules that inhibit bacterial translation, chloramphenicol was putatively identified from a crude fraction of Strain G006 via the characterization of inhibitors of protein synthesis (ChIPS)[1] process and confirmed by high-resolution mass-spectrometry (HR-MS) and ^{1}H NMR spectroscopy. The ChIPS process is the combination of two biochemical assays designed to identify small molecule inhibitors of protein synthesis and predict the possible mechanism of action based on comparison to known translation inhibitors.

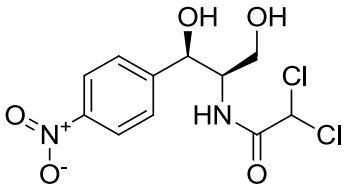
In collaboration with the laboratory of Dr. Alexander Mankin, our fraction library was screened using the ChIPS process. The assay consists of two parts. Resistant mutants of strain SQ110 that were resistant to secondary metabolite fractions, from G006 in particular, were sequenced in order to identify the location of small molecule binding sites, most likely located in the 16S and 23S rRNA gene sequence. SQ110 is an engineered strain of *Escherichia coli* that contains single

[†] This section was reproduced in part from publication: Cédric Orelle, Skylar Carlson, Bindiya Kaushal, Mashal M. Almutairi, Haipeng Liu, Anna Ochabowicz, Selwyn Quan, Van Cuong Pham, Catherine L. Squires, Brian T. Murphy, and Alexander S. Mankin, Tools for characterizing bacterial protein synthesis inhibitors. *Antimicrob. Agents and Chemother*. **2013**, 57, 5994-6004.

copies of all ribosomal RNA and protein genes. This engineered strain allows for rapid selection and identification of ribosomal resistance mutations.

Second, we showed patterns of drug-induced ribosome stalling on mRNA, as monitored by primer extension inhibition, indicating that the mechanism of translation inhibition was similar to that of chloramphenicol. The presence of chloramphenicol (**4**) in fraction 242 was confirmed by HR-MS and ^{1}H NMR analysis (Figure A28 and A29).

The ChIPS process was able to rapidly identify the mode of action of a molecule within a secondary metabolite fraction from strain G006; we subsequently identified the molecule responsible for the observed inhibition as having a mechanism of action similar to chloramphenicol. The sensitivity of the assay was demonstrated by identifying chloramphenicol-like mechanism of action from a crude fraction. These analyses can be performed within a few days and provide a rapid and efficient approach for prioritizing hits in natural product libraries that exhibit protein synthesis inhibitory activity.



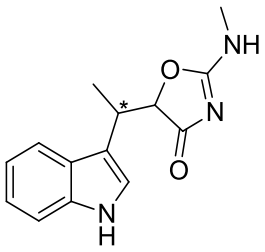
4

A.3 Inhibitor of bacterial translation from strain D085

D085 was isolated from a sediment sample obtained in the summer of 2010 collected from Back Beach, near Rockport, MA by skin diving and was not identified by sequencing the gene that encodes for the 16S rRNA.

In a follow up screening to the ChIPS process, our secondary metabolite fraction library was screened for bacterial translation inhibitors by James Marks, a graduate student in the Mankin laboratory. Secondary metabolite fraction F3 from strain D085 displayed a minimum inhibitory concentration (MIC) of 0.62 μg/mL against the *E. coli* strain SQ110 ΔtolC. This strain of SQ110 contains an additional mutation that has deleted the gene for the multi drug efflux pump TolC. The activity was verified by the same primer extension inhibition experiment in the ChIPS process. Following sequencing, the resistance mutations were localized to the trpRS and trpR genes, suggesting that the mechanism of action was likely targeting the tryptophan tRNA synthetase, similar to the antibiotic indolmycin (**5**).

The antimicrobial activity of the fraction led to the isolation of a bioactive molecule with the molecular weight of 257. This molecule was putatively identified as a mixture of two indolmycin stereoisomers based on analysis of the HR-MS data and the ^{1}H NMR spectrum (Figure A2 and Figure A29) (no further data that would allow stereochemical characterization were collected).



5

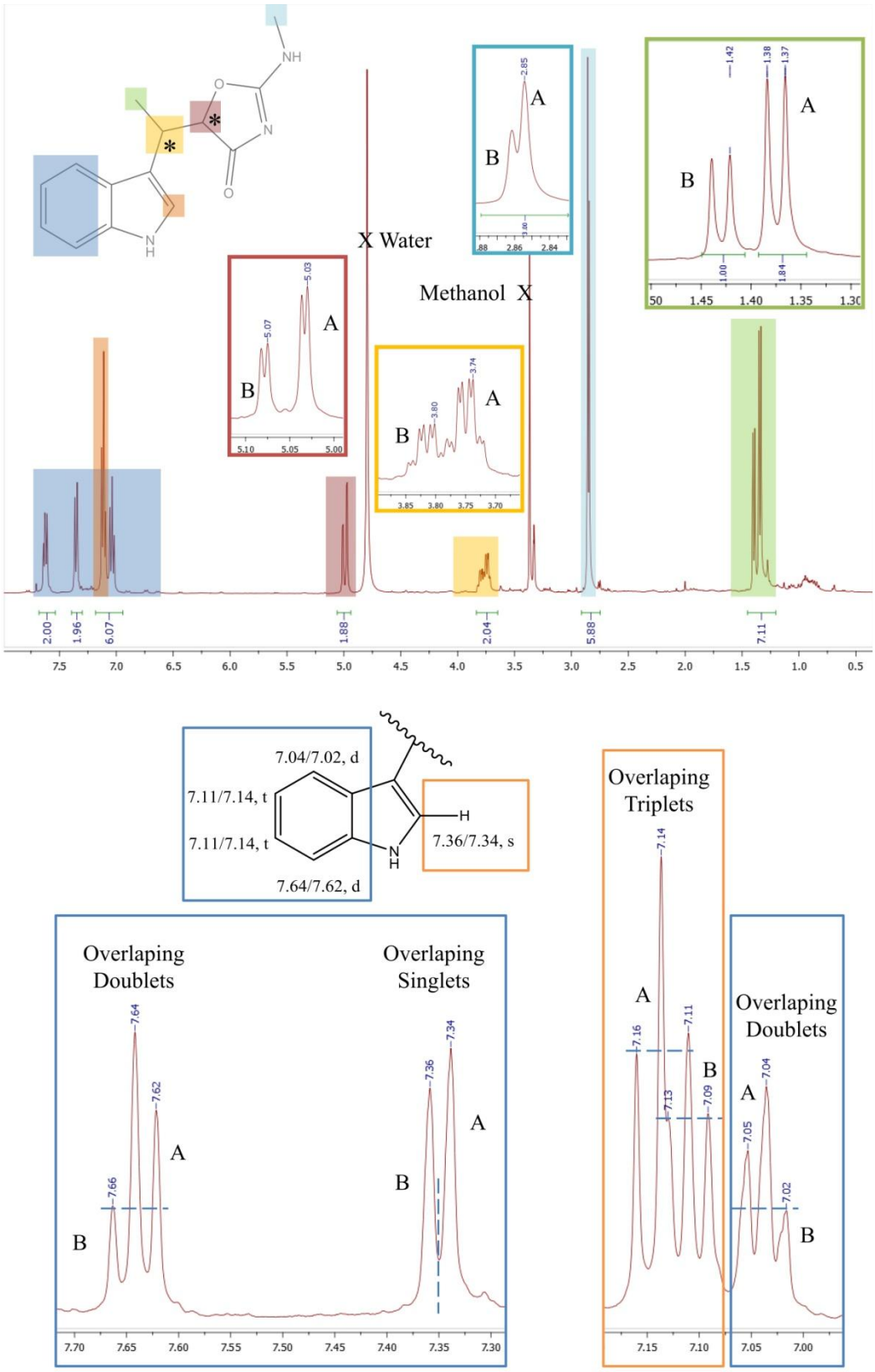


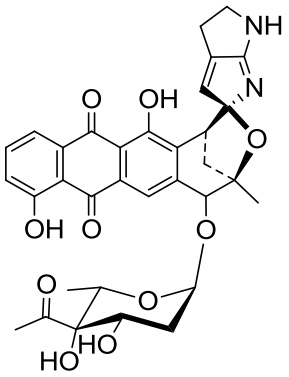
Figure A2 Identification of indolmycin (**5**) using ^{1}H-NMR in MeOD. This sample contains two stereoisomers of indolmycin, labeled A and B.

A.4 Kosinostatin identified from Strain D077, a *Micromonospora* sp.

Strain D077 was isolated from sediment collected from Old Garden Beach near Rockport, MA and was identified as a strain of *Micromonospora humi* by comparison of the sequence of the 16S ribosomal rRNA gene sequence, to the sequence of type strains in the NCBI GenBank database. The polar fractions from this strain, fraction 9 of 10 total fractions from a silica column eluting with 50% EtOAC in MeOH, displayed potent antimicrobial activity against *Enterococcus faecalis* (MIC of <2 μg/mL) and an LC_{50} of 3.12 μg/mL against the ovarian cancer cell line OVCAR5. Ovarian cancer cell line cytotoxicity data was provided by Eoghainin Ó hAinmhire in the laboratory of Dr. Joanna Burdette.

A portion (6 mg) of F9 was separated using a semi-preparative Phenomenex C18 (2) Luna column. The column eluent was collected every 30 seconds or 1 mL per well, in a 96 deep-well plate and concentrated *in vacuo*. The content of each well was resuspended using DMSO and transferred to a 96 well plate for qualitative antimicrobial bioassays. The resultant single-dose (~ 50 μg/mL) biological activity was plotted against the UV absorbance at 254 nm. In Figure A3, the resulting qualitative data for *Pseudomonas aeruginosa* inhibition is shown in red, *E. coli* inhibition is shown in green, and *E. faecalis* is shown in blue. The fraction that displayed the observed biological activity corresponded to a chromatographic peak with a UV profile that was then compared to our in-house UV data base. From this comparison we were unable to identify molecule as kosinostatin. Following the acquisition of ^{1}H-NMR (Figure A30), DEPT, COSY, HSQC, and HMBC spectra the structure was putatively identified as kosinostatin (**6**) (no further data that would allow stereochemical characterization were collected), a quinocycline anthracycline with several unique structural features.

The bicyclic amidine, or pyrrole-pyrrole-like moiety, connected to the anthracycline core via N,O-spiro carbon are unique to the quinocyclines; this has been the study of synthetic[2] and biosynthetic[3] studies. Kosinostatin and isoquinocylcine B, the spiro carbon isomer, are broad spectrum antibiotics[4-5] expected to be acting via DNA intercalation via the COMPARE analysis and is reported to inhibit DNA topoisomerases I and IIα.[6]



6

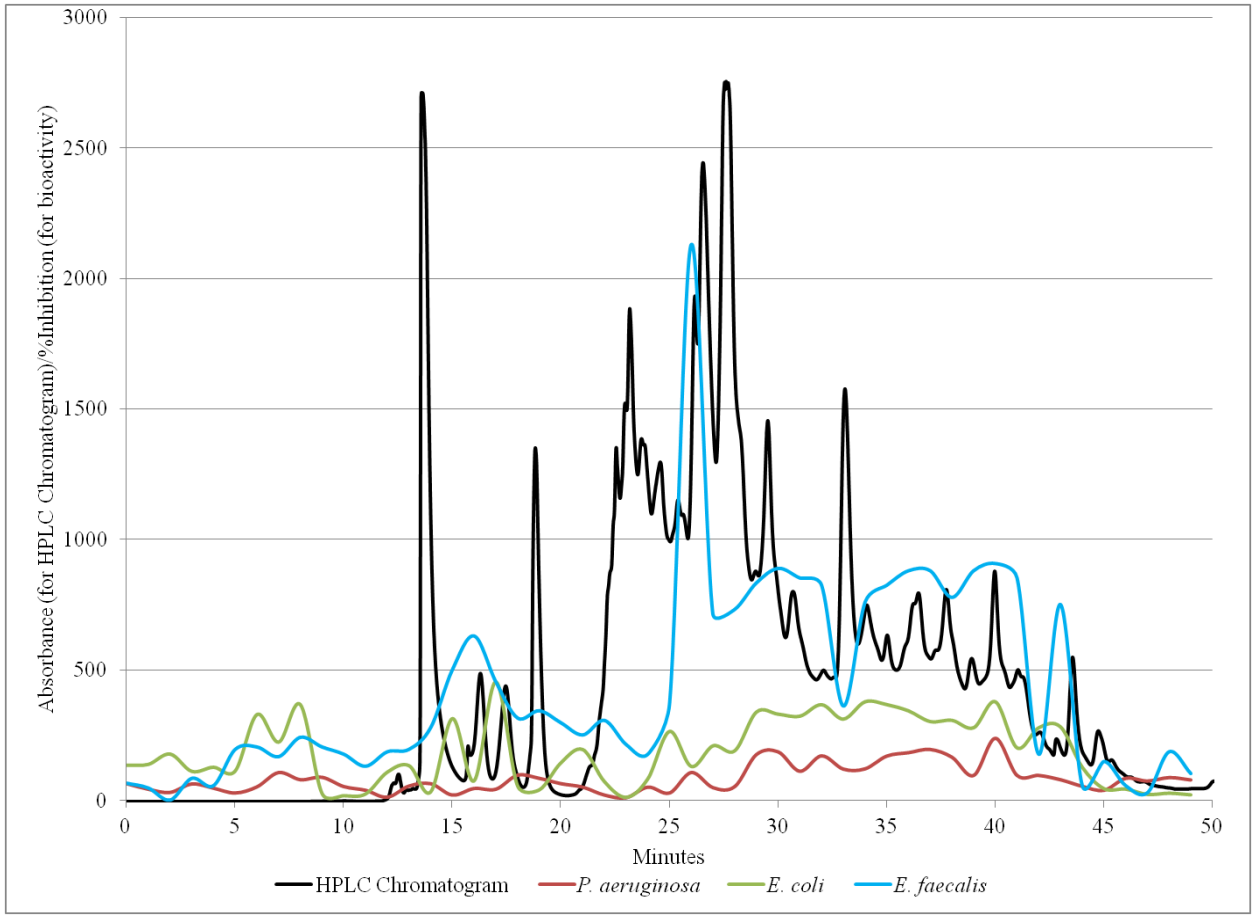
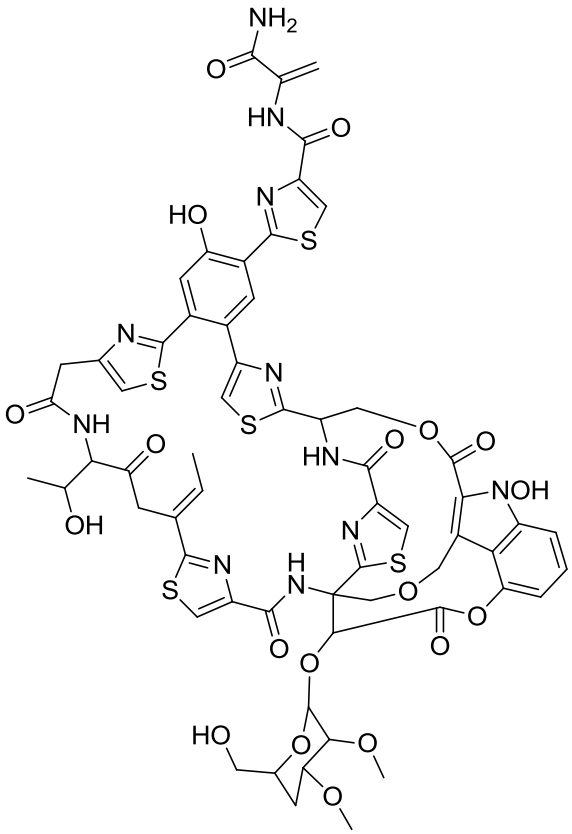


Figure A3 Biochromatogram of D077-F4 in the in-house antimicrobial bioassay.

A.5 Thiazolyl peptides identified from strain D081

Strain D081 was also isolated from sediment collected from Old Garden Beach near Rockport, MA and was identified as *Micromonospora tulbaghiae* by comparison of the sequence of the 16S ribosomal rRNA, to type strains in the NCBI GenBank database.

When the fraction library was screened for inhibitors of *E. faecalis*, F3 and F4 from a silica column from (5 or 50% MeOH in EtOAc, respectively) D081 exhibited MIC values of <2 μg/mL. The most abundant UV peak in the fraction was not active in the biological assay, yet a peak with insufficient weight for further identification was biologically active. The strain was then fermented in 30 L of nutrient rich A1-media and the secondary metabolites were extracted and fractionated over silica gel. Fractions were then tested in the in-house antimicrobial bioassay that indicated that fractions 8 and 9, of 10 fractions from a silica gel column, had MIC values of <2 μg/mL. These fractions were further separated and assayed for biological activity. Subfraction F87 (Figure A4) was analyzed using a Shimadzu triple quadrupole in the UIC Mass Spectrometry, Metabolomics, and Proteomics Facility (UIC MMPF). The ~ 1500 molecular weight and UV maxima at 290 and 375 nm allowed for the putative identification of four thiazolyl peptides (Figure A4). The most abundant of which was putatively identified as S 54832A-I (**7**). The ^{1}H NMR spectrum of a fraction putatively identified as containing a thiazolyl peptide is shown in Figure A31. These cyclic peptides are antibiotics[7] that have been extensively studied and as of 2013, there were 90 published analogs.[7] In order to determine the stereochemistry of a thiazolyl peptide, a large amount of material would be required for the appropriate stereochemical derivitization experiments. Due to the large number of known analogs and the lengthy derivitization required to determine the full structure, this project was not pursued further.



7

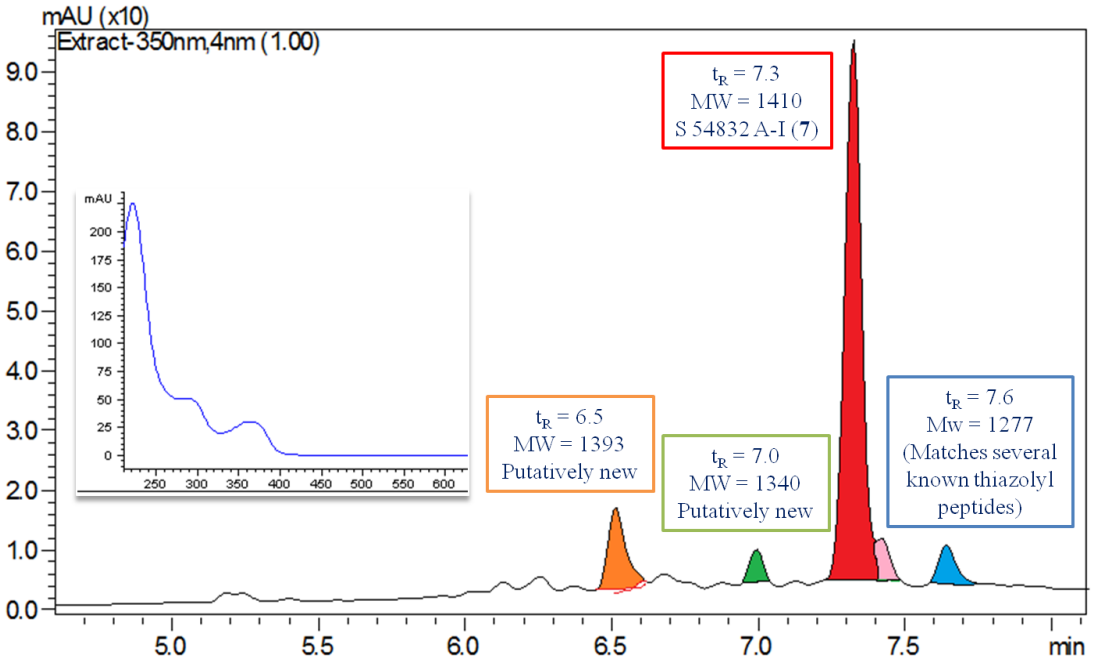


Figure A4 HPLC chromatogram of fraction D081-F87 at 350 nm. Putative identification of novel thiazolyl peptides from D081-F87 are shown in the color coded boxes and UV peaks. The UV profile (inset) and structure of S 54832 A-I (**7**) are also shown. Molecular weights were obtained on a Shimadzu triple quadrupole at the UIC MMPF.

A.6 Strain B034 produces resistomycin and bafilomycin analogs

In collaboration with the Institute for Tuberculosis Research (ITR) our fraction library was screened for inhibitors of non-replicating *Mycobacterium tuberculosis* via the low oxygen recovery assay (LORA) assay.[8] Fraction 4, the most polar fraction from a silica column, from strain B034 displayed an MIC value less than 0.6 μg/mL in the LORA assay. The fraction also displayed activity against the MABA assay. Strain B034 was isolated from Lake Michigan sediment collected at 57 m via PONAR and identified as a strain of *Streptomyces griseoflavus* by comparison of the sequence of the 16S ribosomal rRNA gene sequence, to the sequence of type strains in the NCBI GenBank database.

In order to determine which chromatographic peaks were responsible for the observed biological activity, a portion (4 mg) of F2 was separated for the purposes of generating a biochromatogram using a semi-preparative Phenomenex C18 (2) Luna column. The resultant qualitative single-dose biological activity (shown in red and green, Figure A5) was plotted against the UV absorbance at 254 nm (shown in blue, Figure A5). No peaks eluted before 30 minutes.

Resistomycin (**3**) was putatively identified based by both the characteristic UV profile and comparison of retention times to a standard, and was ultimately confirmed by HR-MS (t_R = 50 minutes, Figure A5).

The biochromatogram highlights the differential activity of the molecules in this fraction. The peaks with retention times between 35 and 42 minutes in Figure A5, displayed LORA activity (red, Figure A5) but not MABA activity (green, Figure A5), and were analyzed using mass spectrometry and evaporative light scattering detection (ELSD) (Figure A6).

The molecular weights of bafilomycin H (**8**) and B_1 (**9**) were identified as 650 and 815, respectively. The UV profile of these peaks matched the in-house UV database and the literature

values reported for the UV maxima. The remaining peaks at 12 minutes and 20 minutes had molecular weights of 649 and 765, respectively. These molecular weights did not match any known bafilomycin analogs. The next experimental step was to determine the relative amounts of each compound in the fraction by using evaporative light scattering detection (ELSD) (Figure A6). The ELSD revealed that the peak putatively corresponding to bafilomycin H was present in the largest amount, indicating the relative amount of new analogs recovered would be very small. The bafilomycins are inhibitors of vacuolar ATP-ases with molecular weights between 550 and 800 amu. There are 30 bafilomycin analogs reported in the literature, and their mechanism of action and structure activity relationships are well studied.[9-10] For these reasons, the project was discontinued.

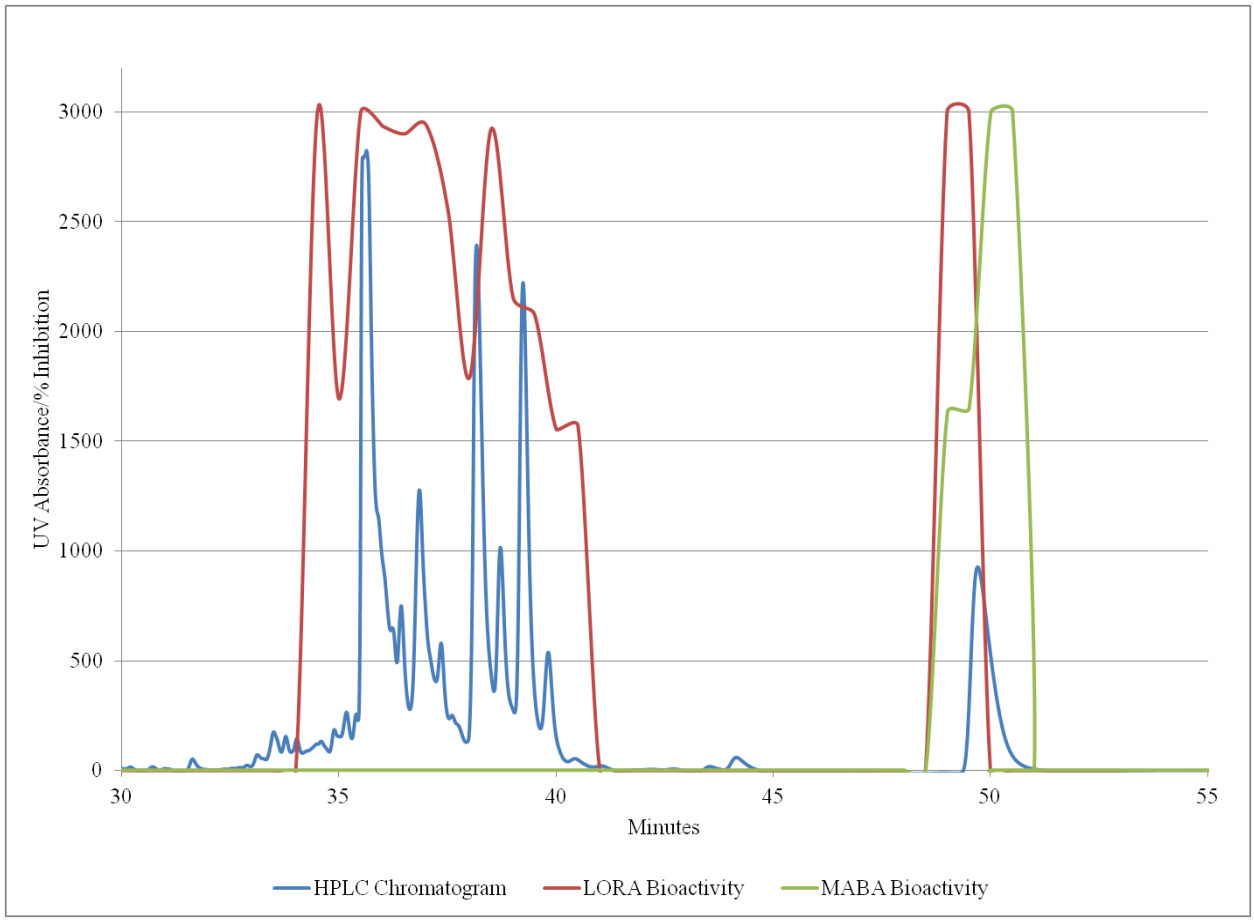


Figure A5 Biochromatogram of B034-F4 in the MABA and LORA bioassays.

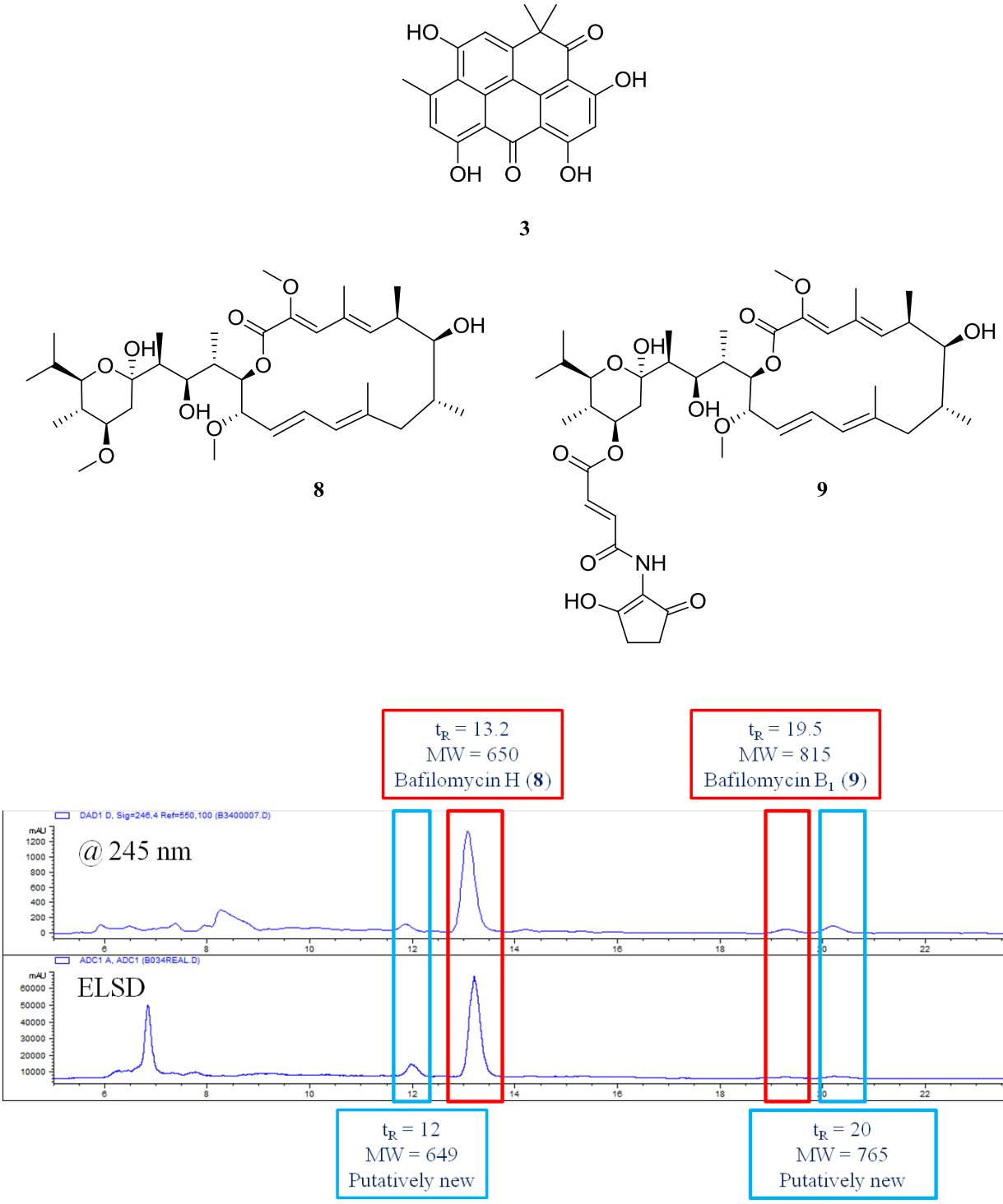


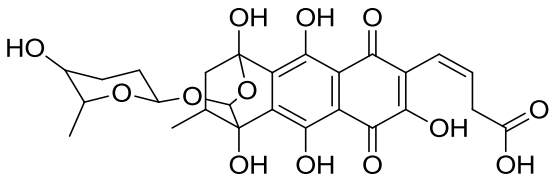
Figure A6 Putative identification of bafilomycin analogs.

ELSD (bottom) compared to the UV absorbance (top) of fraction 4 of Strain B034 at 254 nm. The peaks in red boxes have molecular weights that matched known bafilomycins. The blue boxes represent bafilomycin UV profiles with molecular weights that do not match any reported bafilomycin analogs.

A.7 Strain D034 produces granatacin analogs

The sediment, from which strain D034 was isolated, was collected from Back Beach, near Rockport, MA by skin diving and was identified as *Streptomyces tacrolimicus* by comparison of the sequence of the 16S ribosomal rRNA to type strains in the NCBI GenBank database.

Strain D034 produced a blue pigment when grown on ISP2-Agar (Figure A7). In the search of non-toxic, naturally occurring, blue pigments, graduate student Andrew Newsome requested an extract of the blue agar. The agar was macerated and shaken overnight in filtered DI water. The water was then extracted with XAD-16N resin. The resin was cleaned with MeOH and concentrated *in vacuo*. The blue pigment could not be identified in the extracts we obtained, however HR-MS identified several known analogs of the granatacin type, one of which was the antibiotic MM 44787 (**10**).[11-12] It is likely that the blue metabolite was not absorbed by the XAD resin or possibly decomposed during extraction. Given the identified molecules were not blue and reported to be cytotoxic, the project was dropped.



10

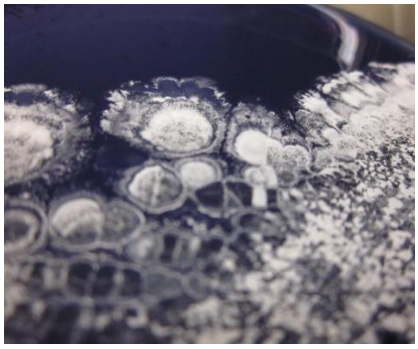


Figure A7 Strain D034 grown on ISP2 agar

A.8 Summary

Each of the actinomycete strains that I worked on during my studies in the Murphy laboratory are listed in Table A1.

Strain	Closest type strain by 16S	Collection site, Depth	Molecular weight	Molecule	Section number	Structure number	Bioactivity that lead to identification
CNJ878	*Micromonospora yangpuensis*	Palau, SCUBA, 25 m	556	juvenimicin C	2.1	1	QR-1 Induction
CNJ878	*Micromonospora yangpuensis*	Palau, SCUBA, 25 m	540	5-O-α-rhamnosyltylactone	2.1	2	QR-1 Induction
B033	*Streptomyces griseoflavus*	Lake Michigan near WI, PONAR, 57 m	333	resistomycin	3.71	3	In-house antimicrobial bioassay
G006	*Micromonospora matsumotoense*	Hao Long Bay, Vietnam PONAR, 22 m	324	chloramphenicol	A.2	4	Bacterial translation inhibition
D085	(not sequenced)	Back Beach, Rockport, MA, skin diving <10 m	257	indolmycin	A.3	5	Bacterial translation inhibition
D077	*Micromonospora humi*	Old Garden Beach, Rockport, MA, skin diving <10 m	616	kosinostatin	A.4	6	In-house antimicrobial bioassay
D081	*Micromonospora tulbaghiae*	Old Garden Beach, Rockport, MA, skin diving <10 m	1410	S54832A-I (thiazolyl peptide)	A.5	7	In-house antimicrobial bioassay
B034	*Streptomyces griseoflavus*	Lake Michigan near WI, PONAR, 57 m	650	bafilomycin H	A.6	8	Ovarian cancer
B034	*Streptomyces griseoflavus*	Lake Michigan near WI, PONAR, 57 m	815	bafilomycin B_1	A.6	9	Ovarian cancer
B034	*Streptomyces griseoflavus*	Lake Michigan near WI, PONAR, 57 m	333	resistomycin	A.6	3	Ovarian cancer
D034	*Streptomyces tacrolimicus*	Back Beach, Rockport, MA, skin diving <10 m	532	MM44787 (granatacin analog)	A.7	10	Ovarian cancer
D034	*Streptomyces tacrolimicus*	Back Beach, Rockport, MA, skin diving <10 m	600	rifamyacin analogs	nr	nr	Ovarian cancer
B007	*Streptomyces griseoflavus*	Lake Michigan near IN, skin diving <10 m	nd	staurosporine analogs	nr	nr	Ovarian cancer
A003	*Streptomyces tendae*	Urrel Beach, Winthrop, MA, skin diving <10 m	nd	macrolide analogs	nr	nr	In-house antimicrobial bioassay

Table A1 Natural products identified

List of all bacterial strains identified by sequencing of the 16S rRNA gene. The collection site and depth are also listed along with the identified compounds.

nd, not determined, nr, not reported.

A.9 References

1. Orelle, C.; Carlson, S.; Kaushal, B.; Almutairi, M. M.; Liu, H.; Ochabowicz, A.; Quan, S.; Pham, V. C.; Squires, C. L.; Murphy, B. T.; Mankin, A. S. Tools for characterizing bacterial protein synthesis inhibitors. *Antimicrob. Agents Chemother.* **2013**, *57*, 5994-6004.
2. Cordes, J.; Harms, K.; Koert, U. Synthesis of the isoquinocycline-pyrrolpyrrole substructure. *Org. Lett.* **2010**, *12*, 3808-3811.
3. Ma, H. M.; Zhou, Q.; Tang, Y.-M.; Zhang, Z.; Chen, Y. S.; He, H. Y.; Pan, H. X.; Tang, M. C.; Gao, J. F.; Zhao, S. Y.; Igarashi, Y.; Tang, G. L. Unconventional origin and hybrid system for construction of pyrrolopyrrole moiety in kosinostatin biosynthesis. *Chem. Biol.* **2013**, *20*, 796-805.
4. Naggar, M. Y. E. Kosinostatin, a major secondary metabolite isolated from the culture filtrate of *Streptomyces violaceusniger* Strain HAL64. *J. Microbiol.* **2007**, *45*, 262-267.
5. Igarashi, Y.; Higuchi, H.; Oki, T.; Furumai, T. NMR analysis of quinocycline antibiotics: structure determination of kosinostatin, an antitumor substance from *Micromonospora* sp. TP-A0468. *J. Antibiot.* **2002**, *55*, 134-140.
6. Furumai, T.; Igarashi, Y.; Higuchi, H.; Saito, N.; Oki, T. Kosinostatin, a quinocycline antibiotic with atitumor activity from *Micromonospora* sp. TP-A0468. *J. Antibiot.* **2002**, *55*, 128-133.
7. Singh, S. B.; Zhang, C.; Zink, D. L.; Herath, K.; Ondeyka, J.; Masurekar, P.; Jayasuriya, H.; Goetz, M. A.; Tormo, J. R.; Vicente, F.; Martın, J.; Gonzalez, I.; Genilloud, O. Occurrence, distribution, dereplication and efficient discovery of thiazolyl peptides by sensitive-resistant pair screening. *J. Antibiot.* **2013**, *66*, 599-607.

8. Cho, S. H.; Warit, S.; Wan, B.; Hwang, C. H.; Pauli, G. F.; Franzblau, S. G. Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **2007**, *51*, 1280-1385.

9. Yousaf, M.; El Sayed, K. A.; Rao, K. V.; Lim, C. W.; Hu, J. F.; Kelly, M.; Franzblau, S. G.; Zhang, F. Q.; Peraud, O.; Hill, R. T.; Hamann, M. T. 12,34-Oxamanzamines, novel biocatalytic and natural products from manzamine producing Indo-Pacific sponges. *Tetrahedron* **2002**, *58*, 7397-7402.

10. Yoshimori, T.; Yamamoto, A.; Moriyama, Y.; Futai, M.; Tashiro, Y. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J. Biol. Chem* **1991**, *266*, 17707-17712.

11. Elson, A. L.; Box, S. J.; Gilpin, M. L. New quinone antibiotics of the granaticin type, isolated from S*treptomyces lateritius* I. Production, isolation and properties. *J. Antibiot.* **1988**, *41*, 540-572.

12. Gilpin, M. L.; Box, S. J.; Elson., A. L. New quinone antibiotics of the granaticin type, isolated from *Streptomyces lateritius*. II. Structure determination. *J. Antibiot.* **1988**, *41*, 512-518.

Appendix II Reference Spectra

Figure A8 ^{1}H NMR spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

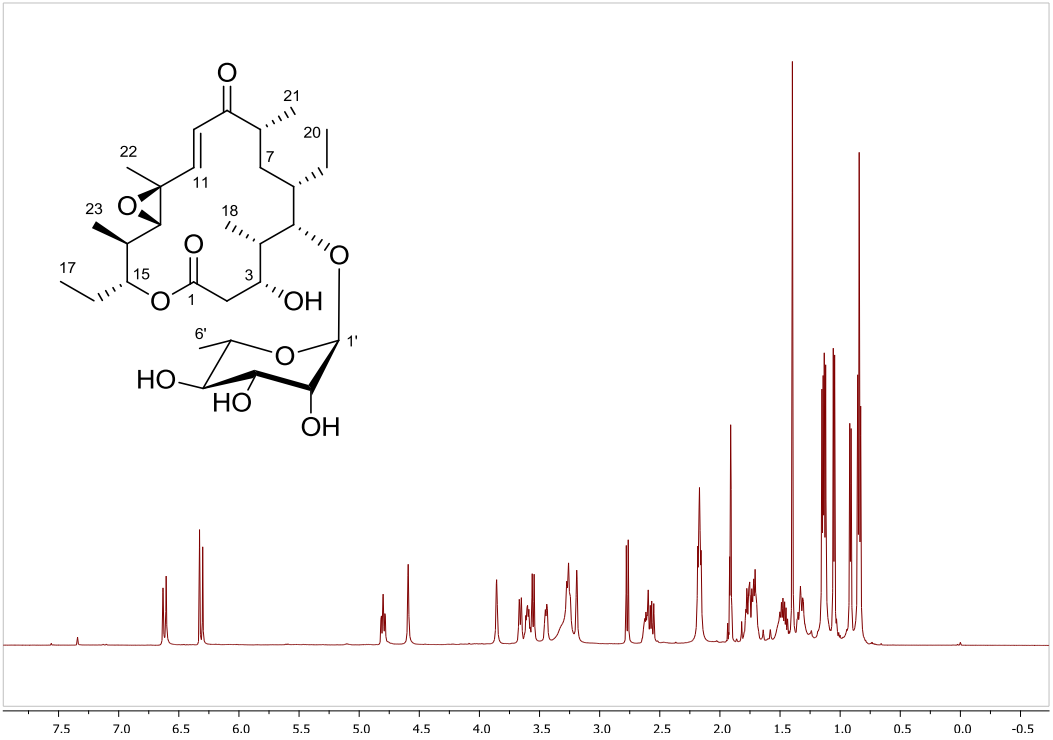


Figure A9 COSY spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

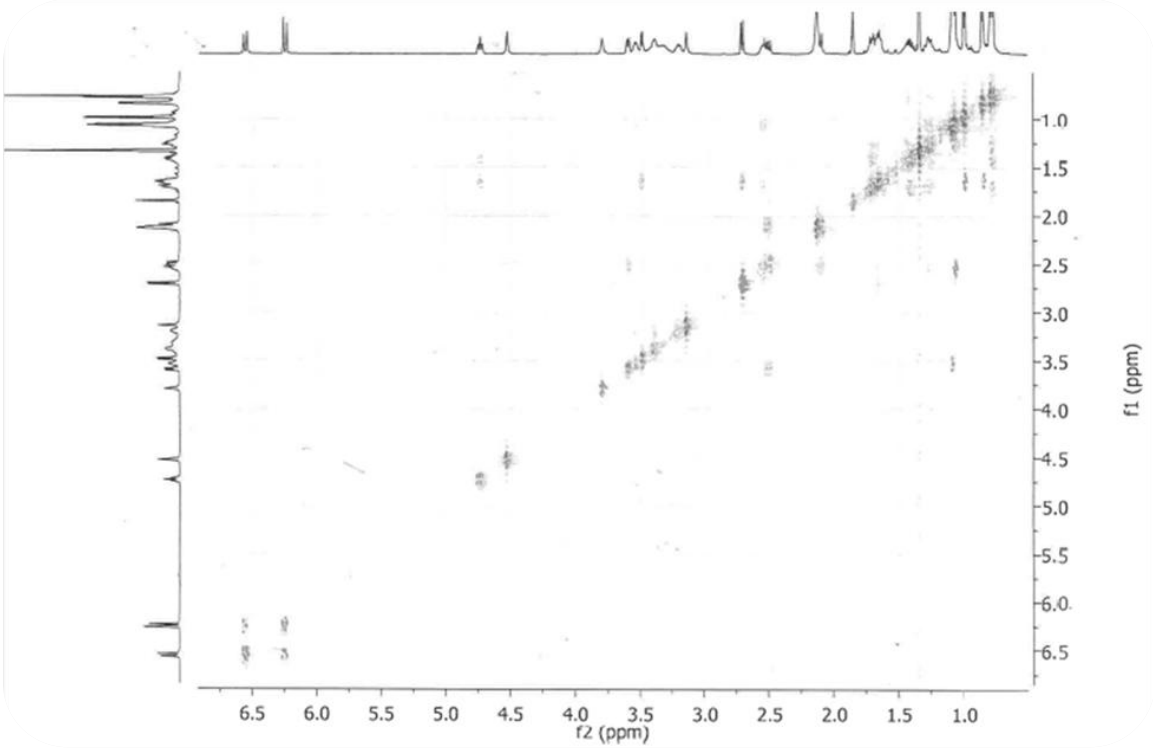


Figure A10 HMBC spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

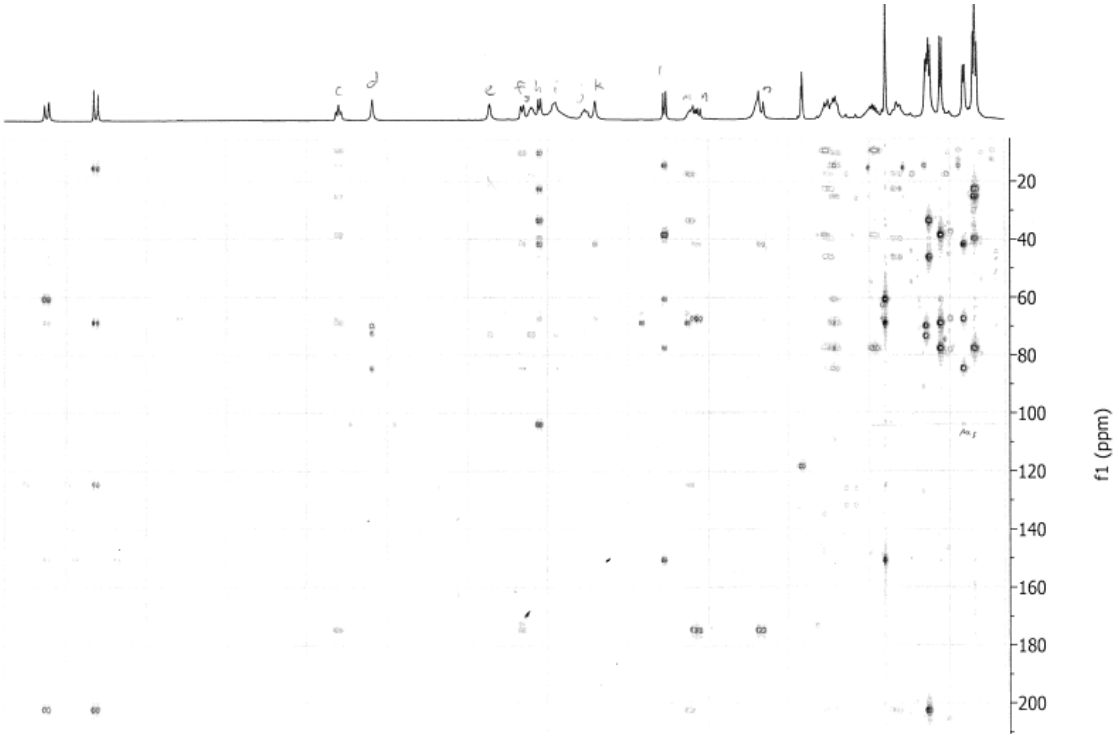


Figure A11 NOESY spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

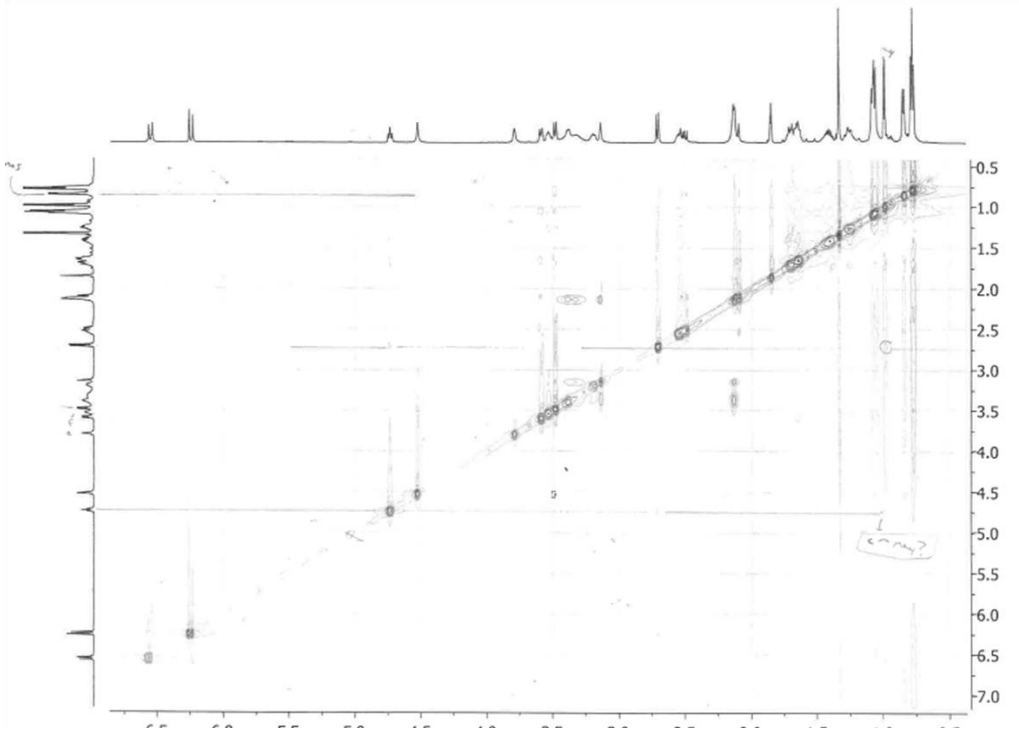


Figure A12 Selective 1D-TOCSY spectrum of H2 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

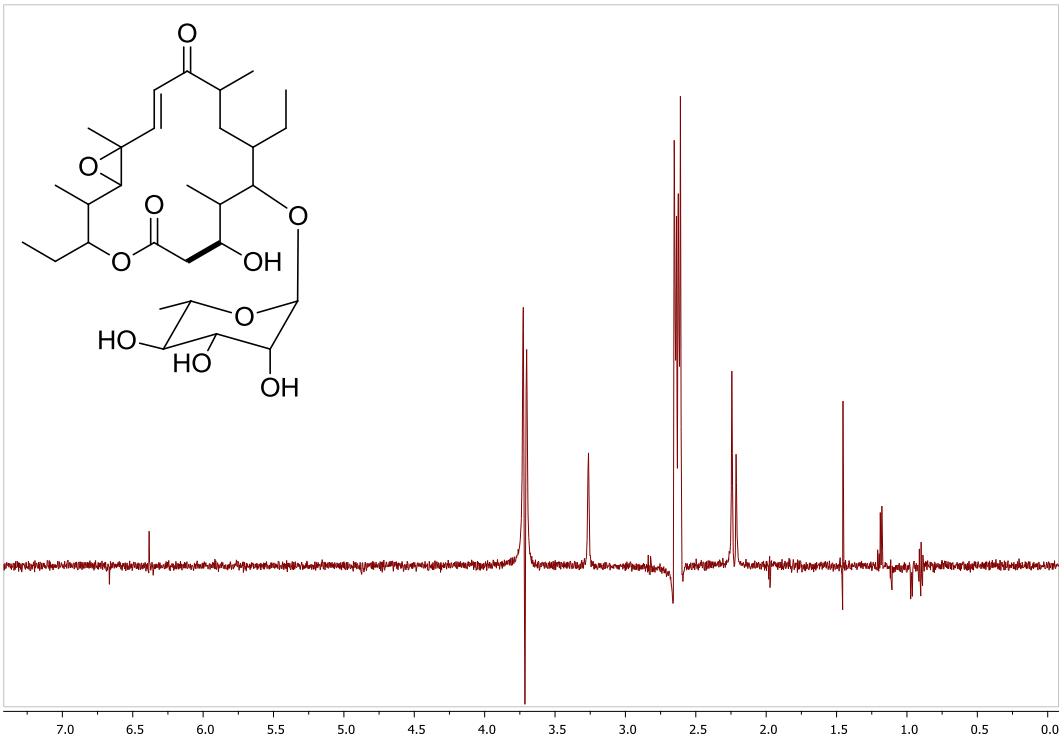


Figure A13 Selective 1D-TOCSY spectrum of H3 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

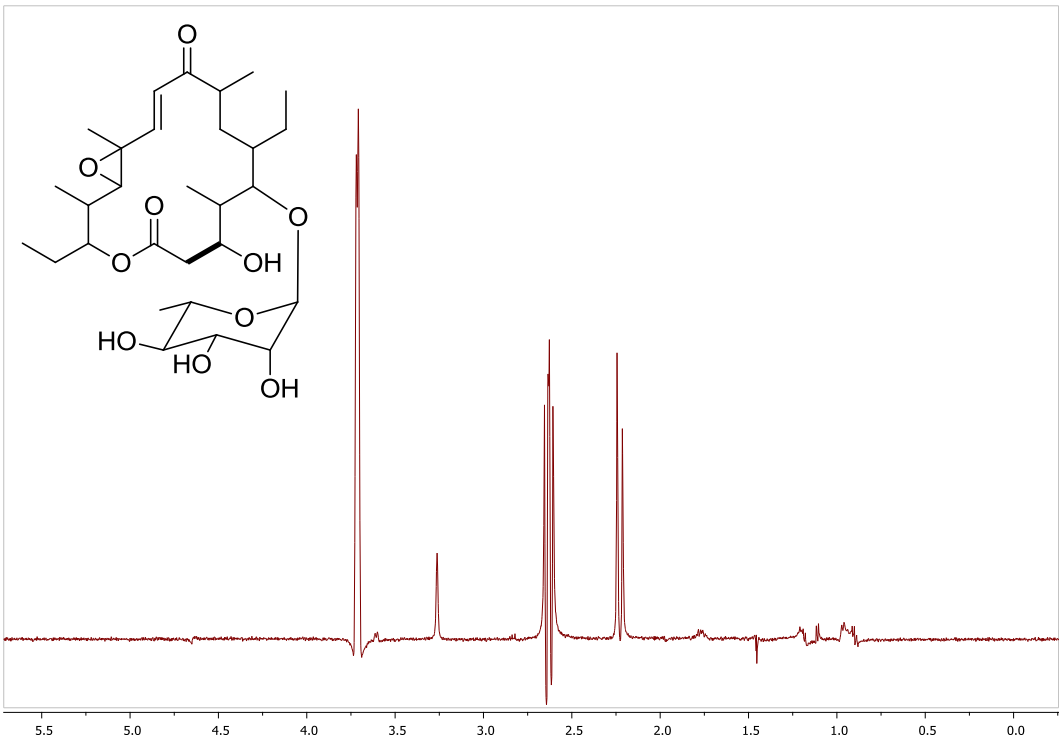


Figure A14 Selective 1D-TOCSY spectrum of H8 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

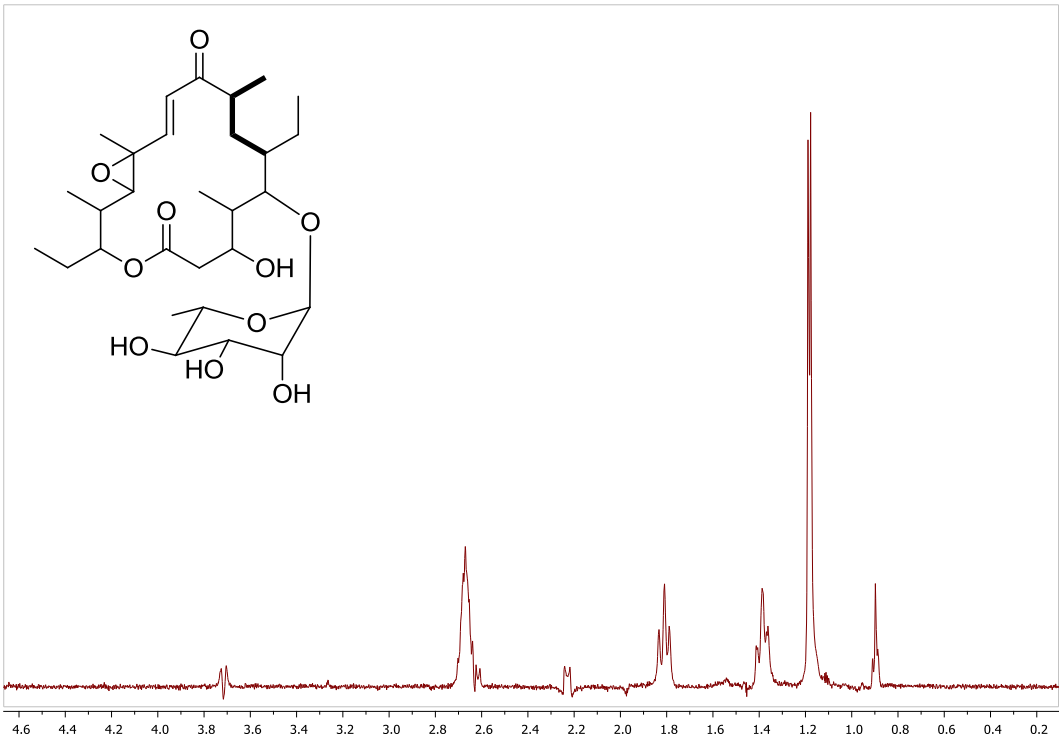


Figure A15 Selective 1D-TOCSY spectrum of H15 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

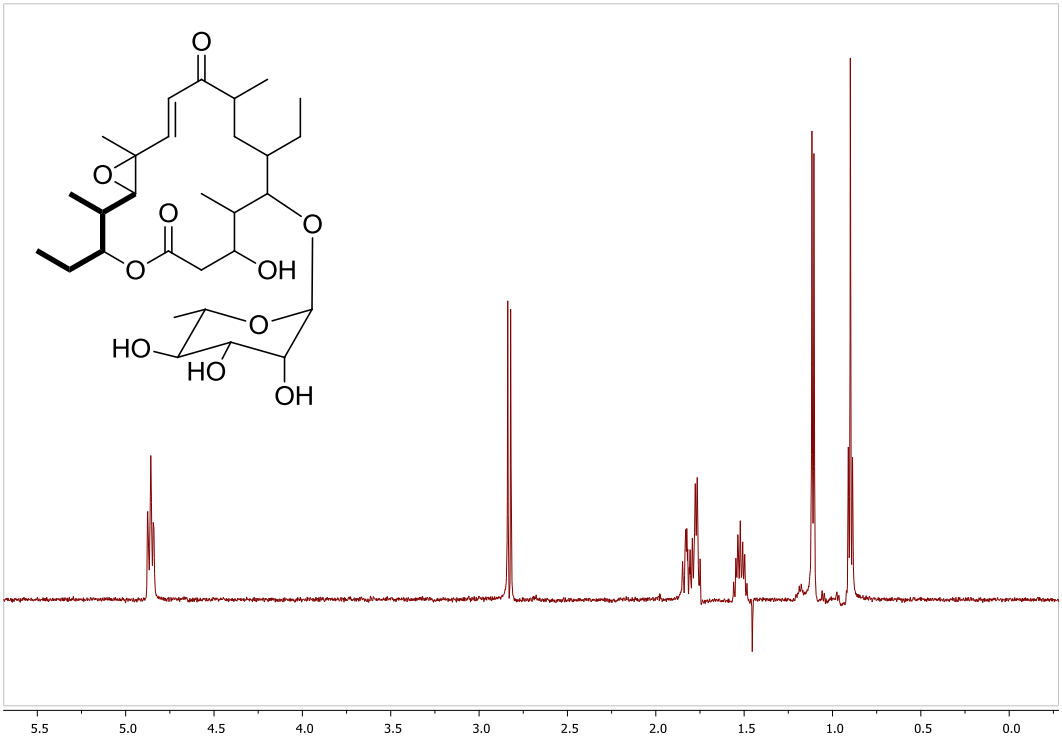


Figure A16 Selective 1D-TOCSY spectrum of H18 (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

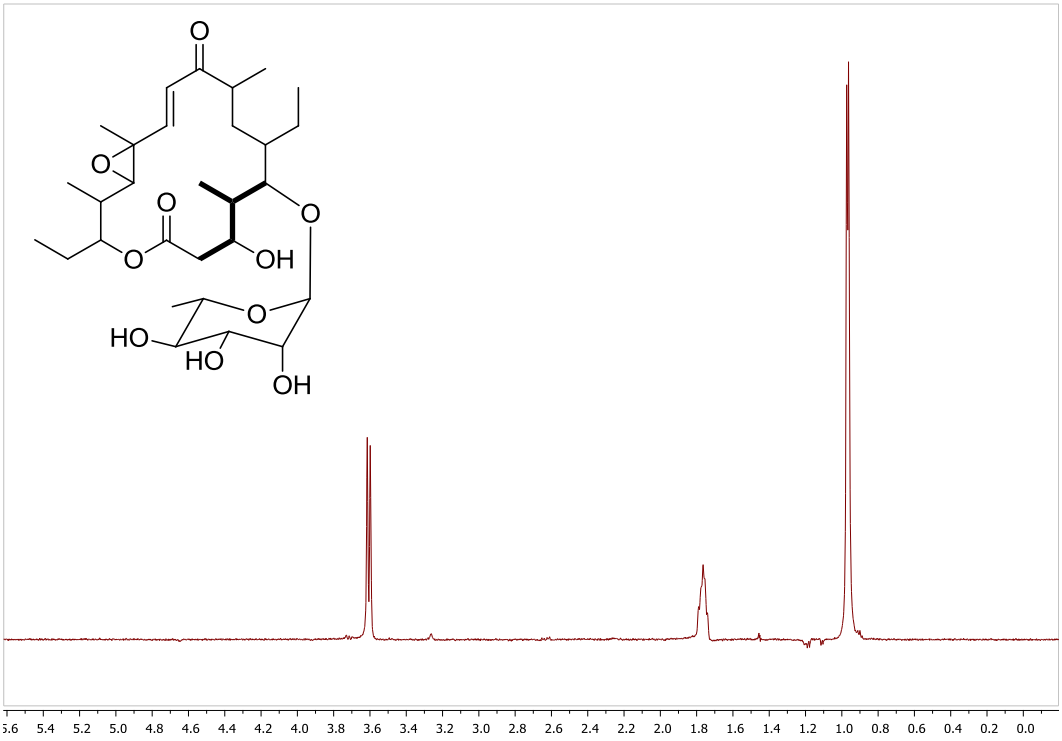


Figure A17 Selective 1D-TOCSY spectrum of H1′ (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

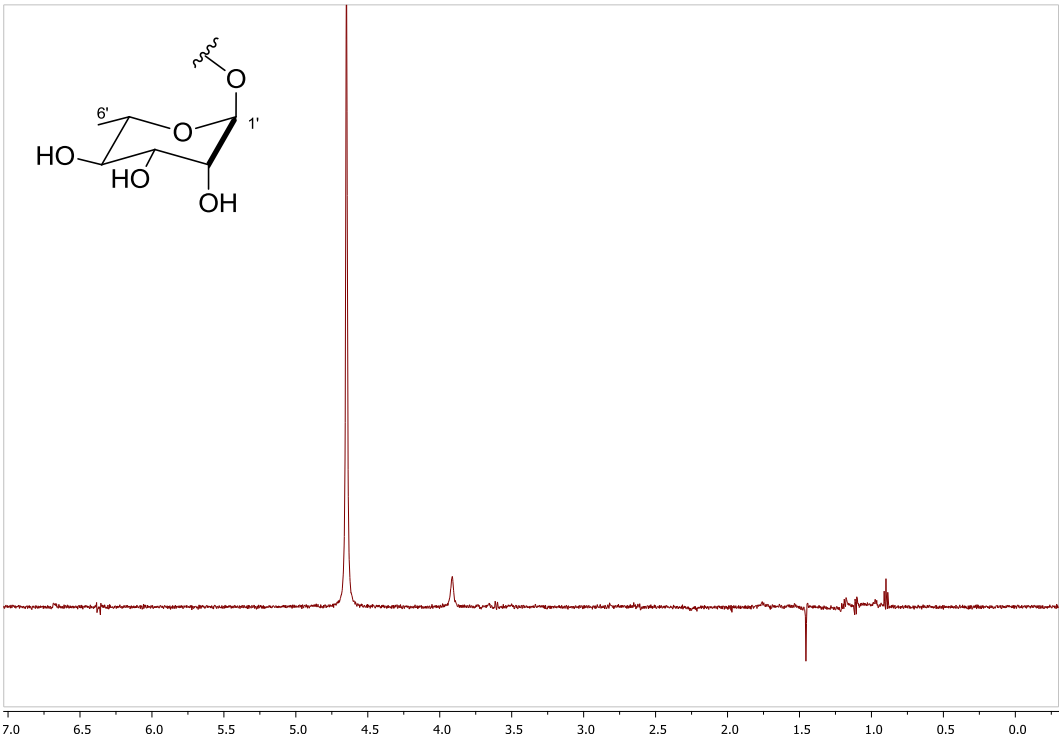


Figure A18 Selective 1D-TOCSY spectrum of H2′ (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

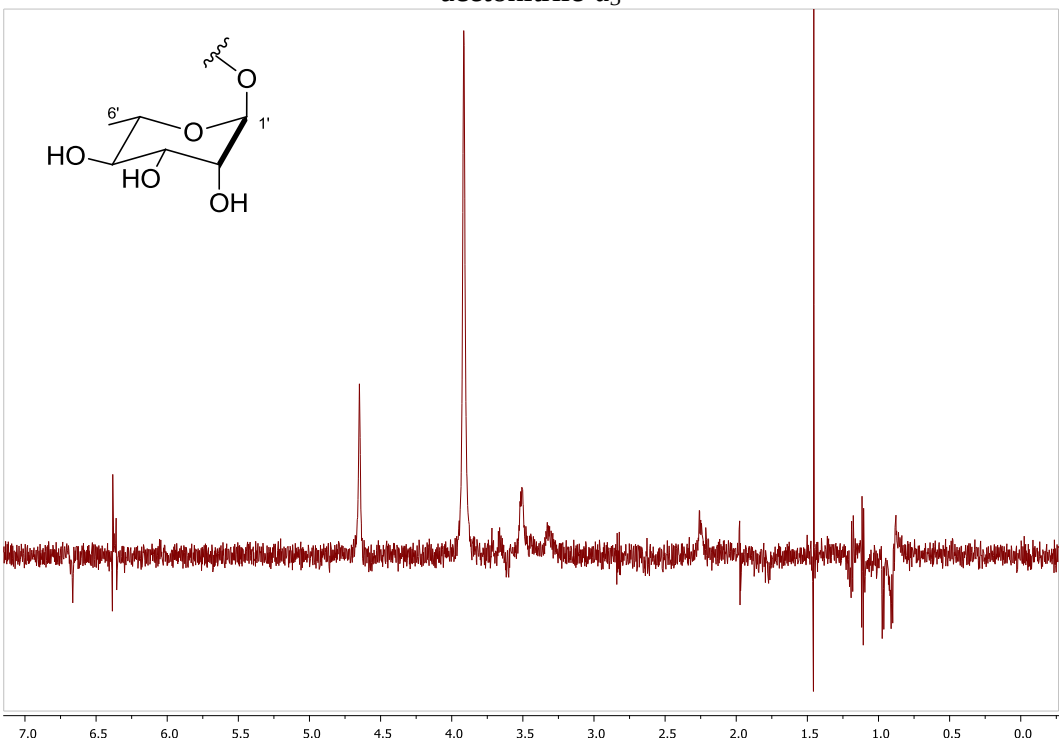


Figure A19 Selective 1D-TOCSY spectrum of H5′ (500 MHz) of juvenimicin C (**1**) in acetonitrile-d_3

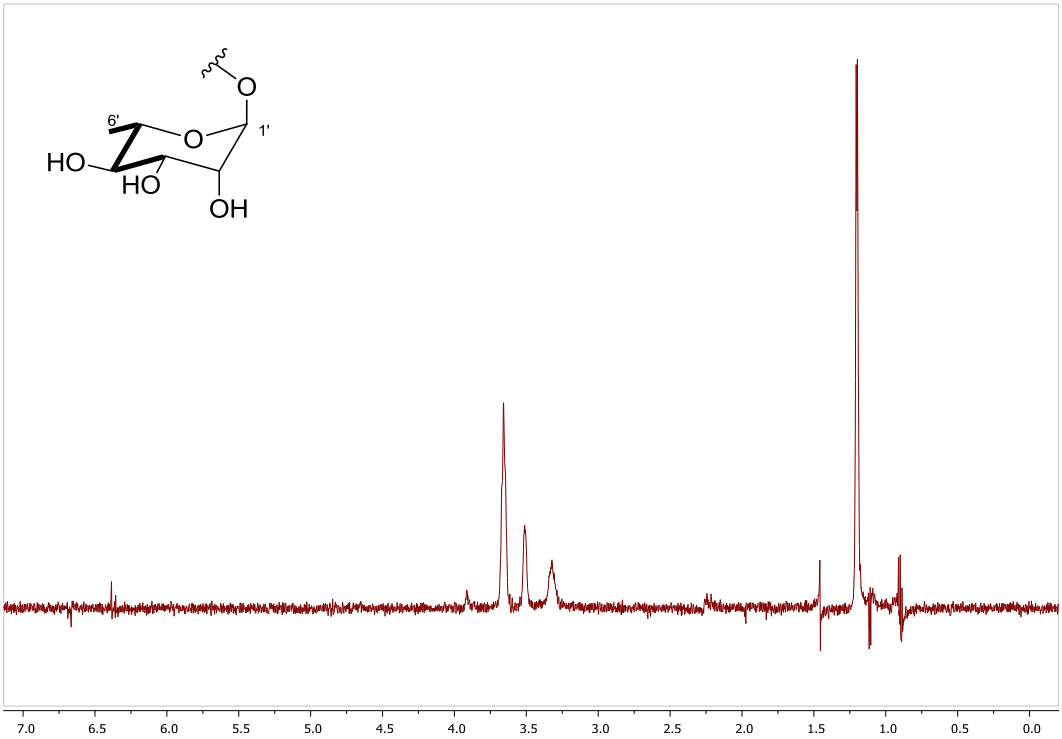


Figure A20 TOCSY spectrum (500 MHz) of juvenimicin C (**1**) in acetonitrile-*d3*

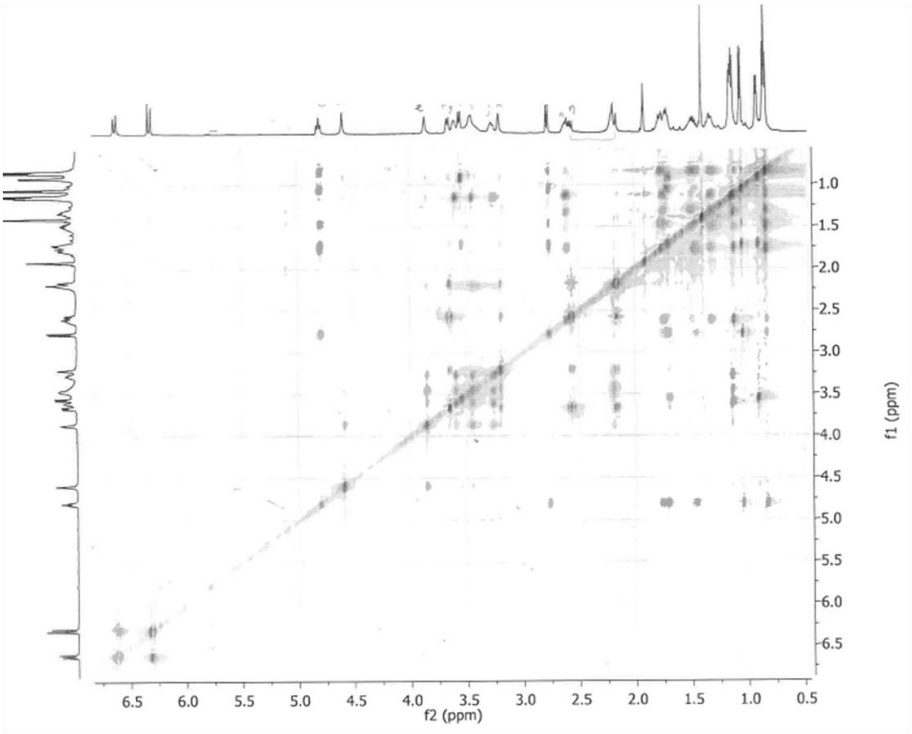


Figure A21 HR-ESI-TOF-MS of juvenimicin C (**1**)

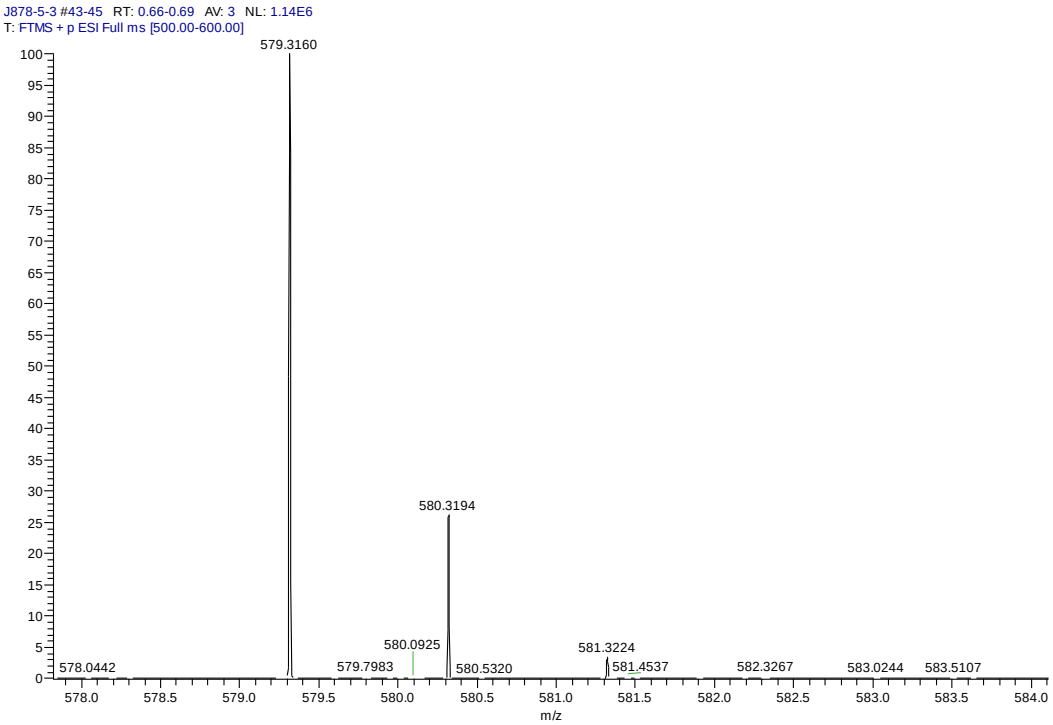


Figure A22 Circular dichroism spectrum of juvenimicin C (**1**)

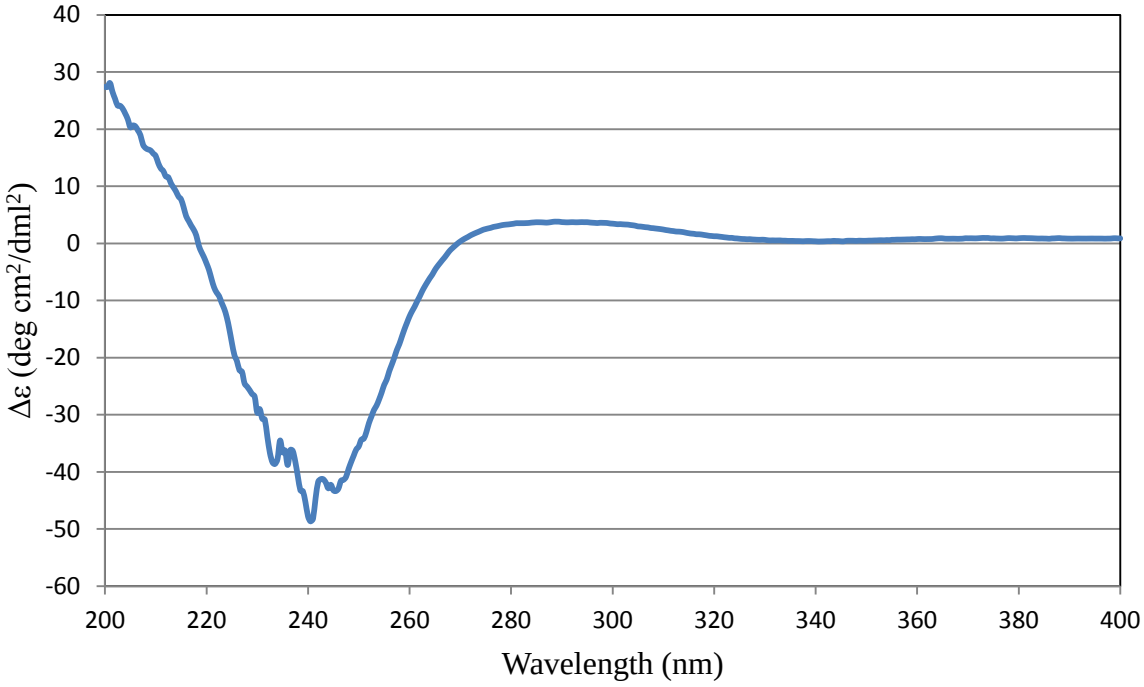


Figure A23 UV spectrum of juvenimicin C (**1**)

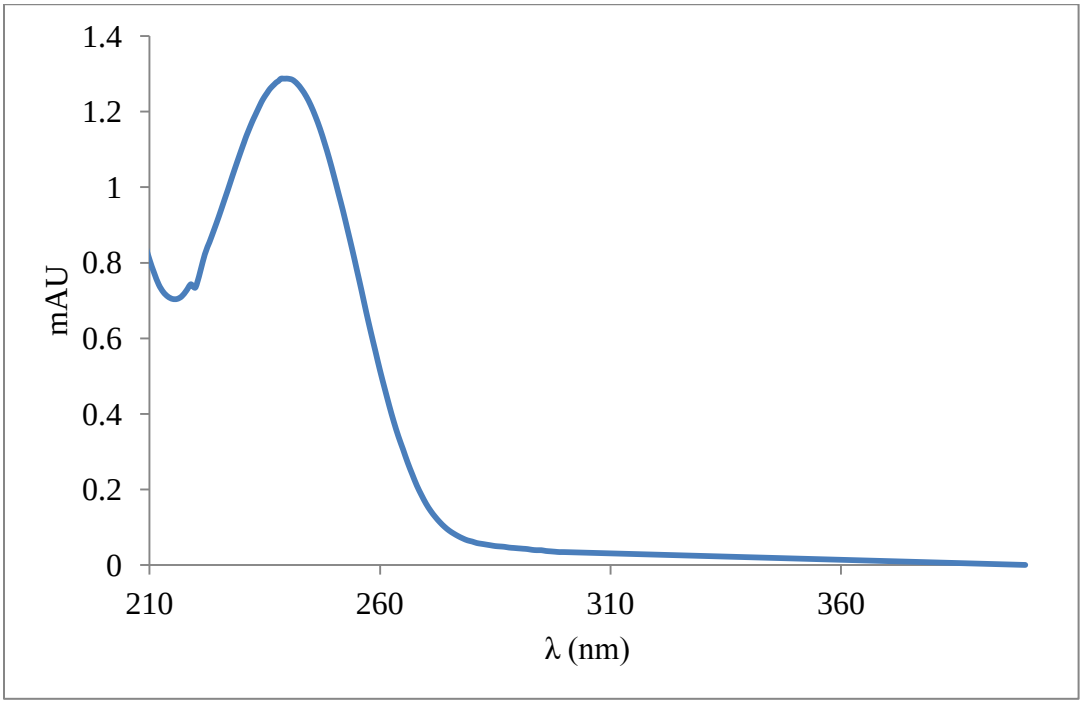


Figure A24 Crystal structure of juvenimicin C (**1**)

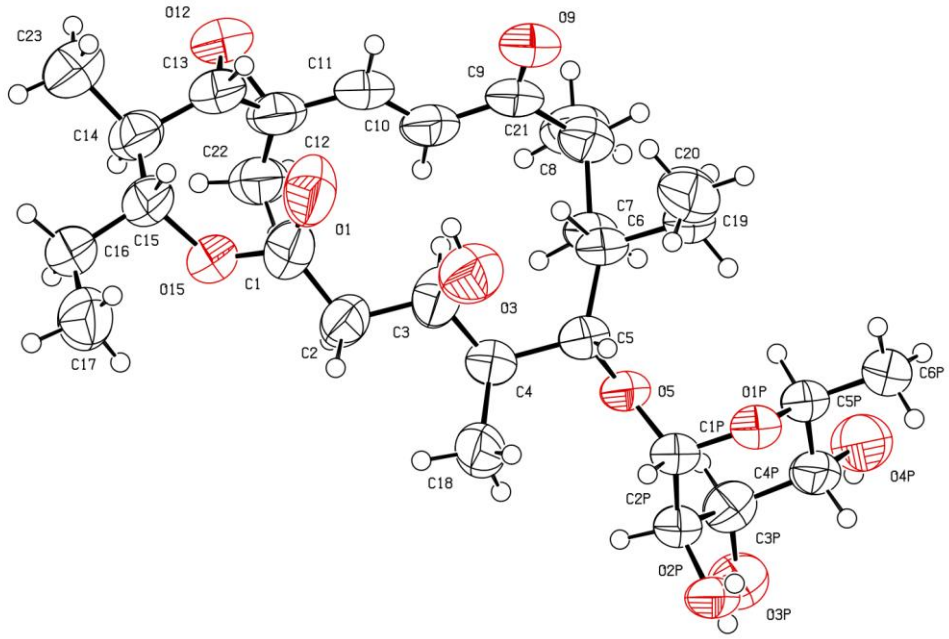


Figure A25 ^{1}H-NMR spectrum (500 MHz) of 20-deoxo-23-deoxy-5-O-(6-deoxy-β-D-glucopyranosyl)-tylonolide (**2**) in acetonitrile-d_3

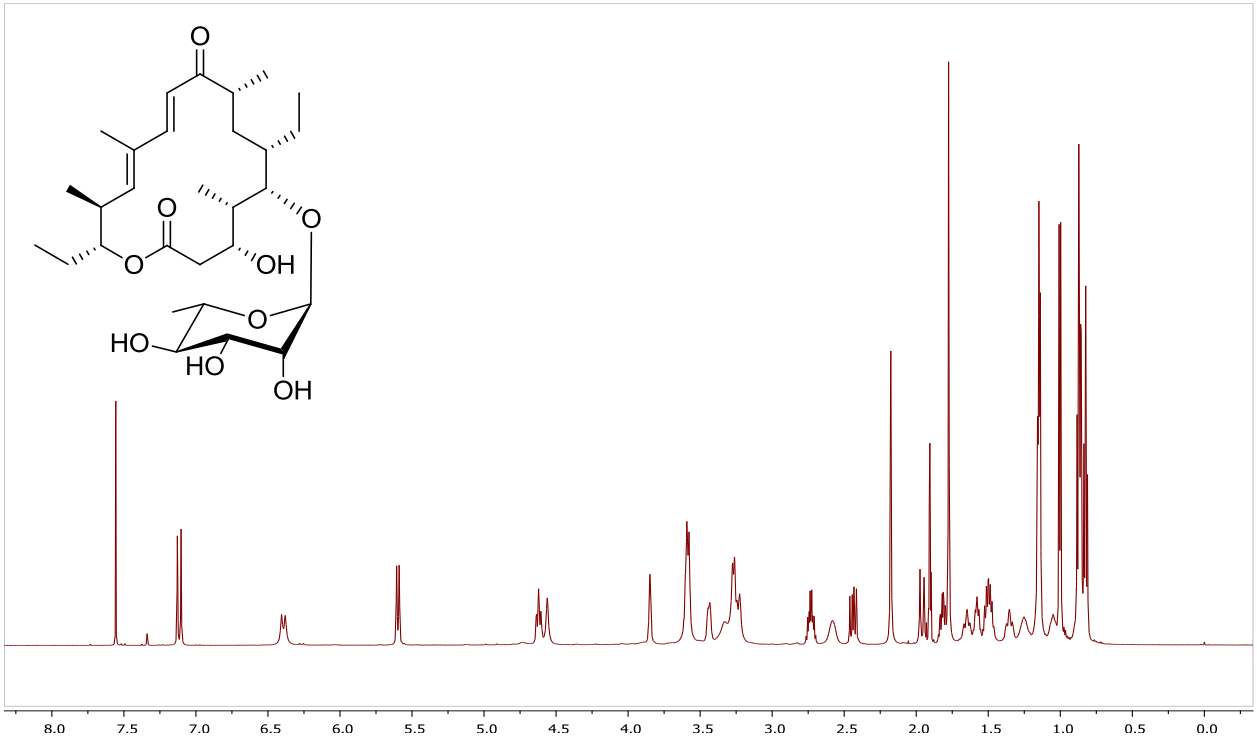


Figure A26 HR-ESI-TOF-MS of 20-deoxo-23-deoxy-5-O-(6-deoxy-β-D-glucopyranosyl)-tylonolide (**2**)

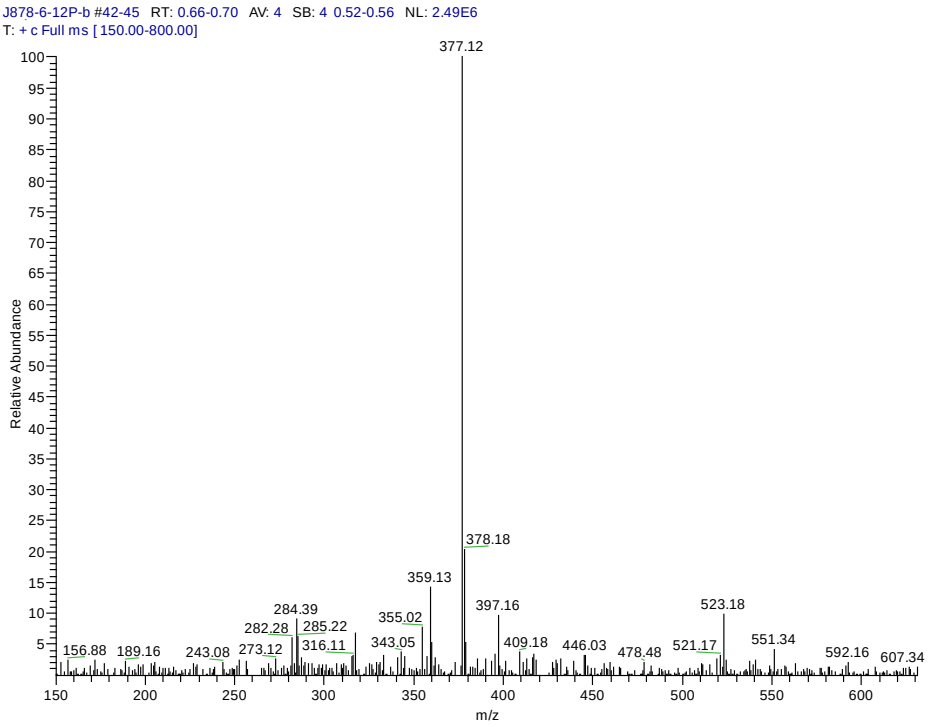


Figure A27 Crystal structure of 20-deoxo-23-deoxy-5-O-(6-deoxy-β-D-glucopyranosyl)-tylonolide (**2**)

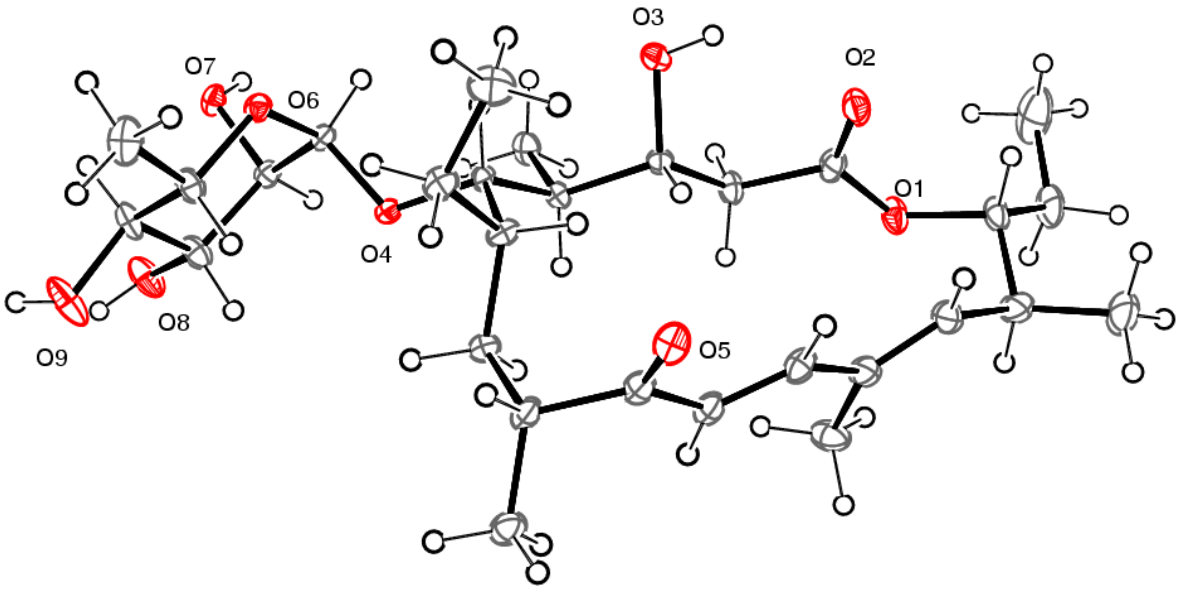


Figure A28 ^{1}H NMR spectrum (600 MHz) of chloramphenicol (**4**) in $CDCl_3$

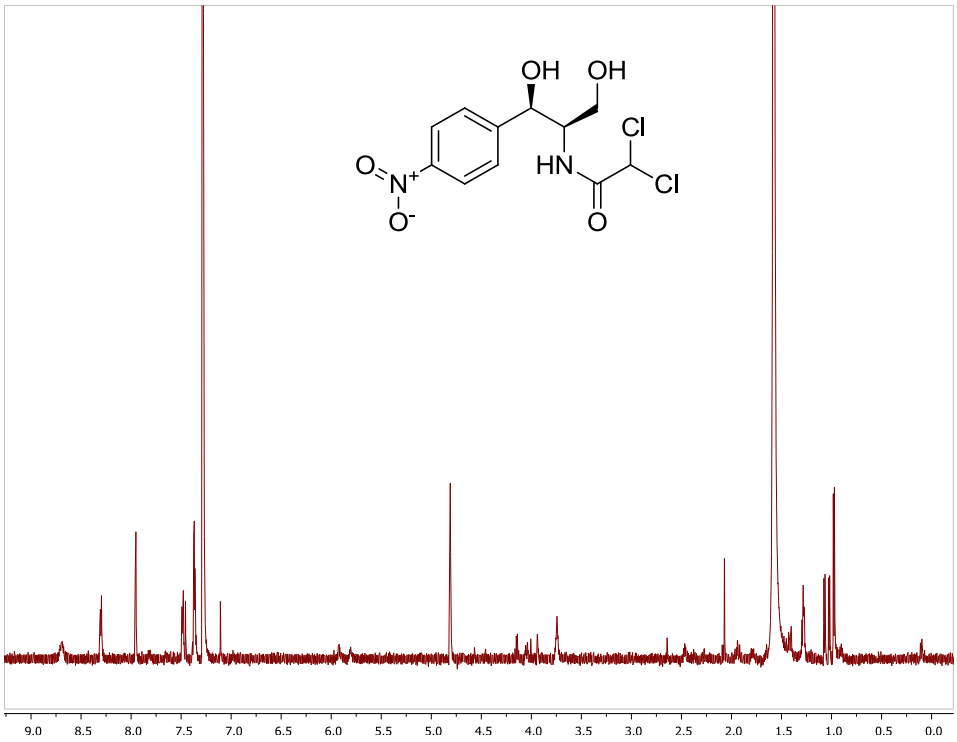


Figure A29 ^{1}H NMR spectrum (600 MHz) of two indolmycin stereoisomers (**5**) in MeOD

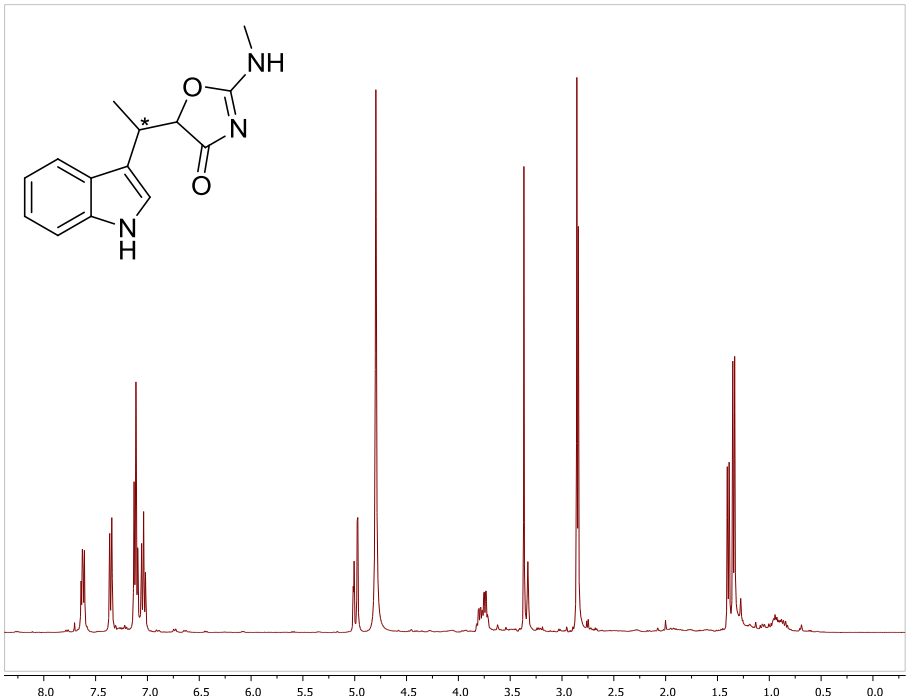


Figure A30 ^{1}H NMR spectrum (600 MHz) of kosinostatin (**6**) in DMSO-d_6

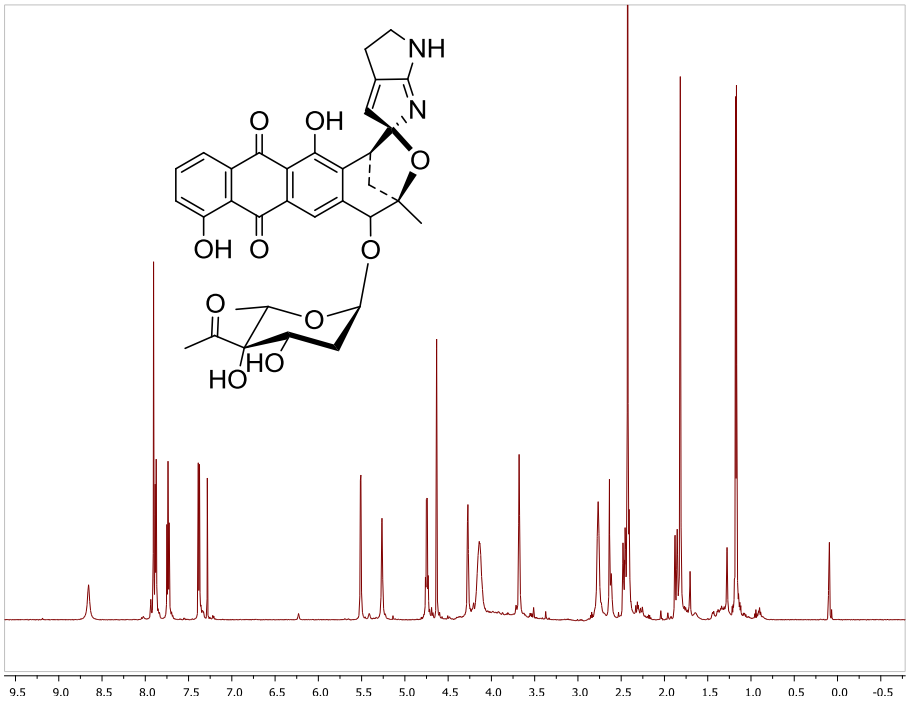


Figure A31 ^{1}H NMR spectrum (600 MHz) of a fraction putatively containing a thiazolyl peptide in DMSO-d_6

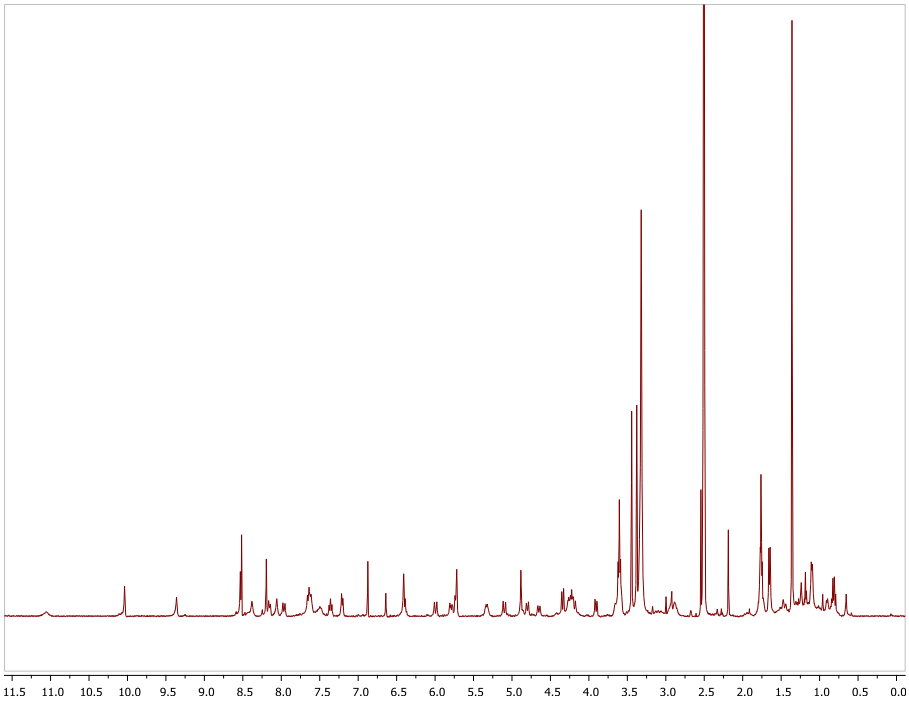
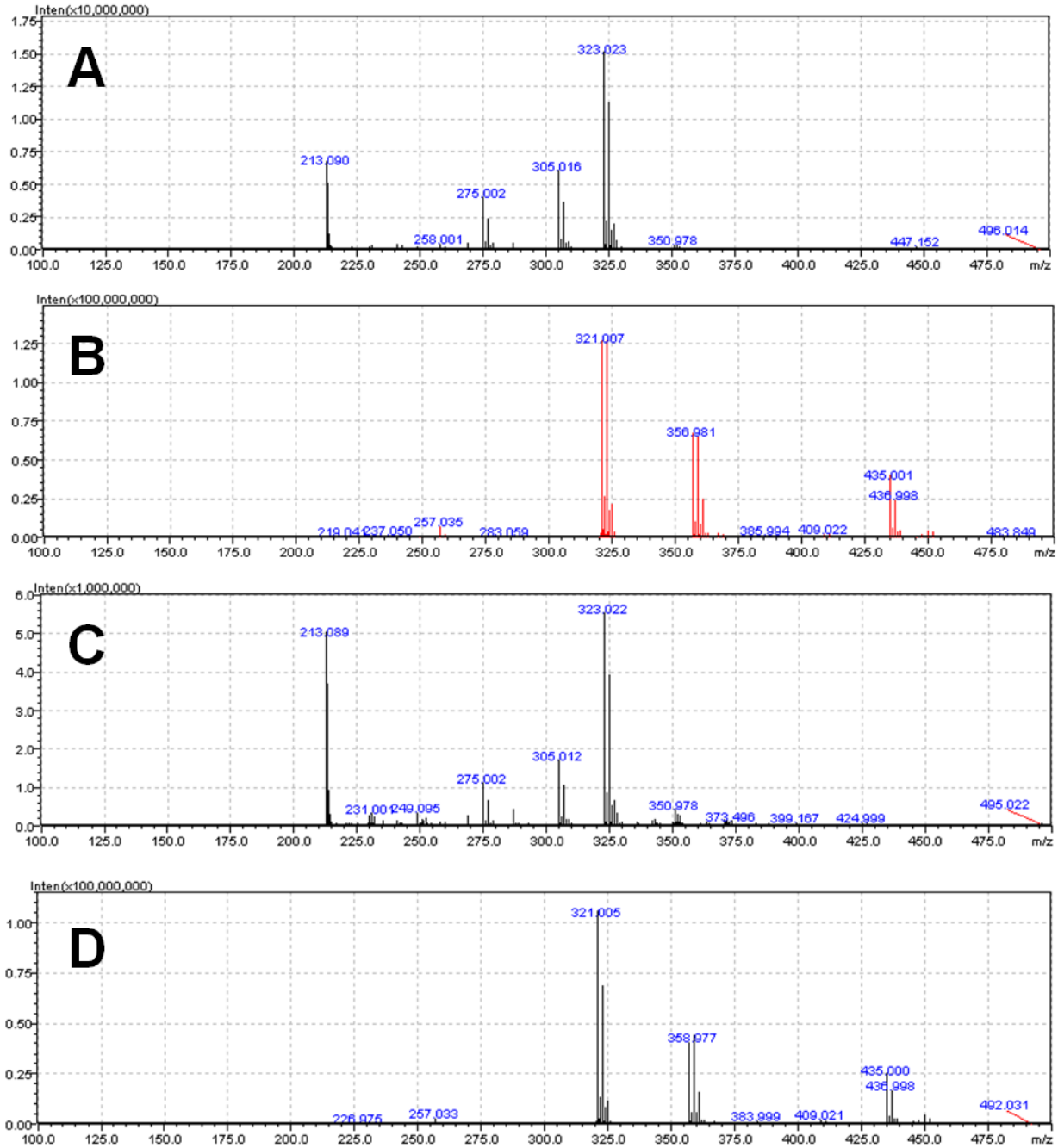


Figure A32 HR-MS of G006-F242 compared to chloramphenicol (**4**) standard



Mass spectrometry (IT-TOF) analysis of the compounds present in the fraction G006-249. The chloramphenicol control (A, B) and fraction G006-249 (C, D) were analyzed under positive and negative ionization modes, respectively.

Table A2 Similarity of Proteobacterial co-culture panel to strains on Health and Human Services Select Agents List (HHS-SA)

HHS-SA **Class** **Family** ***Genus species***	**HHS-SA mimics** **Class** **Family** ***Genus species***
α-Proteobacteria Brucellaceae *Brucella abortus, B. melitensis, B. suis*	α-Proteobacteria Brucellaceae *Brucella neotomae* (ATCC 23459)
β-Proteobacteria Burkholderiaceae *Burkholderia mallei, B. pseudomallei* *Ralstonia solanacearum*	β-Proteobacteria Burkholderiaceae *Burkholderia vietnamiensis* (ATCC BAA-248)
γ-Proteobacteria Enterobacteriaceae *Yersinia pestis* Xanthomonadaceae *Xanthomonas oryzae* *Xylella fastidiosa*	γ-Proteobacteria Enterobacteriaceae *Yersinia pestis* A1122 Xanthomonadaceae *Xanthomonas axonopodis* (ATCC 8718)

The left column lists strains that are currently classified as bioterrorism agents by HHS. The right column lists strains that we chose to mimic their corresponding bioterrorism agents to the left.

Brucella neotomae 5K33 was selected to represent the α-Proteobacteria HHS-SA. In addition to its compatibility with high-throughput BSL-2 screening (most virulent *Brucella* strains require BSL-3 conditions), its genetic homogeneity to zoonotic *Brucella* species has facilitated promising results for its potential use as a brucellosis vaccine.[1] This disease is caused by several *Brucella* species and has been found to infect both humans and marine mammals, indicating that strains within this genus regularly exist in both terrestrial and marine environments.[2-3] Although the two β-Proteobacteria HHS-SA include the genera *Burkholderia* and *Ralstonia*, both share a close phylogenetic relationship when compared with other branches of this phylum in the 16S rDNA-based phylogenetic tree.[4] *Burkholderia vietnamiensis* LMG 10929, a strain that is prevalent in *B. cepacia* complex that commonly infects the lungs of cystic fibrosis patients and

has been isolated from environmental samples (rice, soil, and aquatic), was selected to represent this class.[5-6]

Due to the relatively large phylogenetic diversity within the γ-Proteobacteria class, multiple induction strains were chosen. *Yersinia pestis* A1122 was chosen to act as an induction strain for the Enterobacteriaceae; this strain is a genetic knockout that lacks a 70 kb plasmid and unstable pgm locus essential for virulence. Though *Y. pestis* is most commonly isolated from animal species, a few close relatives (*Y. pseudotuberculosis* and *Y. ruckeri*)[7-8] are regularly isolated from aquatic environments.[9] Finally, *Xanthomonas axonopodis* (ATCC 8718) represents the plant pathogens *X. oryzae* and *Xylella fastidiosa* (Xanthomonadaceae). *X. axonopodis* (ATCC 8718) is also pathogenic toward plants, causing citrus cancers and black rot, though its status as a BSL-1 pathogen makes it an attractive candidate for high-throughput cultivation studies.

References

1. Moustafa, D.; Garg, V. K.; Jain, N.; Sriranganathan, N.; Vemulapalli, R. Immunization of mice with gamma-irradiated *Brucella neotomae* and its recombinant strains induces protection against virulent *B. abortus, B. melitensis*, and *B. suis* challenge. *Vaccine* **2011**, *29*, 784-794.
2. Sohn, A. H.; Probert, W. S.; Glaser, C. A.; Gupta, N.; Bollen, A. W.; Wong, J. D.; Grace, E. M.; McDonald, W. C. Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. *Emerging Infect. Dis.* **2003**, *9*, 485-488.
3. Cutler, S. J.; Whatmore, A. M.; Commander, N. J. Brucellosis--new aspects of an old disease. *J. Appl. Microbiol.* **2005**, *98*, 1270-1281.

4. Kersters, K.; Vos, P. D.; Gillis, M.; Swings, J.; Vandamme, P.; Stackebrandt, E., *Introduction to the Proteobacteria*. 2006; Vol. 5, p 3-37.

5. Menard, A.; Monnez, C.; Estrada de Los Santos, P.; Segonds, C.; Caballero-Mellado, J.; Lipuma, J. J.; Chabanon, G.; Cournoyer, B. Selection of nitrogen-fixing deficient *Burkholderia vietnamiensis* strains by cystic fibrosis patients: involvement of nif gene deletions and auxotrophic mutations. *Environ. Microbiol.* **2007**, *9*, 1176-1185.

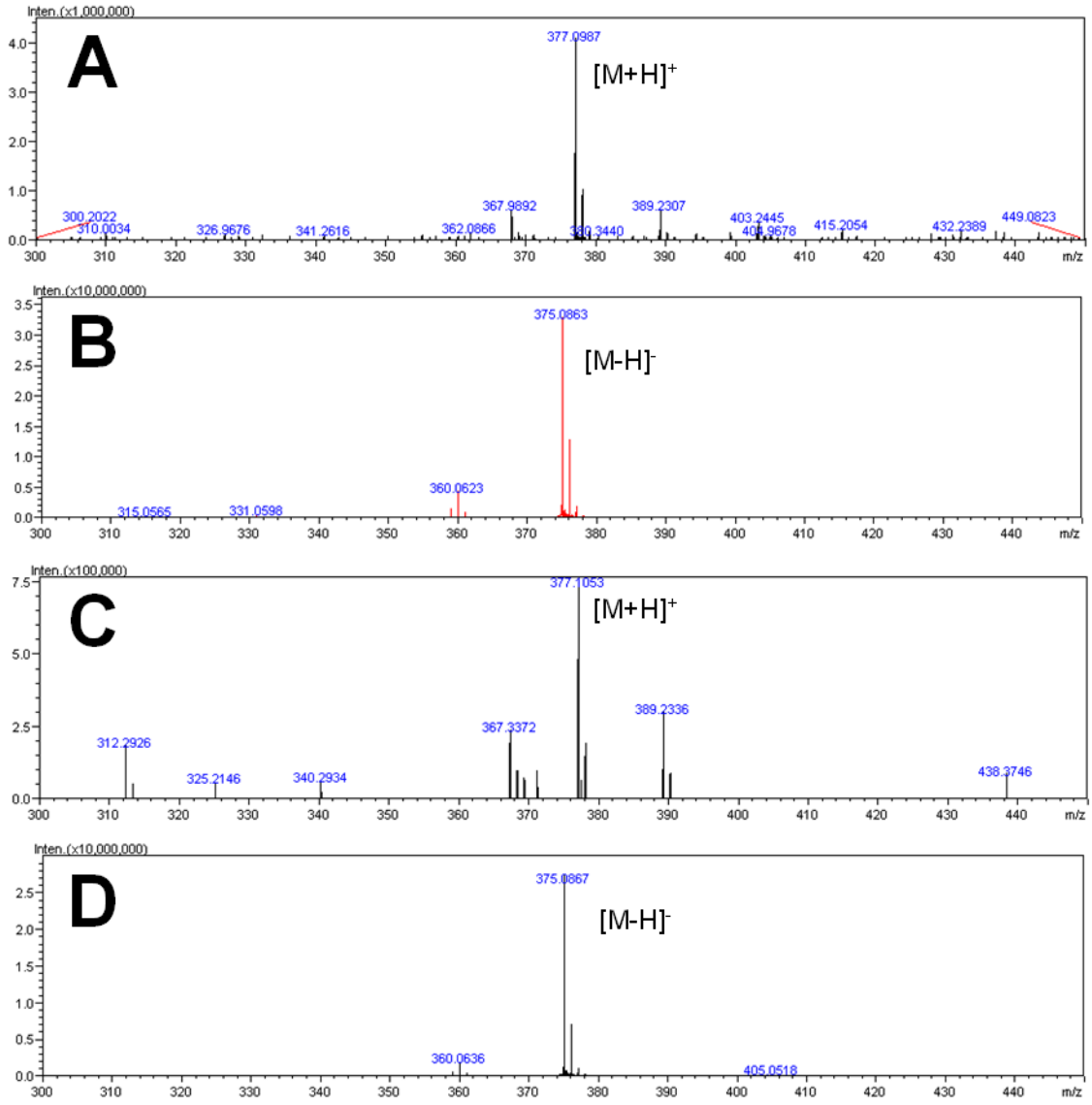
6. Conway, B. A.; Greenberg, E. P. Quorum-sensing signals and quorum-sensing genes in *Burkholderia vietnamiensis*. *J. Bacteriol.* **2002**, *184*, 1187-1191.

7. Welch, T. J.; Fricke, W. F.; McDermott, P. F.; White, D. G.; Rosso, M. L.; Rasko, D. A.; Mammel, M. K.; Eppinger, M.; Rosovitz, M. J.; Wagner, D.; Rahalison, L.; Leclerc, J. E.; Hinshaw, J. M.; Lindler, L. E.; Cebula, T. A.; Carniel, E.; Ravel, J. Multiple antimicrobial resistance in plague: an emerging public health risk. *PLoS ONE* **2007**, *2*, e309.

8. Chain, P. S.; Carniel, E.; Larimer, F. W.; Lamerdin, J.; Stoutland, P. O.; Regala, W. M.; Georgescu, A. M.; Vergez, L. M.; Land, M. L.; Motin, V. L.; Brubaker, R. R.; Fowler, J.; Hinnebusch, J.; Marceau, M.; Medigue, C.; Simonet, M.; Chenal-Francisque, V.; Souza, B.; Dacheux, D.; Elliott, J. M.; Derbise, A.; Hauser, L. J.; Garcia, E. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci.* **2004**, *101*, 13826-13831.

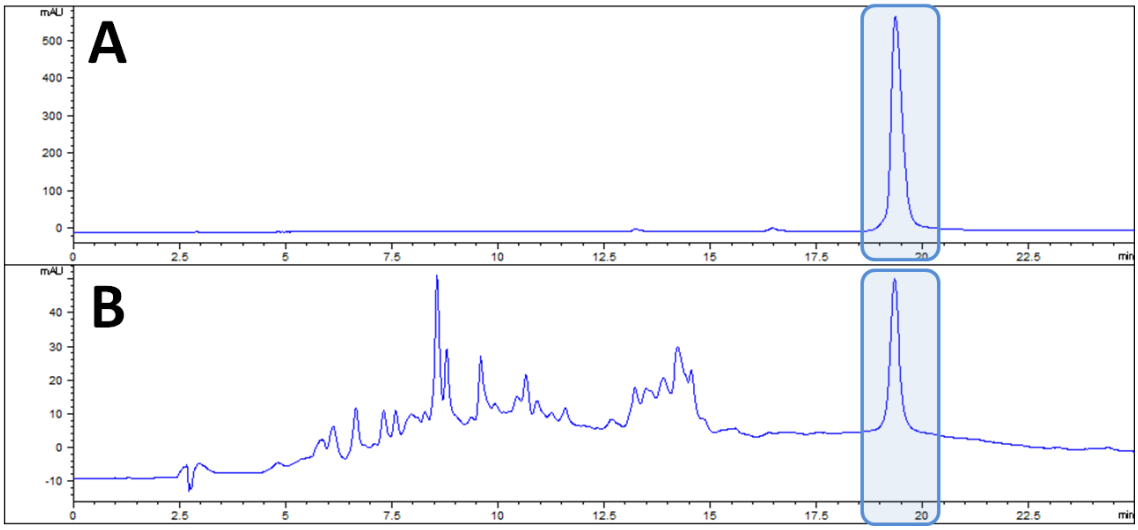
9. Janda, M. J. A., S. L. , *The Family Enterobacteriaceae. In 2nd Edition*. 2 ed.; 2009; Vol. 5.

Figure A33 High-resolution mass spectrometry (IT-TOF) analysis of resistomycin (**3**)



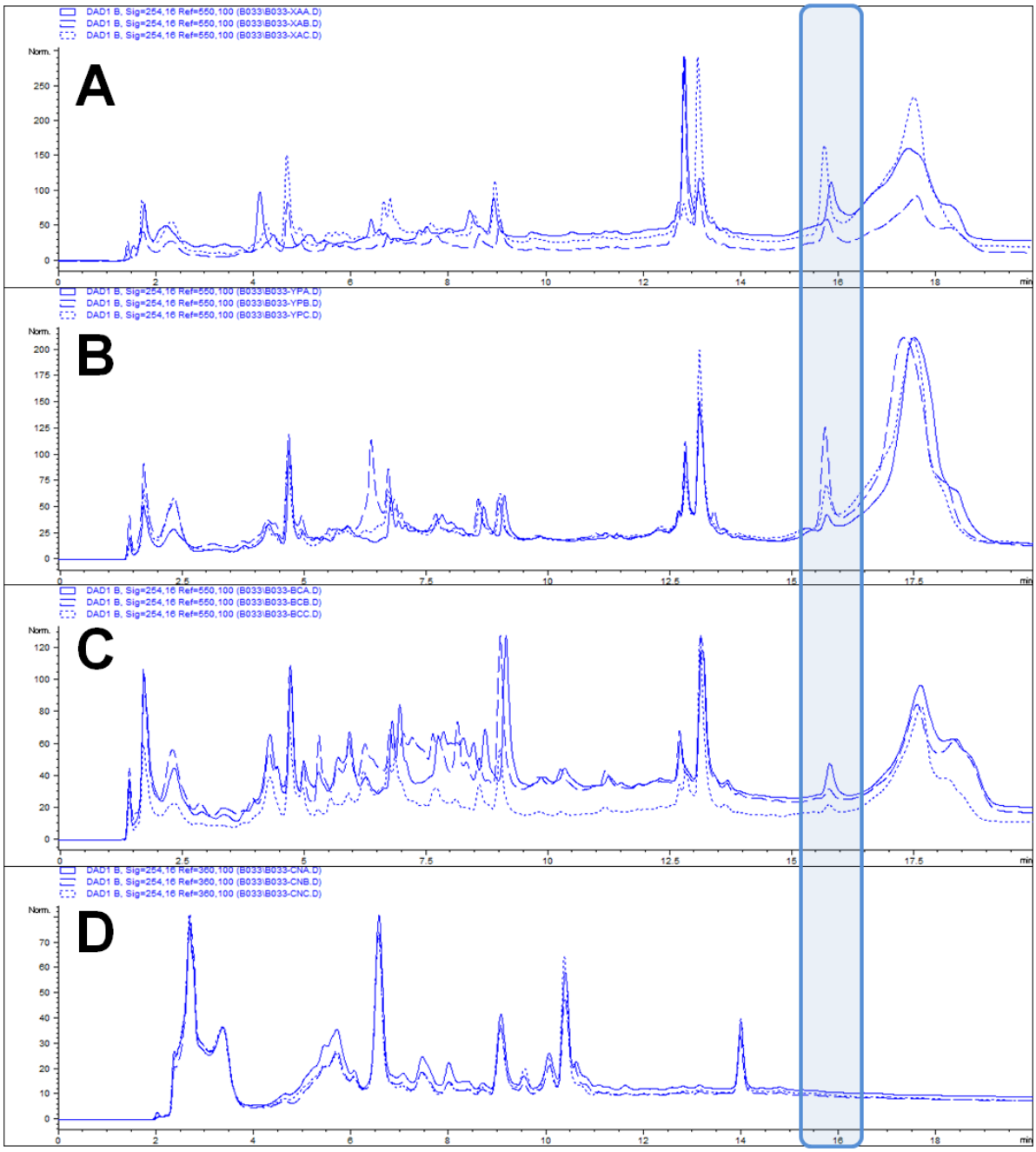
High-resolution mass spectrometry (IT-TOF) analysis of the presence of resistomycin in the co-culture extract of B033-*B. vietnamiensis*. Panel A and B are from the co-culture extract of B033-*B. vietnamiensis* at t_R= 17.9 min, with ionization under positive (A) and negative (B) modes. Panel C and D were generated from a resistomycin standard under positive (C) and negative (D) ionization modes (red color indicates detector saturation). The mass difference between the resistomycin standard and the resistomycin observed in the chromatogram is Δ *m/z* = 0.0116 for the positive mode and Δ *m/z* = 0.0197 for the negative mode.

Figure A34 Identification of resistomycin in B033-*B. vietnamiensis* co-culture extracts



Panel A is the HPLC chromatogram of a resistomycin standard and Panel B is the HPLC chromatogram of the B033-*B. vietnamiensis* co-culture extract.

Figure A35 Resistomycin identified in extracts of all Proteobacteria-B033 cultivation experiments



HPLC chromatograms of the co-culture extracts of B033-*Xanthomonas axonopodis* (A), B033-*Yersinia pestis* (B), and B033-*Brucella neotomae* (C) observed at 254 nm, and cultivated in triplicate. Panel D is the monoculture extract of B033. The shaded box indicates the presence of resistomycin in the co-cultures. The B033-*B. vietnamiensis* experiment is depicted in Figure A34.

Table A3 Ingredients of media types used in nutrient study

	Components per liter
Freshwater-based ISP2	10 g malt extract, 4.0 g yeast extract, 4.0 g dextrose, 500 mL filtered Lake Michigan water, 500 mL deionized water
High starch	10 g starch, 0.3 g casein, 2.0 g potassium nitrate, 2.0 g sodium chloride, 0.05 g dipotassium phosphate, 0.02 g calcium carbonate, 0.01 g iron sulfate heptahydrate, 500 mL filtered Lake Michigan water, 500 mL deionized water
Minimal freshwater-based A1	1.0 g starch, 0.4 g yeast extract, 0.2 g peptone, 500 mL filtered Lake Michigan water, 500 mL deionized water
Lake water	Filtered Lake Michigan water
DI water	Autoclaved deionized water
Freshwater-based A1	10 g starch, 4.0 g yeast extract, 2.0 g peptone, 500 mL filtered Lake Michigan water, 500 mL deionized water
Nutrient rich A1	30 g Instant Ocean ® (Purchased from Pet Co. Animal Supplies, Inc.) , 10 g starch, 4.0 g yeast extract, 2.0 g peptone 1L deionized water

Strain B033 was cultivated using five different nutrient conditions and two nutrient-deprived conditions in an attempt to induce resistomycin production from strain B033 in monoculture.

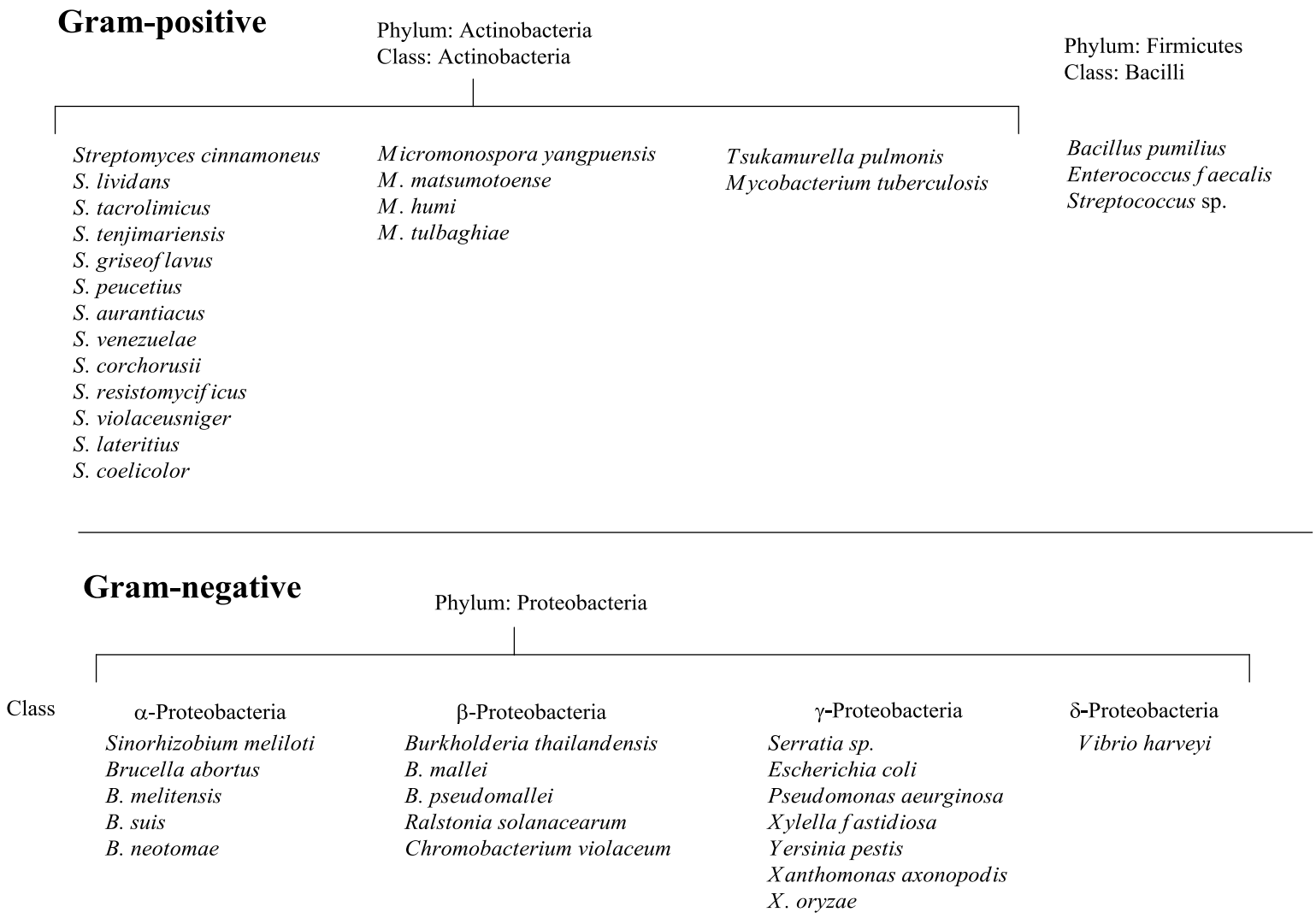
Table A4 Panel of Proteobacteria used in follow-up cultivation studies

Rm obs	Strain ID	GenBank Accession number	Collection Site	Phylum	Class	Genus	Species
no	B912	KM678251	L. Michigan	Actinobacteria	Actinobacteria	*Arthrobacter*	sp.
yes	E906	KM678263	Florida Keys	Actinobacteria	Actinobacteria	*Microbacterium*	sp.
no	D916	KM678268	Massachusetts	Actinobacteria	Actinobacteria	*Microbacterium*	sp.
no	B017	KM678241	L. Michigan	Actinobacteria	Actinobacteria	*Micromonospora*	*aurantiaca*
no	B005	KM678240	L. Michigan	Actinobacteria	Actinobacteria	*Micromonospora*	*chokoriensis*
no	K910	KP203951	Iceland	Actinobacteria	Actinobacteria	*Rhodococcus*	sp.
no	D918	KM678270	Massachusetts	Actinobacteria	Actinobacteria	*Salinibacterium*	sp.
no	B906	KM678246	L. Michigan	Actinobacteria	Actinobacteria	*Sanguibacter*	*antarcticus*
no	B950	KM678258	L. Michigan	Actinobacteria	Actinobacteria	*Sanguibacter*	*antarcticus*
no	B035	KM678243	L. Michigan	Actinobacteria	Actinobacteria	*Streptomyces*	*flavogriseus*
no	B025	KM678242	L. Michigan	Actinobacteria	Actinobacteria	*Streptomyces*	*koyangensis*
no	B917	KM678254	L. Michigan	Bacteroidetes	Flavobacteriia	*Elizabethkingia*	*miricola*
no	B914	KM678252	L. Michigan	Bacteroidetes	Flavobacteriia	*Elizabethkingia*	sp.
no	B916	KM678253	L. Michigan	Bacteroidetes	Flavobacteriia	*Elizabethkingia*	sp.
no	K918	KM678264	Iceland	Firmicutes	Bacilli	*Bacillus*	*amyloliquefaciens*
no	K927	KM678275	Iceland	Firmicutes	Bacilli	*Bacillus*	*hwajinpoensis*
yes	K940	KM678279	Iceland	Firmicutes	Bacilli	*Bacillus*	*muralis*
no	C915	KM678266	L. Tampier	Firmicutes	Bacilli	*Bacillus*	*pumilus*
no	K932	KM678276	Iceland	Firmicutes	Bacilli	*Bacillus*	sp.
no	J908	KM678272	L. Huron	Firmicutes	Bacilli	*Bacillus*	sp.
no	J910	KM678273	L. Huron	Firmicutes	Bacilli	*Bacillus*	sp.
no	J915	KM678274	L. Huron	Firmicutes	Bacilli	*Bacillus*	sp.
no	C905	KM678259	L. Tampier	Firmicutes	Bacilli	*Bacillus*	sp.
no	C916	KM678260	L. Tampier	Firmicutes	Bacilli	*Bacillus*	sp.
no	D905	KM678261	Massachusetts	Firmicutes	Bacilli	*Bacillus*	sp.
no	D913	KM678267	Massachusetts	Firmicutes	Bacilli	*Bacillus*	sp.
no	D917	KM678269	Massachusetts	Firmicutes	Bacilli	*Bacillus*	sp.
no	B932	KM678257	L. Michigan	Firmicutes	Bacilli	*Brevibacillus*	sp.

Rm obs	Strain ID	GenBank Accession number	Collection Site	Phylum	Class	Genus	Species
no	*E. faecalis*		ATCC 29212	Firmicutes	Bacilli	*Enterococcus*	*faecalis*
no	E901	KM678271	Florida Keys	Firmicutes	Bacilli	*Paenibacillus*	sp.
no	D920	KP203953	Massachusetts	Firmicutes	Bacilli	*Paenibacillus*	sp.
no	B931	KP203952	L. Michigan	Proteobacteria	α-Proteobacteria	*Brevundimonas*	sp.
yes	B911	KM678250	L. Michigan	Proteobacteria	α-Proteobacteria	*Brevundimonas*	*vesicularis*
yes	B908	KM678247	L. Michigan	Proteobacteria	α-Proteobacteria	*Brevundimonas*	sp.
yes	*B. neotomae*		ATCC 23459	Proteobacteria	α-Proteobacteria	*Brucella*	*neotomae*
yes	K929	KP203954	Iceland	Proteobacteria	α-Proteobacteria	*Caulobacter*	*henricii*
yes	K934	KM678277	Iceland	Proteobacteria	α-Proteobacteria	*Caulobacter*	sp.
no	K935	KM678278	Iceland	Proteobacteria	α-Proteobacteria	*Caulobacter*	sp.
yes	K936	KM678265	Iceland	Proteobacteria	α-Proteobacteria	*Caulobacter*	sp.
no	B903	KM678245	L. Michigan	Proteobacteria	α-Proteobacteria	*Sphingomonas*	sp.
yes	B910	KM678249	L. Michigan	Proteobacteria	α-Proteobacteria	*Sphingomonas*	sp.
yes	B918	KM678255	L. Michigan	Proteobacteria	α-Proteobacteria	*Sphingomonas*	sp.
no	B929	KM678256	L. Michigan	Proteobacteria	α-Proteobacteria	*Sphingomonas*	sp.
yes	*B. vietnamiensis*		ATCC BAA-254	Proteobacteria	β-Proteobacteria	*Burkholderia*	*vietnamiensis*
no	D907	KM678262	Massachusetts	Proteobacteria	γ-Proteobacteria	*Cobetia*	*marina*
yes	*E. coli*		ATCC 25922	Proteobacteria	γ-Proteobacteria	*Escherichia*	*coli*
no	*P. aeruginosa*		ATCC 27853	Proteobacteria	γ-Proteobacteria	*Pseudomonas*	*aeruginosa*
yes	B901	KM678244	L. Michigan	Proteobacteria	γ-Proteobacteria	*Pseudomonas*	sp.
no	B909	KM678248	L. Michigan	Proteobacteria	γ-Proteobacteria	*Pseudomonas*	sp.
yes	*X. axonopodis*		ATCC 8718	Proteobacteria	γ-Proteobacteria	*Xanthomonas*	*axonopodis*
yes	*Y. pestis*		A1122	Proteobacteria	γ-Proteobacteria	*Yersinia*	*pestis*

Environmental isolates used in follow-up co-culture studies with strain B033 (*Streptomyces* sp.). Strains were identified on the basis of 16S rRNA gene sequence identity with the most closely related type strain. Rm obs = resistomycin observed. Strains used in the follow-up study were identified on the basis of their 16S ribosomal RNA gene. The corresponding sequences have been uploaded to NCBI GenBank with the accession numbers indicated above.

Figure A36 Listing of bacterial strains mentioned in this dissertation



LICENSE TO USE PUBLISHED WORKS

ACS Publications
Most Trusted. Most Cited. Most Read.

Title:	Phylum-Specific Regulation of Resistomycin Production in a Streptomyces sp. via Microbial Coculture
Author:	Skylar Carlson, Urszula Tanouye, Sesselja Omarsdottir, et al
Publication:	Journal of Natural Products
Publisher:	American Chemical Society
Date:	Dec 1, 2014

Logged in as:
Skylar Carlson
LOGOUT

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms & Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from (COMPLETE REFERENCE CITATION). Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your request. No additional uses are granted (such as derivative works or other editions). For any other uses, please submit a new request.

AMERICAN SOCIETY FOR MICROBIOLOGY

Title:	Tools for Characterizing Bacterial Protein Synthesis Inhibitors
Author:	Cédric Orelle, Skylar Carlson, Bindiya Kaushal et al.
Publication:	Antimicrobial Agents and Chemotherapy
Publisher:	American Society for Microbiology
Date:	Dec 1, 0001

Permissions Request

Authors in ASM journals retain the right to republish discrete portions of his/her article in any other publication (including print, CD-ROM, and other electronic formats) of which he or she is author or editor, provided that proper credit is given to the original ASM publication. ASM authors also retain the right to reuse the full article in his/her dissertation or thesis. For a full list of author rights, please see: http://journals.asm.org/site/misc/ASM_Author_Statement.xhtml

SKYLAR CARLSON

Education

B.S. Biochemistry, 2006-2010, Florida State University, Tallahassee, FL
Research topics: Prolonged odorant stimulation sculpts olfactory sensory neurons, Advisor: Dr. Debra Ann Fadool; and Epothilone E total synthesis: a critical literature review, Advisor: Dr. Mark Kearley

PhD, Pharmacognosy, 2010- 2015, University of Illinois at Chicago, Chicago, IL
Title of Dissertation: Secondary metabolite regulation and drug lead discovery from aquatic actinomycetes. Advisor: Dr. Brian T. Murphy

Awards and Honors

Chancellor's Student Service and Leadership Award, 2015
Invited lecturer at Chicago State University, 2013-2015
Poster Award, Third Place, David J. Slatkin Symposium, November 14, 2014
Travel Award, American Society of Pharmacognosy, July 14, 2013
Van Doren Scholar, UIC College of Pharmacy, February 22, 2013
Poster Award, First Place, David J. Slatkin Symposium, October 19, 2012
Graduate College Travel Award, UIC Graduate College, June 9, 2011

I. Research

Publications

Mullowney, M.; Hwang, C; Newsome, A; Wei, X; Tanouye, U; Wan, B; Carlson, S; Barranis, N; Krishnamoorthy, K; White, J; Blair, R; Lee, H; Rathod, P ; Parish, T; Cho, S; Franzblau, S; Murphy, B. Diaza-anthracene antibiotics from a freshwater-derived actinomycete that selectively inhibit *M. tuberculosis*. **ACS Infectious Diseases** Article ASAP DOI: 10.1021/acsinfecdis.5b00005

Carlson, S.; Tanouye, U; Omarsdottir, S; and Murphy, B. T. Phylum-Specific Regulation of Resistomycin Production in a *Streptomyces* sp. via Microbial Co-Culture. **Journal of Natural Products** (2014) Article ASAP DOI: 10.1021/np500767u.

Orelle, C; Carlson, S; Kaushal, B; Almutairi, MM; Liu, H; Ochabowicz, A; Quan, S; Pham, VC; Squires, CL; Murphy, BT; Mankin, A, (2013). Tools for characterizing bacterial protein synthesis inhibitors. **Antimicrobial Agents and Chemotherapy** 57, 5994-6004.

Carlson, S; Marler, L; Nam, SJ; Santarsiero, BD; Pezzuto, JM; and Murphy, BT (2013). Potential chemopreventive activity of a new macrolide antibiotic from a marine-derived *Micromonospora* sp. **Marine Drugs** 11, 1152-1161.

Publications in Preparation or submitted

M. P. Puglisi; J. Sears; K. Ferguson; A. Nelson; T. Nguyen; U. Tanouye; S. Carlson; B. T. Murphy; T. Weyna; and J. Kwan. Effects of Florida algal extracts on environmental strains of bacteria from Florida and other regions of the world. **Applied and Environmental Microbiology** (In preparation).

SKYLAR CARLSON

I. Research

Research Laboratory of Dr. Brian T. Murphy, PhD laboratory
University of Illinois at Chicago, Chicago, IL January 2011- 2015

CHEMISTRY

- Biological and chemical profiling of bacterial co-culture experiments (sample size N=1,000).
- Extensive dereplication using Antimarin, SciFinder, Reaxys and an in-house UV library.
- Advanced reversed phase chromatography with routine use of TLC, SPE, open column chromatography, analytical, semi-preparative and preparative systems.
- Experienced at mass spectrometry analysis utilizing high and low resolution, ESI and CI MS, as well as triple-quadrupole and ionization transfer-time of flight on HPLC and UPLC.
- Well versed in structure elucidation including the acquisition and data processing of 1D and 2D NMR spectra.
- Knowledgeable in chemical derivitization and spectroscopic means (CD, OR, ROESY and NOSEY) for the determination of stereochemistry.
- Routine maintenance and repair of HPLC systems.

MICROBIOLOGY

- Developed a co-culture method to study the induction of antibiotic production by environmental bacteria.
- Isolation, cryo-preservation, cultivation, and extraction methods for both Gram-negative and Gram-positive bacteria. Independently built a library of 125 Gram-negative and Gram-positive bacterial isolates and identified using 16S rRNA sequencing.
- Optimized and routinely performed an in-house *in vitro* growth inhibition bioassays for 6 Gram-negative and 2 Gram-positive BSL-2 pathogens.

COLLECTION EXPERIENCE

- Organized a mobile laboratory for the first collection trip for my laboratory. Worked with my PI to assemble all the neccessary "travel-size" items for our first collection trip.
- Assisted in the sampling of PONAR and preservation of samples for further analysis by metagenomic techniques. Field preparation of sediment and plating while collecting.
- Helped to organize a month long collection trip trip across all five of the Great Lakes.
- Completed primary contacts and literature research on best collection sites for freshwater sponges from Lake Michigan.

LEADERSHIP IN A LABORATORY

- First graduate student in my laboratory and subsequently trained future graduate students.
- Wrote protocols and standard operating procedures for the laboratory.
- Extensive mentorship experience with all levels of students on research projects.

RESEARCH PROPOSALS SUBMITTED

- Fall 2011, National Science Foundation Graduate Research Fellowship Program "Culture independent mycobacterial studies to guide tuberculosis drug discovery efforts."
- Spring 2012, (Preliminary examination proposal, National Institutes of Health R21 format) "Co-culture based approach to induce antibiotics to combat biodefense pathogens."

SKYLAR CARLSON

I. Research

Research Laboratory of Dr. Neil Kelleher
Northwestern University July 2013

- Using the PRiSM system, we examined the expression of biosynthetic gene clusters at the protein level in *Streptomyces* sp. strain B033 during co-culture.
- High resolution mass spectrometric profiling of protein digests and database mining of identified peptide fragments using sequencing software.
- Protein precipitation, quantification, and digestion for LCMS analysis.

Research Laboratory of Dr. Guido F. Pauli
University of Illinois at Chicago, Chicago, IL Fall 2010

- Determined Log P of pure compounds isolated from Trifolium pratense
- Analyzed the differences in Log P of pure compounds in various solvent systems utilizing mechanical techniques and HPLC to optimize determination.

Oral Presentations (‡ represents competitively selected)

1.‡ Carlson S, Tanouye U, Murphy BT. The Induction of Antibiotics to Combat Biodefense Pathogens. American Society of Pharmacognosy Annual Meeting, St. Louis, MO. July 14, 2013.
2.‡ Carlson S, Tanouye U, Murphy BT. The Induction of Antibiotics to Combat Biodefense Pathogens. Minnesota-Iowa-Kansas-Illinois Medicinal Chemistry Meeting, Minneanapolis, MN. April 13, 2013.
3. Carlson S, Tanouye U, Murphy BT. A co-culture based approach toward the induction of antibiotics to combat biodefense pathogens. UIC Center for Pharmaceutical Biotechnology, Chicago, IL. January 9, 2013.
4. Carlson S, Tanouye U, Murphy BT. Enterococcus faecalis inhibitor(s) from marine actinomycete strain A003. UIC Center for Pharmaceutical Biotechnology, Chicago, IL. January 25, 2012.

Poster Presentations

1. Carlson S, Tanouye U, Murphy BT. Phylum-specific regulation of an antibiotic in a Streptomyces sp. via microbial co-culture. UIC College of Pharmacy Research Day, Chicago, IL. February 27, 2015.
2. Carlson S, Tanouye U, Murphy BT. Phylum-specific regulation of an antibiotic in a Streptomyces sp. via microbial co-culture. David J. Slatkin Symposium, Chicago, IL. November 15, 2014.
3. Carlson S, Tanouye U, Murphy BT. Phylum-specific regulation of an antibiotic in a Streptomyces sp. via microbial co-culture. Small Molecule Discovery in Academia, Chicago, IL. October 9, 2014.
4. Carlson S, Tanouye U, Murphy BT. Phylum-specific regulation of an antibiotic in a Streptomyces sp. via microbial co-culture. Midwest Microbial Pathenogensis Conference, Chicago, IL. September 14, 2014.

I. Research

Poster Presentations (continued)

5. Carlson S, Tanouye U, Murphy BT. A co-culture based approach toward the induction of antibiotics to combat biodefense pathogens. 2014 Molecular Biology Research Building (MBRB) retreat, Chicago, IL. June 3, 2014.
6. Carlson S, Tanouye U, Murphy BT. A co-culture based approach toward the induction of antibiotics to combat biodefense pathogens. UIC College of Pharmacy Research Day, Chicago, IL. February 22, 2013.
7. Carlson S, Tanouye U, Murphy BT. A co-culture based approach toward the induction of antibiotics to combat biodefense pathogens. David J. Slatkin Symposium, Chicago, IL. October 20, 2012.
8. Carlson S, Wei X, Tanouye U, Hilliard T, Burdette J, Murphy BT. Marine Actinomycetes as a Source of Novel Ovarian Cancer Drug Leads. Small Molecule Discovery in Academia, Chicago, IL. July 9, 2012.
9. Carlson S, Wei X, Tanouye U, Hilliard T, Burdette J, Murphy BT. Marine Actinomycetes as a Source of Novel Ovarian Cancer Drug Leads. Center for Molecular Innovations in Drug Discovery, Chicago, IL. October 12, 2011.
10. Carlson S, Wei X, Tanouye U, Hilliard T, Burdette J, Murphy BT. Marine Actinomycetes as a Source of Novel Ovarian Cancer Drug Leads. Central Regional Meeting of American Chemical Society, Indiannapolis, IN, June 9, 2011.
11. Carlson S, Wei X, Tanouye U, Hilliard T, Burdette J, Murphy BT. Marine Actinomycetes as a Source of Novel Ovarian Cancer Drug Leads. Minnesota-Iowa-Kansas-Illinois Medicinal Chemistry Meeting, Lawrence, KS, April 8-10, 2011.

II. Teaching

Teaching Development

University of Illinois at Chicago, Course, Spring 2014, Foundations of College Teaching
- Studied the basics of teaching theory and best practices in modern secondary education.
- Developed teaching portfolio (available upon request)

University of Illinois at Chicago, Year-long Course, 2011-2012, Recipient/Participant
- "Graduate Student-Clinical Fellows/Residents Teaching Certificate Program"
- Cross disciplinary education on teaching at a college of pharmacy.

SKYLAR CARLSON

II. Teaching

Teaching Experience

Midtown/Metro, Present, 10 students, 2011- current, Math and Science Tutor
- Tutored inner-city girls in math and science
- Served as a role model to young inner city women as both an academic researcher and a woman in science and secondary education.

Chicago State University, 15 students, Spring 2015, PHAR6532 Chemistry of Natural Products
- Delivered one, 120 minute lecture to PharmD students
- "Inducing Secondary Metabolism Through Bacterial Co-culture"
- Introduced grant writing and the academic research process.

Chicago State University, 30 students, Spring 2014, PHAR6532 Chemistry of Natural Products
- Delivered two, 120 minute lectures to PharmD students
- "Methods in Natural Products Drug Discovery"
- "Inducing Secondary Metabolism Through Bacterial Co-culture"

Chicago State University, 10 students, Fall 2013, PHAR6532 Chemistry of Natural Products
- Delivered two, 120 minute lectures to PharmD students
- "Methods in Natural Products Drug Discovery"
- "Inducing Secondary Metabolism Through Bacterial Co-culture"

Mentorship as a Graduate Student in the Research Setting

Dates of Mentorship	Student, Education at time of Mentorship	Current Education
Summer 2011	Gennaro Paolella, Undergraduate	Pharmacy student, PY1 University of IL at Chicago
Summer 2012	Matthew Hennings, High school student	School of Engineering U. Nebraska-Lincoln
2012-2013	Grace Li, IMSA High school student	Chemical Engineering Cooper Union, NY
Summer 2013	Taylor Highland, Research Assistant	Pharmacy student, PY4 Chicago State University
Fall 2014	Taylor Highland, Research Assistant	Pharmacy student, PY4 Chicago State University
Spring 2015	Flora Dong, Pharmacy Masters Student	Masters Candidate University of Iceland

SKYLAR CARLSON

III. Service

2013	Contribution to American Society of Pharmacognosy quarterly newsletter: Carlson, S. (2013). Coates Delivers Inaugural Farnsworth Lecture at UIC. The ASP Newsletter 49, 4.
2013-2015	Student representative to the Dean for Research and Graduate Education's Counsil at UIC College of Pharmacy. The counsil is responsible for events for the graduate students across the college.
2013-2014	UIC Green Labs Committee Member
2013-2014	Chair for the 52nd Annual Minnesota-Iowa-Kansas-Illinois regional medicinal chemistry meeting held at the UIC College of Pharmacy. The MIKI meeting is a student run and student organized event for 225 individuals. Being chair required attention to detail, teamwork, fundraising, and working with the diverse group of individuals.
2013-2014	College of Pharmacy's Diversity Strategic Thinking and Planning Committee – Recruitment and Retention, Promotion and Tenure Subcommittees.
2014	Moderator for the David J. Slatkin Symposium, November 14, 2014 at the Chicago State University College of Pharmacy.
2014-2015	Founding president of the Medicinal Chemistry and Pharmacognosy Graduate Student Association.
2015	Chair, Program Committee of the Chicago Section of the American Chemical Society