July 2017

Chemical Investigations of Fungal Natural Products for Drug Discovery

Danielle H. Demers
University of South Florida, DHDemers@gmail.com

Follow this and additional works at: https://digitalcommons.usf.edu/etd

Part of the Chemistry Commons

Scholar Commons Citation

This Dissertation is brought to you for free and open access by the USF Graduate Theses and Dissertations at Digital Commons @ University of South Florida. It has been accepted for inclusion in USF Tampa Graduate Theses and Dissertations by an authorized administrator of Digital Commons @ University of South Florida. For more information, please contact digitalcommons@usf.edu.
Chemical Investigations of Fungal Natural Products

for Drug Discovery

by

Danielle H. Demers

A dissertation submitted in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Chemistry
College of Arts and Sciences
University of South Florida

Major Professor: Bill J. Baker, Ph.D.
Lindsey N. Shaw, Ph.D.
James W. Leahy, Ph.D.
Edward Turos, Ph.D.

Date of Approval:
June 27, 2017

Keywords: screening, epigenetic modification, *Phomopsis, Penicillium*, ESKAPE, leishmaniasis

Copyright © 2017, Danielle H. Demers
Dedication

This work is wholeheartedly dedicated to my incredible family. To say I wish to thank my parents, Chris and Sharon, hardly sounds like enough. In my entire life, these two have never doubted me for one second. I would not be the person I am today without their ceaseless support, encouragement, sacrifice, and love. Truly, this body of work would not exist were it not for all the phone calls, packages, emails, and visits that made Florida feel a little less far from home over the past 6 years. I am so lucky to have them, and the amazing family they have built, in my corner.

To my brothers, Luke and CJ, and my “older sister” Cyndi, thank you for your love, friendship, and patience with me over these years. To the friends and extended family members that are far too numerous to list here (such is the blessing and the curse of such a beautifully large, crazy clan), thank you for your continued interest, compassion, and support. And finally, to Kieryn Emilee, I dedicate this to you, and hope you always remember that nothing and no one can keep you from your dreams.

“Keep moving forward”

~ Walt Disney
Acknowledgements

This dissertation is built on the shoulders of giants and the work of an army of scientists. I must first and foremost thank Dr. Bill Baker for sharing his knowledge, financial support, and for telling me “no” when I did not want to hear it. I was blessed with amazing experiences, opportunities, and life lessons during my years here, and none of it would have been possible without you.

To my committee, past and present Baker Lab members, collaborating teams at USF and beyond, and my many undergrads: thank you for your help, enthusiasm, and dedication to this work. No scientist works alone and I am so grateful to have met and worked with so many excellent researchers.

To the colleagues who became friends, and the friends who became family: I would not have survived without you. There are so many people who deserve to be acknowledged for their comradery over these past 6 years, and though I cannot possibly list you all, please know that your contributions to my life here at USF do not go unrecognized. Thank you for turning Florida into my second home. I cannot wait to see what comes next for all of us.
# Table of Contents

List of Tables ........................................................................................................................................ iii

List of Figures ......................................................................................................................................... iv

List of Schemes ...................................................................................................................................... ix

List of Abbreviations and Units ............................................................................................................. x

Abstract .................................................................................................................................................. xiii

Chapter 1. An Introduction to Marine Natural Product Drug Discovery .............................................. 1
  1.1. Why Natural Products ...................................................................................................................... 1
  1.2. Sources of Marine Natural Products ............................................................................................... 2
    1.2.1. Marine Macro-organisms .............................................................................................................. 2
    1.2.2. Marine Micro-organisms ............................................................................................................. 3
    1.2.3. Marine Plant Endophytes ........................................................................................................... 4
    1.2.4. Collection Locations and Non-marine Sources ......................................................................... 5
      1.2.4.1. Antarctica ................................................................................................................................. 5
      1.2.4.2. Florida ....................................................................................................................................... 7
      1.2.4.3. Terrestrial and fresh water environments ............................................................................. 8
  1.3. Approaches Towards the Isolation of Microbial Natural Products ................................................ 9
    1.3.1. Collection and Isolation .............................................................................................................. 9
    1.3.2. Culture Techniques .................................................................................................................... 10
  1.4. Drug Discovery Targets ................................................................................................................... 11
    1.4.1. The ESKAPE Pathogens ............................................................................................................. 13
    1.4.2. Leishmania donovani .................................................................................................................. 13
    1.4.3. Mycobacterium tuberculosis ..................................................................................................... 13
    1.4.4. Clostridium difficile .................................................................................................................... 14
    1.4.5. Naegleria fowleri ....................................................................................................................... 14
    1.4.6. Cancer Targets ............................................................................................................................ 15
  1.5. Future Directions ............................................................................................................................. 15
  1.6. References ...................................................................................................................................... 16

Chapter 2. New Bioactive Meroterpenes from Phomopsis sp. ................................................................. 23
  2.1. BB11-2 Isolation ............................................................................................................................... 23
  2.2. Initial Bioactivities and Scale-up .................................................................................................... 24
  2.3. Compound Isolation and Structure Elucidation .............................................................................. 25
  2.4. Bioactivities .................................................................................................................................... 38
  2.5. Future Directions ............................................................................................................................. 40
  2.6. References ..................................................................................................................................... 40
Chapter 3. The Creation of a Large Fungal Isolate and Extract Library .................................................. 42
  3.1. Curating a Fungal Library ................................................................................................................. 42
  3.2. Screening Protocols, Accomplishments ......................................................................................... 45
  3.3. Metabolomic Analysis ....................................................................................................................... 47
  3.4. Training Set Results ......................................................................................................................... 50
  3.5. Future Directions ............................................................................................................................. 52
  3.6. References ....................................................................................................................................... 52

Chapter 4. A New Citreohybriddione Discovered Via Epigenetic Modification .......................... 54
  4.1. Citreohybridones and Citreohybriddiones ....................................................................................... 54
  4.2. KML12-14MG-B2a Isolation ........................................................................................................... 55
  4.3. Initial Bioactivities, Scale-up, Epigenetic Modification, and Identification ............................... 57
  4.4. Compound Isolation and Structure Elucidation .............................................................................. 59
  4.5. Future Directions ............................................................................................................................. 63
  4.6. References ....................................................................................................................................... 64

Appendix A: Experimental and Supporting Data for Chapter 2 .................................................. 65

Appendix B: Experimental and Supporting Data for Chapter 3 .................................................. 97

Appendix C: Experimental and Supporting Data for Chapter 4 .................................................. 103
List of Tables

Table 2.1. 1D and 2D data for phomopsichromin A (1) in CDCl₃. ........................................26

Table 2.2. 1D and 2D data for phomopsichromin B (2) in CDCl₃. .................................30

Table 2.3. 1D and 2D data for phomopsichromin C (3) in CDCl₃. .................................33

Table 2.4. 1D and 2D data for phomopsichromin D (4) in DMSO-d₆. ............................36

Table 2.5. 1D and 2D data for phomopsichromin E (5) in DMSO-d₆. ............................38

Table 3.1. Collection details for collection trips from Fall 2011 to Spring 2017.............44

Table 3.2. Weekly sequence of events for continuous production of fungal isolates and
extract libraries. .................................................................................................................46

Table 4.1. Screening results for KML12-14MG-B2a. ..............................................57

Table 4.2. 1D and 2D data for citreohybriddione D (1) in CDCl₃. ..............................61

Table A1. The forward and reverse primers used for the fungal strain identification of
BB11-2. .............................................................................................................................66

Table A2. Pure compound bioactivities of 1-6. ...............................................................96
List of Figures

Fig. 1.1. The Antarctic continent as seen from space on Google Earth with common collection sites for natural products featured. ................................................................. 6

Fig. 1.2. The Swiss Polar Institute’s ACE cruise plan, December 2016- March 2017. ............... 7

Fig. 1.3. Left panel: Mangroves growing in highly developed intercoastal waterways in Dunedin, FL; Right panel: Mangroves growing in the protected, pristine Everglades National Park, FL. ................................................................. 8

Fig. 2.1. Chemical structures of new compounds 1-5 and known compound 6. ................. 26

Fig. 2.2. NP HPLC chromatogram of fraction D_C (Scheme 2.1) from the control growth treatment of Phomopsis sp. ................................................................. 27

Fig. 2.3. Important COSY, HMBC, and NOE correlations in phomopsichromin A (1). ....... 29

Fig. 2.4. Important COSY, HMBC, and NOE correlations in phomopsichromin B (2). ....... 31

Fig. 2.5. Methylation reaction of 2 (stereochemistry unknown at the time) to yield 7 with diazomethane. ......................................................................................... 32

Fig. 2.6. Important COSY and HMBC correlations in phomopsichromin C (3). ............... 34

Fig. 2.7. Proposed biosynthetic pathway towards the cyclohexane substructure of 1-6 from Tanabe and Suzuki, 1974. ................................................................. 34

Fig. 2.8. Proposed stereocenters in the cyclohexane ring of 3 sent for ECD calculations. ...... 35

Fig. 2.9. Important COSY, HMBC, and NOE correlations in phomopsichromin D (4). ....... 37

Fig. 2.10. Important COSY and HMBC correlations in phomopsichromin E (5). ............... 37

Fig. 3.1. a) Representative pie chart from bioassay data, in this case, ESKAPE activity, that shows the activity boosting effects of the epigenetic modification; b) Representative pie chart from bioassay data, in this case, ESKAPE activity, that demonstrates distribution of activity across the three treatment conditions. ...... 47

Fig. 3.2. An MDS plot of the Bray Curtis Similarity matrix of square root transformed data for 123 extracts (replicates averaged) from the screening program. ............... 48
Fig. 3.3. An MDS plot of the Bray Curtis Similarity matrix of square root transformed data for 9 randomly selected organisms from those represented in Figure 3.2........49

Fig. 3.4. Selectivity of active extracts.................................................................51

Fig. 4.1. Some of the previously reported citreohybridones, citreohybiddiones A-C, and the new citreohybiddione D (1).................................................................55

Fig. 4.2. Google Maps satellite view of collection areas around Keys Marine Lab, Long Key Florida.................................................................56

Fig. 4.3. The NP MPLC chromatogram of KML12-14MG-B2a HDACi. ...............59

Fig. 4.4. The NP HPLC chromatogram of KML12-14MG-B2a HDACi- F...............60

Fig. 4.5. Important COSY, HMBC, and NOESY correlations in Citreohybiddione D (1).................................................................62

Fig. 4.6. A 3D depiction of 1 for stereochemical review........................................62

Fig. A1. NCBI nucleotide blast results for BB11-2 forward primers. .......................66

Fig. A2. NCBI nucleotide blast results for BB11-2 reverse primers. .......................67

Fig. A3. NP MPLC chromatogram of BB11-2 Control EtOAc partition.....................68

Fig. A4. A second NP MPLC of BB11-2 Control fraction D from the first MPLC separation. Fraction C (bottles 4 & 5) contained the phomopsichromins..........69

Fig. A5. NP MPLC chromatogram of BB11-2 HDACi EtOAc partition.....................70

Fig. A6. NP MPLC chromatogram of BB11-2_DNMTi EtOAc partition.....................71

Fig. A7. NP HPLC chromatogram (black) and ELSD trace (blue) of BB11-2_D_C. ......72

Fig. A8. $^1$H NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin A (1)........74

Fig. A9. $^{13}$C NMR Spectrum (125 MHz, CDCl$_3$) of Phomopsichromin A (1)........75

Fig. A10. $^{1}$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin A (1) ........................................................................................................75

Fig. A11. $^{1}$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin A (1) ........................................................................................................76
Fig. A12. $^1$H-$^1$H gCOSY NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin A (1).................................................................76

Fig. A13. $^1$H-$^1$H NOESY NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin A (1).................................................................77

Fig. A14. $^1$H NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin B (2).................................78

Fig. A15. $^{13}$C NMR Spectrum (125 MHz, CDCl$_3$) of Phomopsichromin B (2).................................79

Fig. A16. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin B (2).................................................................79

Fig. A17. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin B (2).................................................................80

Fig. A18. $^1$H-$^1$H gCOSY NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin B (2).................................................................80

Fig. A19. $^1$H-$^1$H NOESY NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin B (2).................................................................81

Fig. A20. $^1$H NMR Spectrum (400 MHz, CDCl$_3$) of methylated phomopsichromin B (7). A new methoxy signal can be seen at $\delta_H$ 3.92.................................................................81

Fig. A21. $^{13}$C NMR Spectrum (500 MHz, CHCl$_3$) of methylated phomopsichromin B (7). 24 $^{13}$C signals can be seen.................................................................82

Fig. A22. $^1$H NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin C (3).................................83

Fig. A23. $^{13}$C NMR Spectrum (125 MHz, CDCl$_3$) of Phomopsichromin C (3).................................84

Fig. A24. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin C (3).................................................................84

Fig. A25. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin C (3).................................................................85

Fig. A26. $^1$H-$^1$H gCOSY NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin C (3).................................................................85

Fig. A27. $^1$H NMR Spectrum (500 MHz, DMSO-$d_6$) of Phomopsichromin D (4).................................86

Fig. A28. $^{13}$C NMR Spectrum (125 MHz, DMSO-$d_6$) of Phomopsichromin D (4).................................87
Fig. A29. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin D (4)........................................................................................................88

Fig. A30. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, DMSO-$d_6$) of Phomopsichromin D (4)........................................................................................................89

Fig. A31. $^1$H-$^1$H gCOSY NMR Spectrum (500 MHz, DMSO-$d_6$) of Phomopsichromin D (4)........................................................................................................90

Fig. A32. $^1$H NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin E (5) ....................92

Fig. A33. $^{13}$C NMR Spectrum (201 MHz, DMSO-$d_6$) of Phomopsichromin E (5) ....................93

Fig. A34. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin E (5) ........................................................................................................93

Fig. A35. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin E (5) ........................................................................................................94

Fig. A36. $^1$H-$^1$H gCOSY NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin E (5) ........................................................................................................94

Fig. A37. $^1$H NMR Spectrum (600 MHz, CDCl$_3$) of LL-Z1272ε (6)........................................95

Fig. A38. $^{13}$C NMR Spectrum (125 MHz, CDCl$_3$) of LL-Z1272ε (6)........................................96

Fig. C1. NCBI nucleotide blast results for KML12-14MG-B2a forward primers. ...............103

Fig. C2. NCBI nucleotide blast results for KML12-14MG-B2a reverse primers. ...............103

Fig. C3. NP MPLC chromatogram of KML12-14MG-B2a_HDAC EtOAc partition..........104

Fig. C4. NP HPLC ELSD trace of KML12-14MG-B2a_Control_D........................................105

Fig. C5. NP HPLC ELSD trace KML12-14MG-B2a_HDAC_F...........................................105

Fig. C6. NP HPLC ELSD trace of KML12-14MG-B2a_DNMT_C........................................106

Fig. C7. $^1$H NMR Spectrum (500 MHz, CDCl$_3$) of citreohybriddione D.......................107

Fig. C8. $^{13}$C NMR Spectrum (200 MHz, CDCl$_3$) of citreohybriddione D.......................108

Fig. C9. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (800 MHz, CDCl$_3$) of citreohybriddione D. ..............................................................................................................109
**Fig. C10.** $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, CDCl$_3$) of citreohybriddione D. ............................................................................................................................................. 110

**Fig. C11.** $^1$H-$^1$H gCOSY NMR Spectrum (600 MHz, CDCl$_3$) of citreohybriddione D............. 110

**Fig. C12.** $^1$H-$^1$H NOESY NMR Spectrum (800 MHz, CDCl$_3$) of citreohybriddione D......... 111
List of Schemes

Scheme 1.1. A generic flow chart for natural products isolation ........................................... 12

Scheme 2.1. Extraction scheme for *Phomopsis* sp. Control. .................................................. 25
List of Abbreviations and Units

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 3D</td>
<td>1, 2, 3-dimensional</td>
</tr>
<tr>
<td>[α]</td>
<td>specific rotation = 100α/lc</td>
</tr>
<tr>
<td>ACE</td>
<td>Antarctic Circumnavigation Expedition</td>
</tr>
<tr>
<td>br d</td>
<td>broad doublet (NMR)</td>
</tr>
<tr>
<td>br t</td>
<td>broad triplet (NMR)</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>C18</td>
<td>octadecyl bonded silica</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>deuterated chloroform</td>
</tr>
<tr>
<td>CDI</td>
<td>Clostridium difficile infection</td>
</tr>
<tr>
<td>CDDI</td>
<td>Center for Drug Discovery and Innovation</td>
</tr>
<tr>
<td>CH₂N₂</td>
<td>diazomethane</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy (NMR)</td>
</tr>
<tr>
<td>d</td>
<td>doublet (NMR)</td>
</tr>
<tr>
<td>DI</td>
<td>deionized (water)</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMSO-d₆</td>
<td>deuterated dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DNMTi</td>
<td>DNA methyltransferase inhibited</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shifts (NMR)</td>
</tr>
<tr>
<td>ECD</td>
<td>electronic circular dichroism</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization (MS)</td>
</tr>
<tr>
<td>ESKAPE</td>
<td>Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter cloacae</td>
</tr>
<tr>
<td>ELSD</td>
<td>evaporating light scattering detector (liquid chromatography)</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>ε</td>
<td>the molar extinction coefficient in UV spectroscopy</td>
</tr>
<tr>
<td>FL</td>
<td>Florida</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>gCOSY</td>
<td>gradient correlation spectroscopy (NMR)</td>
</tr>
<tr>
<td>gHMBCAD</td>
<td>gradient heteronuclear multiple bond connectivity adiabatic decoupling (NMR)</td>
</tr>
<tr>
<td>gHSQCAD</td>
<td>gradient heteronuclear single quantum correlation adiabatic decoupling (NMR)</td>
</tr>
<tr>
<td>H</td>
<td>hydrogen</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HDACi</td>
<td>histone deacetylase inhibited</td>
</tr>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond connectivity (NMR)</td>
</tr>
</tbody>
</table>
HPLC   high performance/ pressure liquid chromatography
HRESIMS  high resolution electrospray ionization mass spectrometry
hr    hour
HTS    high throughput screening
IC₅₀  inhibitory concentration 50%
IR    infrared spectroscopy
J    coupling constant
LC/MS  liquid chromatography mass spectrometry
LC-QToF-MS  liquid chromatography quadrupole time of flight mass spectrometry
LD₅₀  lethal dose
λmax  maximum absorption wavelength (UV)
m    multiplet (NMR)
MBC₉₉  minimum bactericidal concentration
MDR-TB  multi-drug resistant tuberculosis
MDS   multidimensional scaling
MeOH  methanol
mg    milligrams
MHz   megahertz
MIC   minimum inhibitory concentration
µg    micrograms
µL    microliters
µM    micromolar
mL    milliliters
MPLC  medium pressure liquid chromatography
MRSA  methicillin resistant *Staphylococcus aureus*
MS    mass spectrometry
MW    molecular weight
m/z   mass/ charge ratio (MS)
NCBI  National Center for Biotechnology Information
NIH   National Institutes of Health
NMR   nuclear magnetic resonance
NOE   nuclear overhauser effect (NMR)
NOESY  nuclear overhauser effect spectroscopy (NMR)
NP    normal phase (liquid chromatography)
NTD   neglected tropical disease
OSMAC  one strain many compounds (fungi)
PAM   primary amoebic meningoencephalitis
PCA   principle component analysis
PCR   polymerase chain reaction
PDB   potato dextrose broth
PKS   polyketide synthase
ppm   parts per million, chemical shifts (NMR)
q    quartet (NMR)
QToF  quadrupole time of flight
RP    reverse phase (liquid chromatography)
rt    room temperature
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>research vessel</td>
</tr>
<tr>
<td>s</td>
<td>singlet (NMR)</td>
</tr>
<tr>
<td>SAR</td>
<td>structure activity relationship</td>
</tr>
<tr>
<td>SCUBA</td>
<td>self-contained underwater breathing apparatus</td>
</tr>
<tr>
<td>SDA</td>
<td>sabouraud dextrose agar</td>
</tr>
<tr>
<td>SDB</td>
<td>sabouraud dextrose broth</td>
</tr>
<tr>
<td>t</td>
<td>triplet (NMR)</td>
</tr>
<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
</tr>
<tr>
<td>USF</td>
<td>University of South Florida</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>extensively drug resistant tuberculosis</td>
</tr>
</tbody>
</table>
Abstract

Natural products have, historically, played an important role in drug discovery. Nevertheless, drug resistance, pathogen evolution, and global climate change threaten human health and nearly all current anti-infective treatments on the market today. It is undeniable that new drug discovery efforts are needed with increasing urgency.

Bolstered by a rich history of discovering treatments in the world around us, natural products chemists continue to look to the environment with increasing understanding and emerging technologies that allow efficient, effective isolation of new chemical entities. This thesis will describe one such endeavor.

Focusing on fungal natural products, herein is described the isolation and structure elucidation of new, bioactive natural products. Further, the development and implementation of a large fungal screening program will be discussed, the results of which stand to advance microbial drug discovery in the Baker lab for years to come.
Chapter 1.

An Introduction to Marine Natural Product Drug Discovery

1.1. Why Natural Products

From single celled protozoa to *homo sapiens*, all living beings are comprised of organic molecules that make up the cellular machinery essential for life. While some of these compounds must be acquired from the organisms’ surroundings, many of these metabolites that are necessary for basic cellular function are produced *de novo* by the cell. These primary metabolites—sugars, proteins, lipids, etc. may vary significantly form one life form to another, but the basic chemical composition of each living thing is the same. While DNA structure and composition surely separates a bacterium from a human being, an investigation of the chemicals found within a single cell from either of those organisms will expose a high level of similarity in the aforementioned primary metabolites. What may differ chemically within this investigation, however, is the myriad of small molecules seemingly unnecessary to primary cellular function. These “secondary metabolites”, or natural products, are generally signaling molecules that allow the organism not only to survive, but to thrive. All of these metabolites—primary and secondary—allow living organisms to live amongst and interact with one another. At a purely chemical level, all of life is competing on the same playing field, and this is the basis for interactions between species.

Given that all life forms have the potential to interact with each other chemically— one organism produces a molecule that interacts with a receptor in another organism— it therefore
follows that the study of these natural products can be of great benefit to human health. For, what is human disease but an interaction between life forms? What is a drug, but a molecule that interacts with a receptor?

With advances in technologies that allow us to visit and sample from all corners of the globe, we now understand that there is immeasurable biological and chemical diversity just waiting to be discovered. This chemical diversity is an incredible source for drug discovery. The creativity of the human mind is nothing compared to the creativity of evolution, and we continue to discover natural products with novel chemical scaffolds never before seen or imagined through synthetic methods. Further, natural products have consistently played a strong role in the development of new drugs for human health, with a full 65% of all small molecule approved drugs since 1981 being inspired by, derivatives of, or directly, natural products.¹

While the natural products of terrestrial communities have long been studied for their applications to human health,¹–⁴ marine environments have added their own repertoire of important compounds to the list over the past few decades.¹,⁵–⁸ With relatively newly accessible regions of the marine environment (namely, deep water and polar communities)⁹–¹¹ the biological and chemical diversity of marine environments poses great potential for bioprospecting.

1.2. Sources of Marine Natural Products

1.2.1. Marine Macro-organisms

The world’s oceans cover more than 70% of the planet. The exceptionally varied environments within these oceans range around the globe from the tropics to the poles. Even in the harshest of these environments, intense biodiversity can be found. Within these biodiversity
hotspots, marine organisms have adapted unique physical and chemical attributes to support their survival and proliferation. Benthic marine macro-organisms- multicellular organisms, specifically those that are sedentary and lack any physical defense mechanisms- have been of interest for their chemistry for some time, both in the chemical ecology and drug discovery arenas\textsuperscript{12–15}.

Many new and novel pharmacophores have been discovered from organisms on the marine benthos,\textsuperscript{1,5} and currently, there are two such compounds that are commercially available as drugs: ziconotide (Prialt\textsuperscript{®}) from the marine cone snail \textit{Conus magnus}\textsuperscript{8}, and trabectedin (Yondelis\textsuperscript{®}) isolated from the tunicate \textit{Ecteinascidia turbinate}\textsuperscript{6,7}. Countless other marine natural products that have not yet made it through the drug development pipeline exhibit early and exciting activities in various bioassays\textsuperscript{5,12,13,16}, inspiring future marine natural products discovery efforts.

1.2.2. Marine Micro-organisms

While marine macro-organisms have been found to contain interesting secondary metabolites,\textsuperscript{1,5} efforts towards any drug development with these compounds are often met with the issue of supply. Many benthic marine organisms cannot be cultured in an artificial setting\textsuperscript{17} and are often originally collected in some of the most remote and difficult to access regions of the world. Harvesting the biomass of these organisms needed for complete drug development would be impossible without great ecological impact. Though they could present a source-independent supply, synthetic routes to some of these complex scaffolds remain extremely difficult.\textsuperscript{18} Therefore, attaining an adequate supply of marine macro-organism derived compounds of interest for the rigorous drug development pipeline can be an extremely daunting challenge.
In fact, faced with this problem, and utilizing advances in genetic sequencing and microbial isolation techniques, it has been discovered that many natural products initially isolated from marine macro-organisms actually originate from invertebrate associated microorganisms.\textsuperscript{5,13} With attention turning towards the density, diversity, and biosynthetic capabilities of marine microorganisms, the collection, isolation, and culture of these important and sometimes elusive marine participants has become an interesting area of study.\textsuperscript{13,19–21}

1.2.3. Marine Plant Endophytes

The discovery of the potent anti-cancer drug paclitaxel (taxol\textsuperscript{®}) from the yew tree, \textit{Taxus brevifolia}, added to the already successful narrative of terrestrial plant natural products as an important source of bioactive compounds.\textsuperscript{22} However, everything changed after paclitaxel was later isolated from the endophytic fungus \textit{Taxomyces andreanae}.\textsuperscript{23} The study of endophytic micro-organisms, specifically endophytic fungi, was elevated significantly, and today, endophytic fungi are considered an important source of natural products.\textsuperscript{24–26}

In the marine realm, mangrove plants have emerged as a significant source of endophytic fungi. Found in the harsh fringe marine environments across the globe, mangrove forests are well known for intense biodiversity, as well as their adaptations to withstand consistently changing temperature and salinity. Endophytic fungi isolated from these environments have been of particular interest,\textsuperscript{26–29} but interestingly, none of the work reported included the mangrove environments in Florida, USA. After a number of collections in Everglades National Park and other mangrove communities in Florida, a proof of concept study by Beau et al. demonstrated that, unsurprisingly, these local mangrove environments were an untouched resource of endophyte biological and chemical diversity.\textsuperscript{30} The work described herein in chapters 3 and 4
seeks to build on these initial studies, hopefully adding to the case for further investigation, appreciation, and protection of these exciting marine environments.

1.2.4. Collection Locations and Non-marine Sources

Perhaps as important as choosing a target organism is choosing a collection location. The marine environment is a vast and variable biome, and much of it still remains to be explored. Even the most well studied marine environments- the tropics, for example, with their easily accessible reef systems and temperate climates- are changing and evolving with climate change and anthropomorphic stressors. With these changes, come changes in the diversity of life and chemistry that can be found there. The same can be said for terrestrial and fresh water environments, and as scientific tools and instrumentation become more advanced the study of natural products from all corners of the globe remains an exciting adventure.

1.2.4.1. Antarctica. Niche environments in the low to middle latitude seas of the world are extremely interesting, for here, currents make possible the diverging and converging of species across the globe. This means, in some instances, that species may have an extremely large distribution and remain relatively similar. Antarctica, however, is an example of an environment in which this is not true. Upon the splitting of the continent from Australia and South America some 30 million years ago, the Antarctic Circumpolar current was formed, effectively cutting the continent and its surrounding waters off from the rest of the globe. These freezing, isolated waters are now home to organisms that have evolved through periods of fluctuating glaciation and without influence from the currents and winds from the tropics. Those organisms living on the benthos in this environment- both macro-organisms and their micro-organism compatriots- have evolved many chemical interactions with one another as a way to survive. These species often exhibit rich biosynthetic potential, and the study of secondary
metabolites produced in this environment is of great interest to many groups- including ours.$^{9,31}$

The Baker lab Antarctic collection features organisms collected by SCUBA and trawling in the areas of Palmer Station and throughout the Scotia Arc (Figure 1.1) over decades of visits to the continent.

![Google Earth image of Antarctica with collection sites](image)

**Figure 1.1.** The Antarctic continent as seen from space on Google Earth with common collection sites for natural products featured. (Image credit: J. L. Fries)$^{34}$

In the winter of 2016-17, we participated in the Swiss Polar Institute’s Antarctic Circumnavigation Expedition (ACE), sampling, in some places for the first time, via Agassiz trawl, ROV, and intertidal collections throughout the sub-Antarctic waters and islands (Figure 1.2).
Samples collected during this expedition include bulk material for chemical investigation as well as tissue samples preserved for the isolation of associated fungi and bacteria. All macroorganisms collected will be genetically identified at the Western Australia Museum by Dr. Nerida Wilson. Illustrating an ideal example of an understudied and interesting niche environment, our Antarctic investigations are bolstered by strong collaborations that help to advance our studies of this wildest of marine frontiers.

1.2.4.2. Florida. Though we travel to the ends of the earth to collect samples, the Baker lab calls the University of South Florida, in Tampa, Florida, home. Fortunately, Florida is
home to a number of unique marine environments that are also ideal collection locations for the isolation of natural products. Florida supports vast mangrove forests in both developed and protected areas (Figure 1.3). These mangrove communities, as mentioned above, are rather understudied, and are ideal for the collection of endophytic fungi and bacteria.

Figure 1.3. Left panel: Mangroves growing in highly developed intercoastal waterways in Dunedin, FL; Right panel: Mangroves growing in the protected, pristine Everglades National Park, FL.

Florida’s warm waters also support beautiful coral reef communities in the Keys and up the west coast into the Gulf of Mexico. With just a short boat ride, SCUBA divers from the lab can collect a vast array of benthic organisms for chemical and microbial investigation. Convenience is not to be taken for granted in the choosing of a site from which to collect material for natural products research, and sometimes you only have to look as far as your own back yard.

1.2.4.3. Terrestrial and fresh water environments. Nowhere is this mantra better proven than in chapter 2 of this manuscript. Sedentary and microbial organisms in all environments- marine, terrestrial, and fresh water- must produce secondary metabolites with which they can interact with the world around them. Micro-environments, such as fresh water
ponds, inner-city forests, or coastal estuaries, to name a few, may be home to countless organisms that must respond to incredibly localized stressors that make no two environments exactly the same. Chapter 2 will tell the fortuitous story of noticing a new organism (a fresh water bryozoan) growing in a neighborhood retention pond that resulted in the isolation of a suite of new, bioactive compounds.

Natural products exist all around us, and nature is the supreme synthetic chemist. Even our own human gut microbiome is currently demanding attention for secondary metabolite production and the potential for therapeutic applications.\textsuperscript{35–37} New chemical structures are emerging all the time from countless environmental sources, and as threats to human health evolve, it could certainly be argued that natural products research is the way forwards.

1.3. Approaches Towards the Isolation of Microbial Natural Products

1.3.1. Collection and Isolation

The majority of marine microbes are still considered “unculturable”, however, much work has gone into shrinking that percentage by investigating new collection, isolation, and culture techniques.\textsuperscript{20,26,38} The fungal library in the Baker lab at the University of South Florida serves as a comprehensive example of microbial isolates obtained using an evolving repertoire of techniques from a variety of sources. Marine organisms such as sponges, tunicates, and corals are collected either by SCUBA or trawling, and small tissue samples are surface sterilized before being plated on a variety of solid media plates featuring varying chemical and nutrient compositions. Mangrove and mangrove-associated plant material is similarly collected and inoculated. Sediment and water column samples are concentrated, or filtered, before being plated on solid media or suspended in liquid media. Collection and isolation techniques are adaptable to
the source organism or targeted isolates. Microbial isolate cultures can be miniaturized and standardized, as will be discussed in chapter 3, for screening purposes, or scaled up and tailored for titer improvement of compounds of interest. By keeping a functional archive of all isolates (stored at -80°F) this microbial library can be investigated again and again as new culture techniques and screening targets emerge.

1.3.2. Culture Techniques

Thanks to genomic advancements, it is clear that micro-organisms cultured in the lab routinely produce only a fraction of the secondary metabolites that are coded for in their DNA. This is accomplished by the regulation of transcription by enzymes activated and deactivated based on environmental factors. In filamentous fungi, we know that most secondary metabolite genes are clustered to allow for the most efficient regulation, and these clusters can be activated or deactivated by culture conditions, resulting in vastly different metabolite production. Once isolated, a micro-organism of interest can be cultured in the lab under any number of easily accessible stressors that can change secondary metabolite production. Culture variations can be as simple as changing the shape of the culture vessel, or as complex as the addition of biological material from another microbe or host organism. In this way, a single strain can produce a multitude of different compounds. While this ‘OSMAC’ (‘One Strain Many Compounds’) strategy is extremely useful in exploiting the full biosynthetic potential of a micro-organism of interest, it is rather intensive in time and consumables.

Rather than systematically changing culture conditions, the biosynthetic potential of a micro-organism of interest can also be explored through whole genome sequencing. Many secondary metabolites are products of known biosynthetic pathways. The ability to ascribe a product to the genes that code for it allows for the unique ability to analyze a whole genome and
predict the metabolites that can be produced. Culture conditions curated to that biosynthetic pathway can then be employed to isolate specific compounds of interest.\textsuperscript{46–51}

Genome mining and the OSMAC approach are both useful techniques for the discovery of the biosynthetic potential of a single organism. If, however, you have a microbial library that you would like to screen, these techniques may not be the most efficient. Epigenetic modification—that is, the use of small molecule enzyme inhibitors to promote the expression and prevent the silencing or downregulation of secondary metabolite gene clusters\textsuperscript{52–55}—can be used as a more ubiquitous technique to exploit the biosynthetic potential of a larger number of microorganisms. Chapter 3 will discuss the use of histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors\textsuperscript{30} as culture additives to epigenetically ‘turn on’ secondary metabolite gene clusters in a library of filamentous fungi for the maximum surveying of bioactive natural product potential therein.

1.4. Drug Discovery Targets

Natural products isolation efforts largely follow the same generic scheme (Scheme 1). Efforts aimed at drug discovery can take place at any of the stages, from extraction to pure compound isolation. There are pros and cons to each approach, though it is generally accepted that the earlier you can prioritize your efforts, the better.
Crude extracts can contain thousands of compounds, however, it is possible to get useful information from that complex mixture in a high-throughput way. Metabolite profiling of crude extracts can be used for initial dereplication and more advanced metabolomic analysis can reveal chemical outliers that may be of interest.\textsuperscript{56,57} High-throughput bioassays that are tolerant of complex mixtures can be used to discover and prioritize activity early in the investigation process. More sensitive and selective bioassays that are not tolerant of complex mixtures would require more purified fractions or pure compounds. It is important, therefore, when embarking on a natural products screening program, to coordinate bioassay capabilities to isolation protocols, in addition to other target selection criteria. The descriptions that follow comprise the panel of targets that will be discussed in Chapter 3. These targets are of great contemporary relevance to

\textbf{Scheme 1.1.} A generic flow chart for natural product isolation.
human health concerns and each feature robust bioassay methodologies that assist in early crude extract level prioritization.

1.4.1. The ESKAPE Pathogens

With growing antibiotic resistance, and a decrease in antibiotic drug discovery, the Infectious Disease Society of America issued a ‘call to arms’ in 2009 to the drug discovery community to combat what they called the ESKAPE pathogens: the gram positive Enterococcus faecium and Staphylococcus aureus, and gram negative Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter cloacae. These clinically relevant, highly drug resistant pathogens represent a continuously growing threat to human health and an important target for drug discovery efforts.

1.4.2. Leishmania donovani

A neglected tropical disease (NTD), Leishmaniasis is a parasitic infection caused by an intramacrophage protozoa that is transmitted to humans by the bite of infected sandflies. The visceral form of this disease, most commonly caused by Leishmania donovani, is typically fatal when left untreated. Upon entering the host, the parasite—in a non-flagellated amastigote life stage—invades macrophage cells to travel through the body and reproduce. Recent advances in infected macrophage in-vitro culture techniques allow for more clinically relevant assays to be performed in a high throughput screening (HTS) context. These advancements will hopefully aid in the discovery of new treatments for this disease in the face of increasing resistance to existing treatments.

1.4.3. Mycobacterium tuberculosis

Tuberculosis (TB) remains a global health crisis, despite the advances of the whole genome sequencing project that revealed the genome of Mycobacterium tuberculosis. This
disease, whose latent form is estimated to infect one third of the world’s population, poses many drug development hurdles. Multi-drug resistant (MDR-TB) and extensively drug resistant (XDR-TB) strains have emerged despite the current course of treatment typically involving combinatorial therapies aimed directly at preventing resistance. Drug discovery efforts, therefore, must address new mechanisms of action or \textit{M. tuberculosis} targets. Additionally, TB drugs have the burden of needing to be compatible in combinatorial treatments for the immunocompromised, particularly those with HIV/AIDS, among whom incidence of this disease are highest.\textsuperscript{66} Natural products based drug discovery against this target have revealed promising results, with many existing treatments coming from natural products. With such a demanding target comes the need to screen a broad swath of chemical space, confirming natural products drug discovery efforts as a promising way forward in the search for treatments of this disease.\textsuperscript{67}

1.4.4. \textit{Clostridium difficile}

The leading cause of healthcare related infection, \textit{Clostridium difficile} is an easily spread, diarrhea causing bacteria that is considered a threat to human health worldwide. The use of antibiotics which upset the human gut microbiome is the primary cause of \textit{C. difficile} infection (CDI), but any immunocompromised individuals are at risk. With increasing incidences of resistance, recurrence, and mortality, the need for discovery of new treatments against this bacteria is imperative. Most challengingly, new drugs to fight CDI must act without impact on the normal human gut fauna. Many novel treatment avenues have been suggested, among which, the discovery and use of bacterial natural products remain of high interest.\textsuperscript{68–70}

1.4.5. \textit{Naegleria fowleri}

\textit{Naegleria fowleri} is a free living, warm-water loving amoeba that causes the nearly always fatal primary amoebic meningoencephalitis (PAM). Diagnosis of PAM is extremely
difficult, and current treatment options are time sensitive and limited to existing drug combinations (e.g. Amphotericin B, fluconazole, and miltefosine).\textsuperscript{71,72} As clinicians start to understand and diagnose PAM better, and the risk of this disease continues to rise with increasing global temperatures, new drugs that specifically target this amoeba are urgently needed.

1.4.6. Cancer Targets

With structures as diverse as their targets, natural products have long played a role in the treatment of various cancers.\textsuperscript{1,22,73} As understanding of the complex physiology of human cells (both healthy and cancerous) continues to grow, assays directed at testing compounds against highly specific cellular targets continue to emerge. Rather than whole cancer-cell assays, these target specific assays can help to exclude compounds that are broadly cytotoxic to all cells in favor of compounds that are active within the specific mechanism of action that is desired.

Autopalmitoylation dysregulation is implicated in many disease states. In a newly developed assay, palmitoylation of proteins can be monitored for modulation by compounds of interest in a HTS manner. This allows compounds to be rapidly screened not for their effect on the whole cell, but rather just on this particular pathway of interest.\textsuperscript{74}

1.5. Future Directions

As I have attempted to illustrate, there are many different techniques available for natural products drug discovery efforts. While exploring the biosynthetic potential of a single organism can be very lucrative, screening efforts are needed in order to identify those “lead” organisms. With a robustly designed screening program, natural product extracts from a multitude of sources can be screened side by side in high-throughput capable bioassays against a wide variety of
disease targets. The resulting diversity of bioactivity information combined with metabolite profiling can afford intense prioritization of extracts at a very early stage (Scheme 1.1), streamlining further chemical investigation to a highly time and cost effective level of efficiency. Focusing on marine fungi, the remaining chapters in this dissertation will put forth evidence in support of one such screening initiative, its successes, lessons, and some recommendations for improvements moving forward. The work discussed herein will present a number of new, bioactive microbial natural products that resulted from this initiative. At the conclusion, I hope to convince the reader that microbial natural products drug discovery can be an efficient, cost effective endeavor towards combating some of the most difficult challenges facing human health today.

1.6. References


(34) Fries, J. L. Chemical Investigation of Antarctic Marine Organisms & Their Role in Modern Drug Discovery, University of South Florida, 2016.


(45) Scherlach, K.; Hertweck, C. Triggering Cryptic Natural Product Biosynthesis in


Chapter 2

New Bioactive Meroterpenes from *Phomopsis* sp.

2.1. BB11-2 Isolation

In the winter of 2011 small colonies of the fresh water bryozoan *Pectinatella magnifica* were noticed on a morning run in a retention pond located in north Tampa, Florida. These colonial organisms were collected for chemical analysis. While collecting, one of the colonies was found to be growing around the end of a tree branch that was partially submerged in the water. A small piece of this branch was collected along with the bryozoan and returned to the lab for processing. Once in the lab, the branch was processed for microbial isolation (procedure in Appendix B). From two SDA plates, 7 fungal isolates were obtained based on morphological features. These organisms were given a name of “BB11” for “Baker’s Backyard 2011” and archived as described in Appendix B.

Following chemical investigation of isolate BB11-2, fungal identification was obtained via Sanger sequencing of the 18S ribosomal spacer region (Appendix A). An NCBI nucleotide blast returned identification as *Diaporthe* or *Phomopsis* sp.; genera that have been determined to be the same but represent different life cycles of these common,\(^1\) chemically rich\(^2\)–\(^7\) endophytic fungi. For purposes of this dissertation, the isolate will be referred to as *Phomopsis* sp.
2.2. Initial Bioactivities and Scale-up

The BB11 fungal isolates were screened in a number of assays and assay development protocols and their extracts were known within the lab to be highly active with low cytotoxicity. Isolate BB11-2 was chosen for its reproducible bioactivity and tolerance towards multiple media types. As a part of a scale-up optimization study, BB11-2 was scaled up on rice media in three growth conditions: control (700g of rice media + 100mL SDB), HDACi (700g of rice media + a 435µM solution of sodium butyrate in 100mL SDB), and DNMTi (700g of rice media + a 435µM solution of 5-azacytidine in 100mL SDB). After 21 days of growth, each extract was extracted in 1:3 MeOH/ EtOAc solution overnight, followed by 2 subsequent 24 hour extractions in 100% EtOAc. The extracts for each of the three culture conditions were dried down and subjected to a H2O: EtOAc partition. The lipophilic partitions were each separated on NP MPLC (Scheme 2.1). The fractions of the control extract were further investigated.
Scheme 2.1. Extraction scheme for *Phomopsis* sp. Control.

2.3. Compound Isolation and Structure Elucidation

The extract of the control culture condition of *Phomopsis* sp. was investigated by NMR and bioactivity guided fractionation (Scheme 2.1) and yielded 5 new meroterpenes: phomopsichromins A-E (1-5), and the known compound LL-Z1272ε (6)⁸ (Figure 2.1).
In the first MPLC separation of the lipophilic partition of the Control extract, the majority of the mass eluted in one large peak in approximately 1:1 n-hexanes: ethyl acetate. This fraction (Fraction D) was further purified via NP MPLC on an extended non-polar gradient (n-hexanes to ethyl acetate). Again, the majority of the mass eluted in a single peak in approximately 1:1 n-hexanes: ethyl acetate (Fraction C). Further purification was accomplished on NP HPLC using a
cyano column (CN capping of the silica particles) and UV detection. Compounds 1-6 (Figure 2.1) were isolated as illustrated in Figure 2.2.

![NP HPLC chromatogram of fraction D_C (Scheme 2.1) from the control growth treatment of Phomopsis sp. Peaks are annotated with their isolated compounds (Figure 2.1). Each compound required some further purification on reverse phase (RP) HPLC for structure elucidation and bioassay purposes, but represented the major component of each of the peaks seen. Black is the UV chromatogram at 320nm, and blue is the ELSD trace.](image)

**Figure 2.2.** NP HPLC chromatogram of fraction D_C (Scheme 2.1) from the control growth treatment of Phomopsis sp. Peaks are annotated with their isolated compounds (Figure 2.1). Each compound required some further purification on reverse phase (RP) HPLC for structure elucidation and bioassay purposes, but represented the major component of each of the peaks seen. Black is the UV chromatogram at 320nm, and blue is the ELSD trace.

Compounds 1-6 all share a sesquiterpene backbone functionalized differentially at C-9. 1-3 share a chromene substructure while compounds 4-6 feature a ring-opened subunit. The
structures of Phomopsichromins A-E (1-5) were elucidated as described below. NMR spectra, IR, UV, and optical rotation data for all compounds can be found in Appendix A.

Table 2.1. 1D and 2D data for phomopsichromin A (1) in CDCl₃.

| Pos | δc ⁺ | δh (m, J(Hz))⁻ | HMBC ⁺⁺⁺  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50.4</td>
<td>2.41 (q, 6.6 x 2, 1 H)</td>
<td>2, 5, 6, 7, 12, 14</td>
</tr>
<tr>
<td>2</td>
<td>213.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>41.5</td>
<td>2.33 (m, 1 H)</td>
<td>2, 4, 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.36 (m, 1 H)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30.8</td>
<td>1.64 (m, 1 H)</td>
<td>3, 5, 6, 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.85 (m, 1 H)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>36.1</td>
<td>1.97 (m, 1 H)</td>
<td>1, 3, 4, 6, 13</td>
</tr>
<tr>
<td>6</td>
<td>43.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>30.6</td>
<td>1.41 (m, 1 H)</td>
<td>1, 5, 6, 8, 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.48 (m, 1 H)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>34.3</td>
<td>1.52 (m, 1 H)</td>
<td>7, 9, 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.77 (m, 1 H)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>79.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>125.8</td>
<td>5.46 (d, 10.2, 1 H)</td>
<td>8, 9, 15, 16, 21</td>
</tr>
<tr>
<td>11</td>
<td>116.9</td>
<td>6.76 (d, 10.2, 1 H)</td>
<td>9, 15, 16, 17, 21</td>
</tr>
<tr>
<td>12</td>
<td>7.5</td>
<td>0.91 (d, 6.8, 3 H)</td>
<td>1, 2, 6</td>
</tr>
<tr>
<td>13</td>
<td>14.9</td>
<td>0.89 (d, 6.8, 3 H)</td>
<td>4, 5, 6</td>
</tr>
<tr>
<td>14</td>
<td>15.4</td>
<td>0.59 (s, 3 H)</td>
<td>1, 5, 6, 7</td>
</tr>
<tr>
<td>15</td>
<td>27</td>
<td>1.44 (s, 3 H)</td>
<td>8, 9, 10, 11</td>
</tr>
<tr>
<td>16</td>
<td>106.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>160.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH</td>
<td></td>
<td>11.74 (s, 1 H)</td>
<td>16, 17, 18, 21</td>
</tr>
<tr>
<td>18</td>
<td>103.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>144.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>111.9</td>
<td>6.24 (s, 1 H)</td>
<td>10, 11, 16, 18, 21, 22, 23</td>
</tr>
<tr>
<td>21</td>
<td>158.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>24</td>
<td>2.55 (s, 3 H)</td>
<td>17, 18, 19, 20, 21, 23</td>
</tr>
<tr>
<td>23</td>
<td>175.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁻¹H NMR recorded at 500 MHz, reported in ppm (multiplicity, J-coupling in Hz, integration);  
⁺⁺⁺¹³C NMR recorded at 125 MHz;  
⁺⁺⁺ recorded from a gHMBCAD experiment at 500 MHz and reported as positions of carbons.

Phomopsichromin A (1), [α]²⁰D +1.2, was isolated as a white powder. The ¹H NMR spectrum of 1 (Table 2.1) notably displayed 3 olefinic protons, peaks for 5 methyl substituents, and a phenol. The ¹³C NMR spectrum indicated the presence of a carboxylic acid (δc 175.18)
and ketone (δC 213.87). Remaining 13C signals were split between the aromatic and alkene regions and implied a high level of substitution on the aromatic ring (as evidenced by the small number of olefinic protons in the 1H NMR spectrum). The chromene substructure was tentatively assigned based on 2D NMR experiments and 13C ppm shifts of carbons 9 and 21. The remaining terpene scaffold was completed based on HMBC and COSY NMR data (Figure 2.3). HRESIMS of 1 (m/z 387.2137 [M + H]+) resembled compounds in the literature8–12 but did not match any exactly, confirming that 1 was a new compound. Using the NMR data from these related compounds, the chromene substructure was confirmed. Relative stereochemical assignments at methyl-bearing carbons 1 (R), 5 (R), 6 (S), and 9 (S) were assigned via 1 and 2D NOE experiments (Figure 2.3) and comparison to the literature11–13. ECD will be used to confirm absolute stereochemistry.

Figure 2.3. Important COSY, HMBC, and NOE correlations in phomopsichromin A (1).
Table 2.2. 1D and 2D data for phomopsichromin B (2) in CDCl₃.

<table>
<thead>
<tr>
<th>Pos</th>
<th>δCᵇ</th>
<th>δH (m, J/Hz)ᵃ</th>
<th>HMBCᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.4</td>
<td>1.43 (m, 1H)</td>
<td>2, 6, 12, 14</td>
</tr>
<tr>
<td>2</td>
<td>73.1</td>
<td>3.85 (q, 2.8, 1H)</td>
<td>3, 4, 6, 12</td>
</tr>
<tr>
<td>3</td>
<td>33.9</td>
<td>1.55 (m, 1H) 1.8 (m, 1H)</td>
<td>1, 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.63 (m, 1H)</td>
<td>1, 2, 4, 5</td>
</tr>
<tr>
<td>4</td>
<td>25.4</td>
<td>1.27 (m, 1H)</td>
<td>3, 5</td>
</tr>
<tr>
<td>5</td>
<td>36.5</td>
<td>1.46 (m, 1H)</td>
<td>6, 7, 14</td>
</tr>
<tr>
<td>6</td>
<td>38.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>30.9</td>
<td>1.35 (m, 2H)</td>
<td>5, 6, 8, 14</td>
</tr>
<tr>
<td>8</td>
<td>34.4</td>
<td>1.45 (m, 1H) 1.63 (m, 1H)</td>
<td>6, 7, 9</td>
</tr>
<tr>
<td>10</td>
<td>126.2</td>
<td>5.46 (d, 10.2, 1H)</td>
<td>8, 9, 15, 16</td>
</tr>
<tr>
<td>11</td>
<td>116.6</td>
<td>6.74 (d, 10.2, 1H)</td>
<td>9, 16, 17, 21</td>
</tr>
<tr>
<td>12</td>
<td>12.3</td>
<td>0.95 (d, 7.1, 3H)</td>
<td>1, 2, 6</td>
</tr>
<tr>
<td>13</td>
<td>15.6</td>
<td>0.82 (d, 6.6, 3H)</td>
<td>4, 5, 6</td>
</tr>
<tr>
<td>14</td>
<td>17.3</td>
<td>0.86 (s, 3H)</td>
<td>7, 6, 5</td>
</tr>
<tr>
<td>15</td>
<td>27.1</td>
<td>1.41 (s, 3H)</td>
<td>8, 9, 10</td>
</tr>
<tr>
<td>16</td>
<td>106.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>160.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH</td>
<td>11.80</td>
<td>11.80 (s, 1H)</td>
<td>16, 17, 18</td>
</tr>
<tr>
<td>18</td>
<td>103.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>144.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>111.9</td>
<td>6.22 (s, 1H)</td>
<td>16, 18, 21, 22</td>
</tr>
<tr>
<td>21</td>
<td>158.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>24.4</td>
<td>2.53 (s, 3H)</td>
<td>18, 19, 20</td>
</tr>
<tr>
<td>23</td>
<td>174.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ¹H NMR recorded at 600 MHz, reported in ppm (multiplicity, J-coupling in Hz, integration); ᵇ¹³C NMR recorded at 125 MHz; ᶜrecorded from a gHMBCAD experiment at 500 MHz and reported as positions of carbons.

Phomopsichromin B (2) was isolated as a white powder, C₂₃H₃₂O₅, HRESIMS m/z 389.2322 [M + H]^⁺ (calculated, 389.2283), [α]²₀D -1.5. Comparison to the HRMS of 1 indicated a difference of 2 protons. The appearance of a broad multiplet at δH 3.85 and the loss of the ketone signal at δC 213.87 inferred a reduction at C-2. The 2D NMR spectra further supported this assignment (Figure 2.4). The stereochemistry at position 2 was determined to be S based on the multiplicity observed in the proton NMR spectrum. The observed J-values of the quartet at
\( \delta_H \) 3.85 most closely matched those expected from the axial orientation of the hydroxyl group. This orientation could also be observed in other related compounds in the literature.\(^{14} \) The remaining chiral centers were determined to be the same as in 1. Again, the stereochemistry will be confirmed via ECD.

Figure 2.4. Important COSY, HMBC, and NOE correlations in phomopsichromin B (2).

Curiously, in multiple 1D NMR analyses of 2 over time, notable shifts in ppm were observed for some peaks (e.g. \( \delta_H \) 6.22, 3.85, and 2.53; \( \delta_C \) 73.17, 103.46, 158.73, and 174.72). The assumption was made that there were diastereoisomers present (further supported by some poorly resolved crystal data), however, various NP and RP HPLC attempts eluted a single peak in all conditions. The beginning, middle, and end of the peak were collected as separate fractions (a, b, and c) in an attempt to separate the diastereoisomers. Clear ppm differences were observed between fractions a and c, but they were chromatographically indistinguishable. The decision was made to methylate the carboxylic acid at C-23 in fractions a and c to reduce the effects of hydrogen bonding in separation attempts and aid in NMR spectral evaluation (Figure 2.5).
Figure 2.5. Methylation reaction of 2 (stereochemistry unknown at the time) to yield 7 with diazomethane.

The resulting methyl derivatives of fractions a and c (7) were confirmed by 1D NMR (Appendix A). All ppm differences between fractions a and c were lost, indicating that all previously noted shifts in ppm had been a concentration-based artifact of hydrogen bonding of 2 to itself in solution. 2 was determined to be a single diastereoisomer and remaining chemical and biological analyses were completed (Appendix A).

Phomopsichromin C (3) was isolated as a white powder, [α]$_{20}^{D}$ +1.3. $^1$H NMR data resembled 1 and 2 in the chromene region, however, the methyl signals at position 12, 13, and 14 were notably shifted downfield and were all overlapping (Table 2.3). New peaks at $\delta_C$ 170.87 and 21.4, with a new methyl signal in the $^1$H spectrum ($\delta_H$ 2.05) indicated added ester functionalization in the molecule. HRESIMS $m/z$ 431.2434 [M + H]$^+$ (calculated, 431.2389) gave a molecular formula of C$_{25}$H$_{34}$O$_6$, confirming the addition of a –CO(O)CH$_3$ group. The available 2D NMR data confirmed that the new functionalization was at C-2 ($\delta_C$ 75.16) (Figure 2.6).
Table 2.3. 1D and 2D data for phomopsichromin C (3) in CDCl₃.

<table>
<thead>
<tr>
<th>Pos</th>
<th>δ_C⁶</th>
<th>δ_H (m, J(Hz))</th>
<th>HMBC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.5</td>
<td>1.55 (m, 1 H)</td>
<td>6, 12, 14</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>75.1</td>
<td>4.97 (q, 2.0, 1 H)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30.9</td>
<td>1.5 (m, 1 H)</td>
<td>1, 2, 4, 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.81 (m, 1 H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>25.8</td>
<td>1.29 (m, 1 H)</td>
<td>2, 3, 5, 6, 13</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 (m, 1 H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>36.2</td>
<td>1.47 (m, 1 H)</td>
<td>3, 4, 6, 14</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>38.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>30.7</td>
<td>1.4 (m, 2H)</td>
<td>1, 5, 8, 9, 14</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>34.4</td>
<td>1.4 (m, 1H)</td>
<td>7, 9, 10, 11, 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.65 (m, 1 H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>80.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>126.1</td>
<td>5.45 (d, 10.2, 1 H)</td>
<td>8, 9, 15, 16, 21</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>116.7</td>
<td>6.74 (d, 10.0, 1 H)</td>
<td>9, 15-17, 21</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>12.0</td>
<td>0.83 (m, 3 H)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>15.6</td>
<td>0.83 (m, 3 H)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>16.7</td>
<td>0.83 (m, 3 H)</td>
<td>1, 5, 7, 8</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>27.1</td>
<td>1.4 (s, 3 H)</td>
<td>8-11</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>106.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>160.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH</td>
<td></td>
<td>11.82 (s, 1 H)</td>
<td>16, 17, 18, 21</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>103.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>144.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>111.9</td>
<td>6.22 (s, 1 H)</td>
<td>11, 16-18, 21-23</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>158.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>24.4</td>
<td>2.53 (s, 3 H)</td>
<td>16-20, 23</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>174.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>170.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>21.4</td>
<td>2.05 (s, 3 H)</td>
<td>2, 24</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) H NMR recorded at 600 MHz, reported in ppm (multiplicity, J-coupling in Hz, integration); \(^b\) C NMR recorded at 125 MHz; \(^c\) recorded from a gHMBCAD experiment at 500 MHz and reported as positions of carbons.
Figure 2.6. Important COSY and HMBC correlations in phomopsichromin C (3).

Due to the overlapping signals of the methyl signals on the cyclohexane ring of 3, elucidating the stereochemistry at C-1, 2, 5, and 6 based on NMR data posed a significant challenge. The methyl group at C-9 was assumed, as in compounds 1 and 2, to be S based on chemical shift. The stereochemistry at C-2 was again determined to be S based on the 1D $^1$H multiplicity. Figure 2.7 illustrates the proposed biosynthetic pathway for the cyclohexane substructure of 1-5 based on work done by Tanabe and Suzuki in 1974 on a related compound.\textsuperscript{13} This pathway indicates that the ketone at C-2 is a part of the precursor molecule, and therefore all reductions at C-2 occur post translationally. This suggests a conserved chirality at the centers at C-1, C-5, and C-6.

Figure 2.7. Proposed biosynthetic pathway towards the cyclohexane substructure of 1-6 from Tanabe and Suzuki, 1974.

Based on these assumptions, a compound with proposed stereochemistry (Figure 2.8) was submitted for ECD.
Phomopsichromin D (4, C_{23}H_{34}O_5, HRESIMS m/z 391.2473 [M + H]^+ (calculated, 391.2440), [α]^{20}_{D} +0.2) was isolated as a white powder from the last and least lipophilic HPLC fraction of MPLC fraction D_C (Figure 2.2). 4 was notably distinct from 1-3 in that it was quite different in polarity (not soluble in CDCl_3) and so all NMR data was obtained in DMSO-\textit{d}_6 (Table 2.4). The olefin region of the proton NMR spectrum of 4 was the most drastically different from compounds 1-3 and there was an additional phenol signal at δ_H 10.02, indicating that 4 lacked the completed chromene substructure.
Table 2.4. 1D and 2D data for phomopsichromin D (4) in DMSO-\textit{d}_6.

<table>
<thead>
<tr>
<th>Pos</th>
<th>$\delta^b_{\text{C}}$</th>
<th>$\delta^a_{\text{H}}$ (m, $J$(Hz))</th>
<th>HMBC$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.0</td>
<td>1.35 (m, 1 H)</td>
<td>2, 5, 6, 12, 14</td>
</tr>
<tr>
<td>2</td>
<td>70.4</td>
<td>3.62 (q, 2.4, 1 H)</td>
<td>4, 6, 12</td>
</tr>
<tr>
<td>3</td>
<td>34.1</td>
<td>1.4 (m, 1 H)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6 (m, 1 H)</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>25.5</td>
<td>1.13 (m, 1 H)</td>
<td>1, 3, 5, 6, 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.53 (m, 1H)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>36.0</td>
<td>1.4 (m, 1 H)</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>38.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>36.0</td>
<td>1.17 (m, 1 H)</td>
<td>1, 5, 6, 8, 9, 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.23 (m, 1 H)</td>
<td>5, 6, 8, 9, 14</td>
</tr>
<tr>
<td>8</td>
<td>32.4</td>
<td>1.69 (m, 1 H)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.75 (m, 1 H)</td>
<td>5, 10, 11, 15</td>
</tr>
<tr>
<td>9</td>
<td>134.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>121.8</td>
<td>5.12 (br t, 6.9, 1 H)</td>
<td>8, 11, 15, 16</td>
</tr>
<tr>
<td>11</td>
<td>21.5</td>
<td>3.16 (br d, 7.2, 2 H)</td>
<td>7, 8, 9, 10, 15, 16, 17, 21</td>
</tr>
<tr>
<td>12</td>
<td>12.7</td>
<td>0.84 (d, 7.2, 3 H)</td>
<td>1, 2, 6</td>
</tr>
<tr>
<td>13</td>
<td>15.7</td>
<td>0.74 (d, 6.6, 3 H)</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>17.3</td>
<td>0.75 (s, 3 H)</td>
<td>1, 6, 7</td>
</tr>
<tr>
<td>15</td>
<td>16.1</td>
<td>1.70 (s, 3 H)</td>
<td>7, 8, 9, 10, 11, 16</td>
</tr>
<tr>
<td>16</td>
<td>112.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>159.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH</td>
<td></td>
<td>12.61 (s, 1 H)</td>
<td>16, 17, 18, 21</td>
</tr>
<tr>
<td>18</td>
<td>103.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>139.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>110.4</td>
<td>6.23 (s, 1 H)</td>
<td>11, 16, 17, 18, 21, 22, 23</td>
</tr>
<tr>
<td>21</td>
<td>162.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH</td>
<td></td>
<td>10.02 (s, 1 H)</td>
<td>16, 17, 20, 21</td>
</tr>
<tr>
<td>22</td>
<td>23.8</td>
<td>2.38 (s, 3 H)</td>
<td>18, 19, 20, 21, 23</td>
</tr>
<tr>
<td>23</td>
<td>174.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$\textsuperscript{1}H NMR recorded at 500 MHz, reported in ppm (multiplicity, $J$-coupling in Hz, integration); $^b$\textsuperscript{13}C NMR recorded at 125 MHz; $^c$ recorded from a gHMBCAD experiment at 500 MHz and reported as positions of carbons.

2D NMR data (Figure 2.9) further supported this, and the “open” carbon skeleton was assigned and confirmed by comparison to literature data on known compound 6. Stereochemistry on the cyclohexane ring was set as in compounds 1-3. The double bond of the open chain was determined to be $E$ based on the comparison of the $J$-value of H-10 to the literature.
Figure 2.9. Important COSY, HMBC, and NOE correlations in phomopsichromin D (4).

Phomopsichromin E (5) was isolated as a white powder, C_{23}H_{32}O_{5}; HRESIMS m/z 389.2363 [M + H]^+, [α]^{20}_D -0.4. By comparing 5 to 4 it was immediately evident that the only difference between the two was the increased oxidation at C-2. Comparison to 1 and 4, along with the 2D data for 5 (Table 2.5), completed the structure (Figure 2.10).

Figure 2.10. Important COSY and HMBC correlations in phomopsichromin E (5).
Table 2.5. 1D and 2D data for phomopsichromin E (5) in DMSO-$d_6$.

<table>
<thead>
<tr>
<th>Pos</th>
<th>$\delta^c_b$</th>
<th>$\delta^b_H$ (m, J(Hz))$^a$</th>
<th>HMBC$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49.2</td>
<td>2.53 (q, 6.7, 1 H)</td>
<td>2, 5, 6, 12, 14</td>
</tr>
<tr>
<td>2</td>
<td>212.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>40.7</td>
<td>2.1 (m, 1 H)</td>
<td>1, 2, 4, 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.4 (m, 1 H)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30.3</td>
<td>1.47 (m, 1 H)</td>
<td>3, 5, 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.76 (m, 1 H)</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>35.0</td>
<td>1.99 (m, 1 H)</td>
<td>3, 4, 6, 13, 14</td>
</tr>
<tr>
<td>6</td>
<td>42.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>35.4</td>
<td>1.23 (m, 1 H)</td>
<td>5, 6, 8, 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.32 (m, 1 H)</td>
<td>1, 6, 8, 9, 14</td>
</tr>
<tr>
<td>8</td>
<td>32.0</td>
<td>1.8 (m, 1 H)</td>
<td>7, 9, 10, 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.91 (m, 1 H)</td>
<td>7, 9, 10, 15</td>
</tr>
<tr>
<td>9</td>
<td>134.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>122.2</td>
<td>5.18 (br t, 6.8, 1 H)</td>
<td>8, 11, 15, 16</td>
</tr>
<tr>
<td>11</td>
<td>21.5</td>
<td>3.17 (d, 7.1, 2 H)</td>
<td>9, 10, 16, 17, 21</td>
</tr>
<tr>
<td>12</td>
<td>7.5</td>
<td>0.76 (d, 6.6, 3 H)</td>
<td>1, 2, 6, 7</td>
</tr>
<tr>
<td>13</td>
<td>14.7</td>
<td>0.81 (d, 6.6, 3 H)</td>
<td>4, 5, 6, 12</td>
</tr>
<tr>
<td>14</td>
<td>15.0</td>
<td>0.46 (s, 3 H)</td>
<td>1, 5, 6</td>
</tr>
<tr>
<td>15</td>
<td>16.1</td>
<td>1.73 (s, 3 H)</td>
<td>8, 9, 10, 16</td>
</tr>
<tr>
<td>16</td>
<td>111.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>159.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH</td>
<td></td>
<td>12.62 (s, 1 H)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>103.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>139.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>110.4</td>
<td>6.23 (s, 1 H)</td>
<td>16, 17, 18, 22</td>
</tr>
<tr>
<td>21</td>
<td>162.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH</td>
<td></td>
<td>10.04 (s, 1 H)</td>
<td>16, 17, 20, 21</td>
</tr>
<tr>
<td>22</td>
<td>23.7</td>
<td>2.39 (s, 3 H)</td>
<td>18, 19, 20, 21</td>
</tr>
<tr>
<td>23</td>
<td>174.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$H NMR recorded at 600 MHz, reported in ppm (multiplicity, J-coupling in Hz, integration); $^b$C NMR recorded at 200 MHz; $^c$recorded from a gHMBCAD experiment at 600 MHz and reported as positions of carbons.

2.4. Bioactivities

Phomopsichromins A (1), B (2), C (3), and E (5) all show reasonable activity against the infected macrophage model of the Leishmania donovani parasite at 3, 1.9, 0.67, and 0.80 µM, respectively. Interestingly, neither phomopsichromin D (4) nor LL-Z1272ε (6) showed any activity towards the parasite or the macrophages.
Phomopsichromin C (3) was the only compound with any notable activity against any of the ESKAPE pathogens, with an MIC against MRSA of 58 µM. In a drug wash-out study, 3 was determined to be bactericidal. Impressively, this compound exhibits an MBC$_{99}$ of 47 µM, which is reasonably close to the control gentamicin (20 µM in this assay). However, 3 also displays notable cytotoxicity towards HepG4 human liver cells, with an LD$_{50}$ of 37 µM. Further supporting its cytotoxicity and lack of specificity, 3 is also active against the macrophage contained *Leishmania donovani* as mentioned above.

1, 2, and 5 all displayed activity against MRSA at extremely high concentrations (> 500 µM) and further cytotoxicity testing may be useful. If these compounds continue to be selective against the *Leishmania donovani* parasite, further biological testing against that, and other parasite targets would be warranted.

While none of the phomopsichromins immediately emerge as promising lead compounds against ESKAPE or *Leishmania donovani*, with a group of compounds of this size, and interesting “natural structure activity relationship (SAR) study” is beginning to take form. It is notable that 4 remains inactive, despite being nothing more than the ring-open form of 2. Meanwhile, 1 and its ring opened form, 5, display similar bioactivities. While six compounds is hardly enough to constitute a true SAR study, one can imagine that with the isolation (or synthesis) of more of these analogs, a pharmacophore may well begin to emerge. A fungal source is ideal for such studies as more material of these, and any other discovered compounds, could be obtained quite easily and in high quantities.
2.5. Future Directions

Our *Phomopsis* sp. isolate proved to be a producer of new, bioactive chemistry, and I believe, may still have biosynthetic potential remaining to be discovered. All six compounds discussed herein were isolated from the extract of the control growth condition. The extracts from the modified growth conditions remain to be explored. Additionally, NMR data suggests that there may be more phomopsichromins (or other known or new derivatives) as minor components of the investigated fractions that were abandoned due to time constraints. This strain remains archived in the Baker lab fungal library, along with the other 6 isolates from the branch piece. These organisms are all known to produce bioactive extracts and would all be interesting targets for further chemical analysis and growth culture optimization.

This genera is known to have great biosynthetic potential and despite being such a well-studied organism, environmental strains such as the one investigated here continue to be producers of new compounds. I believe that the work discussed herein stands as another layer of support for environmental microbial isolates as a promising source of new chemistry for drug discovery.

2.6. References


(4) Isaka, M.; Jaturapat, A.; Rukseree, K.; Danwisetkanjana, K.; Tanticharoen, M.;


Chapter 3
The Creation of a Large Fungal Isolate and Extract Library

3.1. Curating a Fungal Library

The Baker lab is currently home to over 7000 microbial isolates. With a focus on filamentous fungi, this library includes micro-organisms isolated from marine and terrestrial environments in Florida and Antarctica. The isolation techniques employed to build this library have evolved over time, inspired by the work of colleagues, techniques found in the literature, and the findings of various protocol optimization experiments. Isolation techniques vary according to source and isolate targets, but can be described by the following general protocol: 1) tissue surface sterilization with a 10% bleach solution and/or isopropyl alcohol; 2) tissue subsampling into 1 cm cross-sections; 3) plating in triplicate onto solid media plates of variable composition; 4) careful monitoring of colony growth on solid media plates for a period of 1-4 weeks in the lab; and 5) transferring individual colonies to new isolation plates of similar composition. The last step is repeated until a pure colony is established for each isolate.

Variations in solid media plate composition are employed to target a wide range of fungi and bacteria, and all tissue samples collected are plated across 6-10 different media types to access the microbial diversity that can be found living within each target organism. Each plate type is designed with the following general ingredients in varying concentrations: a nutrient source, agar, salt, and small molecule antibacterial and antifungal agents in sub-lethal doses to discourage the growth of fast-growing organisms. Available nutrient mixtures include: Sabaraud
Dextrose Broth (SDB), Potato Dextrose Broth (PDB), Tryptic Soy Broth (TSB), Malt Broth, Actino Agar, and glycerol. Small molecule additives include: nystatin, cycloheximide, and chloramphenicol. Solid media types are curated for each collection expedition based on source and microbial targets.

Collection locations and source organisms represented in the isolate library are varied. Some examples of organisms commonly sampled for microbial isolation include: Floridian mangroves (*Rhizophora mangle, Avicennia germinans*, and *Laguncularia racemose*), mangrove associated trees (*Conocarpus erectus, and Coccoloba uvifera*), and benthic marine invertebrates including sponges, tunicates, and corals. Collection date, location, and source organism identification is all carefully recorded in field notebooks during each expedition.

To accommodate different field conditions, two different collection techniques have been developed. The first technique is field plating, in which tissue samples are surface sterilized and directly plated on location onto solid media plates (“field plates”), which are then transferred back to the lab for monitoring of growth. The second technique is glycerol cryotube preservation, in which tissue samples are surface sterilized, frozen and stored for transit in a 20% glycerol solution in cryotubes before being plated in the lab. This allows for microbial collections to take place around the world, preserving tissues and micro-organisms until they can be processed in the lab. Table 3.1 illustrates collection details for all collection trips by the Baker lab between 2011 and 2017.
Table 3.1. Collection details for collection trips from Fall 2011 to Spring 2017.

<table>
<thead>
<tr>
<th>Season, Year</th>
<th>Location</th>
<th>Target Source Organism(s)</th>
<th>Target Micro-Organism(s)</th>
<th>Collection Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring, 2012</td>
<td>Everglades National Park, FL</td>
<td>MG</td>
<td>F, B</td>
<td>FP</td>
</tr>
<tr>
<td>Summer, 2012</td>
<td>Keys Marine Lab, FL</td>
<td>MG, MA</td>
<td>F, B, A, M</td>
<td>FP</td>
</tr>
<tr>
<td>Fall, 2013</td>
<td>Inter-coastal Islands, Dunedin, FL</td>
<td>MG</td>
<td>F</td>
<td>FP</td>
</tr>
<tr>
<td>Spring, 2014</td>
<td>Tampa Bay, St. Petersburg, FL</td>
<td>MG</td>
<td>F</td>
<td>FP</td>
</tr>
<tr>
<td>Summer, 2014</td>
<td>Keys Marine Lab, FL</td>
<td>MG, MA</td>
<td>F</td>
<td>FP</td>
</tr>
<tr>
<td>Summer, 2014- Fall, 2015</td>
<td>Inter-coastal Islands, Dunedin, FL</td>
<td>MG</td>
<td>F, B</td>
<td>L</td>
</tr>
<tr>
<td>Summer, 2015</td>
<td>R.V. Weatherbird, Gulf of Mexico</td>
<td>MA</td>
<td>F, B</td>
<td>GC</td>
</tr>
<tr>
<td>Spring 2016</td>
<td>Palmer Station, Antarctica</td>
<td>MA</td>
<td>F, B</td>
<td>GC</td>
</tr>
<tr>
<td>Fall 2016- Spring 2017</td>
<td>R.V. Akademik Troyshnikov, ACE, Antarctica</td>
<td>MA</td>
<td>F, B</td>
<td>GC</td>
</tr>
<tr>
<td>Spring, 2017</td>
<td>Palmer Station, Antarctica</td>
<td>MA</td>
<td>F, B</td>
<td>GC</td>
</tr>
</tbody>
</table>

MG= mangrove and mangrove associate plant material; MA= benthic marine macro-organisms (ie, sponges, tunicates, corals); F= fungi; B= bacteria; A= actinobacteria; M=myxobacteria; FP= field plating; GC= glycerol cryotube preservation; L= whole tissue samples returned to lab (not frozen) to follow isolation protocol (utilized for sampling sites close to the lab).

After establishing a pure isolate, all micro-organisms are archived in a 20% glycerol solution at -80 °C. Bacterial cells are suspended in the solution, while fungal material is stored as small cubes of growth cut from SDA isolation plates. Isolates are identified according to the following nomenclature: “Collection Location, Year- Source Organism Number- Isolation Plate Type-Isolate Number”. In this way, isolates can easily be grouped and retrieved according to any one of the collection or isolation parameters. Nearly 75% of the existing fungal isolates in this library were subjected to an epigenetics based high throughput screening (HTS) project.
3.2. Screening Protocols, Accomplishments

In accordance with two NIH R21 grants (NIH AI103673 and AI103715) a high throughput, epigenetics based fungal screening program was designed. A culture miniaturization and modification protocol was developed to accommodate the use of 20mL scintillation vials and a brown rice media. The histone deacetylase (HDAC) inhibitor sodium butyrate, and the DNA methyltransferase (DNMT) inhibitor 5-azacytidine were employed at concentrations of 100µM for epigenetic modification. With a target of screening 500 organisms in 3 growth conditions (Control, HDACi, and DNMTi) each month for 12 months, goal-oriented timelines and protocols (Table 3.2) were developed and put in place. Screening was completed with full-time staffing by 2 graduate and approximately 20 undergraduate students. Trainings and proper protocol regulations were employed to ensure uniformity in the extracts generated by the program. A full methodology for the culture, extraction and related procedures can be found in Appendix B. By implementing the developed protocols, a library of nearly 10,000 fungal extracts was produced over a time period of 18 months. At the height of productivity, 1,500 extracts were being produced and screened each month.
Table 3.2. Weekly sequence of events for continuous production of fungal isolates and extract libraries.

<table>
<thead>
<tr>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ Make rice media ≥ 4 boxes (400 vials)</td>
<td>□ Harvest, start extraction of 21 day old samples</td>
<td>□ Decant extracts</td>
<td>□ Weigh extracts</td>
<td>□ Get hit report, slant &amp; organize hits</td>
</tr>
<tr>
<td>□ Make isolation plates (types A,B,D &amp; TSA =1L; C &amp; SDA ≥ 2L)</td>
<td>□ Inoculate rice ≥ 4 boxes (400 vials)</td>
<td>□ Dry extracts under air</td>
<td>□ Weigh samples</td>
<td>□ Plate extracts for bioassay and storage (CDDI)</td>
</tr>
<tr>
<td>□ Make 200mL x 3 SDB for Eppendorf</td>
<td>□ Wash vials</td>
<td>□ Weigh samples</td>
<td>□ Create plate maps</td>
<td>□ Wash vials</td>
</tr>
<tr>
<td>□ Make glycerol cryotubes</td>
<td>□ Pre-weigh vials</td>
<td>□ Wash vials</td>
<td>□ Wash vials</td>
<td>□ Isolate/ describe/ archive fungi</td>
</tr>
<tr>
<td>□ Fill eppendorfs (SDB, HDAC, DNMT)</td>
<td>□ Isolate/ describe/ archive fungi</td>
<td>□ Isolate/ describe/ archive fungi</td>
<td>□ Plate extracts for bioassay and storage (CDDI)</td>
<td>□ Isolate/ describe/ archive fungi</td>
</tr>
<tr>
<td>□ Wash vials</td>
<td>□ Get hit report, slant &amp; organize hits</td>
<td>□ Get hit report, slant &amp; organize hits</td>
<td>□ Plate extracts for bioassay and storage (CDDI)</td>
<td>□ Plate extracts for bioassay and storage (CDDI)</td>
</tr>
</tbody>
</table>

The resulting extract library was dispersed for screening and archived in DMSO at a concentration of 10mg/mL in 96-well plate format. Beyond the scope of the original two grant funded screening targets (the ESKAPE pathogens and *Leishmania donovani*), extracts have been distributed for screening in a number of additional targets, resulting in a body of data that can inform high level prioritization of active extracts. Additional screening is ongoing, but extract data to date includes bioactivities against the ESKAPE pathogens, *Leishmania donovani*, J774 macrophage cells, *Mycobacterium tuberculosis*, *Clostridium difficile*, and *Naegleria fowleri*. The
library was additionally screened against a number of cell-based cancer targets and in a yeast based multiplex assay for anti-helminthics. Some active extracts were investigated by metabolomic analysis.

3.3. Metabolomic Analysis

Extracts from a subset of organisms producing bioactivities were subjected to metabolomics analysis to investigate and confirm chemical diversity of the extract library and effectiveness of epigenetic modification techniques. Bioassay results showed desirable distribution of active extracts among the three culture conditions, and in each assay, organisms displaying activity only after modification accounted for 15-30% of all activity observed (Figure 3.1).

![Figure 3.1](image.png)

*Figure 3.1. a) Representative pie chart from bioassay data, in this case, ESKAPE activity, that shows the activity boosting effects of the epigenetic modification; b) Representative pie chart from bioassay data, in this case, ESKAPE activity, that demonstrates distribution of activity across the three treatment conditions. This is the overall trend amongst all testing completed to date.*

To support and further investigate this diversity of activity, 123 extracts from 41 active organisms were analyzed via LC-QToF-MS. This subset of samples included active and non-active extracts (for instance, in the case that an organism only displayed activity after
modification, the control extract was still included in the analysis). It included a wide range of isolates from different collections, isolation media, and source organisms. The LC-QToF-MS data were summarized into a list of ‘chemical entities’ (HRESIMS @ retention time) for each sample using Agilent Qualitative Analysis and Mass Profiler Professional software (full protocols in Appendix B). Each sample, identified by its list of chemical entities, could then be statistically compared using Multidimensional Scaling (MDS). Each sample was also identified by a number of additional factors, including biological activity, culture treatment, and isolate identity. Using Primer 6 software, MDS plots could be generated and annotated with any of these factors. This analysis allowed for a visual representation of the chemical similarity of the isolates and extracts to one another, and verified the chemical diversity of both the fungal library as well as the different culture treatments (Figure 3.2 and 3.3).

Figure 3.2. An MDS plot of the Bray Curtis Similarity matrix of square root transformed data for 123 extracts (replicates averaged) from the screening program. Chemically unique samples (i.e. sample 93 in the top right quadrant) are clearly visible.
Figure 3.3. An MDS plot of the Bray Curtis Similarity matrix of square root transformed data for 9 randomly selected organisms from those represented in Figure 3.2. Each organism is denoted by an individual color, while each culture treatment is represented by a shape (control = triangle, HDAC = circle, DNMT = square). Full shapes represent extracts with ESKAPE activity, empty shapes represent no activity. Similarity contours highlight cluster analysis across organisms and treatments.

In Figure 3.3 we can see that some organisms share less than 20% similarity with the other analyzed organisms (e.g. KML12-14MG-B2a). Additionally, we can see that for some organisms, different culture conditions induce an extract dissimilarity as high as 60% (e.g. EG10-47C-1). This data illustrates not only chemical uniqueness between different fungal isolates- validating our collection and isolation protocols- but also allows organisms in which the epigenetic modification has had a large impact on the metabolite profile to be identified.
Organisms whose modified extracts exhibit unique chemistry and biological activities would be of high interest for scale up and chemical analysis.

Through the processing for MDS analysis, this data is also ideally prepared for dereplication efforts. With a robustly annotated database of known cytotoxins, nuisance compounds could be quickly identified and their extracts de-prioritized.

This data represents an exercise in metabolomics analysis of a small subset of screened extracts, but could be scaled up and performed on the entire extract library for high-level analysis of all available chemistry.

3.4. Training Set Results

Another subset of the extract library (1305 extracts, 13% of the total library) was screened fully against *Mycobacterium tuberculosis* (TB), the ESARKE pathogens, *Leishmania donovani*, *Naegleria fowleri*, and the J774 murine macrophage cell line. Using stringent definitions of 'active' to moderate the hit rate, 16% of these 1305 extracts were determined to be active. While this number is quite large, this is a result of the fact that 77% of the hits were hits in only one assay. Within this subset, accounting for all bioactivities, the previously observed trends in each of the individual assays (Figure 3.1) were verified; the three culture treatments were equally effective in producing active extracts, and 39% of active fungi only produced hits in the HDACi and DNMTi culture conditions.

To analyze this data in the most meaningful way, strict activity cut-offs were set for each bioassay. For simplicity, the ESARKE pathogens’ MICs were reported as a single scaled score for all 6 pathogens; an extract was considered ‘active’ in this assay if it had a scaled score ≥ 7. For highest clinical relevance, only extracts exhibiting an IC50 value < 1 μg mL-1 in the infected
macrophage model of the *Leishmania donovani* parasite were included. TB activity was included when ≥ 85%, and activity against *Naegleria fowleri* was defined as inhibition of >33% at either concentration tested (50 and 5 µg/mL). Any cytotoxicity against the J774 macrophage cells up to 20 µg/mL was also included. Results can be seen in Figure 3.4.

![Figure 3.4. Selectivity of active extracts.](image)

Only 48 active extracts (23% of total active, 4% of total screened) were not specific to a single target organism. 17 extracts (8%, 1%) were active only against *N. fowleri*, 53 (25%, 4%) against the ESKAPE panel, 37 (17%, 3%) against *Mycobacterium tuberculosis*, 41 (20%, 3%) against the *L. donovani* infected macrophage, and 14 (7%, 1%) were cytotoxic only against the J774 macrophages. When the definition of ‘active’ was relaxed in a secondary hit, (i.e. an extract was only considered ‘selective’ if it had reported activity only one of the 4 assays) 100 extracts, 48% of active extracts, retained their qualification as ‘selective’.
This is a unique set of data featuring extracts of diverse fungal origin screened against a wide range of eukaryotic and prokaryotic disease causing organisms. The specificity of bioactivity in this data set suggests unique underlying chemical profiles and, gratifyingly, provides strong support to our screening program design. With this data, we have demonstrated that vigorous front-end investigation (multiple bioassays, metabolomic analyses, dereplication, etc.) of a library of extracts can inform scale-up prioritization in a highly effective way for the greatest chance of isolating new, bioactive natural products. Scale-up efforts are underway to further validate these methods.

3.5. Future Directions

With such promising data from the extract library, scale-ups of bioactive fungi have commenced. A scale-up protocol was developed (Appendix B) and samples prioritized according to data available at the time. With the completed analysis of the training set, newly prioritized organisms have been identified and are available for chemical investigation in future. Extracts that feature specific, potent, and induced bioactivities can be scaled up and subjected to chemical analysis including new dereplication protocols as updated databases become available. With the plethora of front-end analysis that has been employed and presented here, organisms can now be chosen for chemical investigation with confidence in the chances of discovering new, bioactive secondary metabolites.

3.6. References Cited


(2) Kjer, J.; Debbab, A.; Aly, A. H.; Proksch, P. Methods for Isolation of Marine-Derived


Chapter 4

A New Citreohybriddione Discovered Via Epigenetic Modification

4.1. Citreohybridones and Citreohybriddiones

The hybrid strain KO 0031 of *Penicillium citreo-viride* B. IFO 6200 and 4692 has been reported to be a prolific producer of meroterpenoid secondary metabolites.\(^1\) Many of these hybrid polyketide-terpenoids are known to be feeding deterrents against the crop pest *Plutella xylostella*.\(^2\)\(^-\)\(^5\) The previously reported citreohybridones and citreohybriddiones are two groups of these fungal natural products (Figure 4.1). Herein, the isolations of a new citreohybriddione (citreohybriddione D, \textbf{1}) from an environmental *Penicillium* sp. fungus will be discussed.
Figure 4.1. Some of the previously reported citreohybridones, citreohybiddiones A-C, and the new citreohybiddione D (I).

4.2. KML12-14MG-B2a isolation

A number of collection trips to the Keys Marine Lab, Long Key, FL have targeted mangrove endophytes. Traveling by canoe and using field plating techniques (as described in Appendix B) samples were collected from areas all around the facility targeting a broad range of
micro-organisms. The Keys Marine Lab is uniquely located with access to a wide variety of mangrove environments (Figure 4.2).

![Google Maps satellite view of collection areas around Keys Marine Lab, Long Key Florida. Location markers: Blue = Keys Marine Lab; Red = developed mangrove areas; Green = protected mangrove areas within Long Key State Park; Yellow = exposed mangrove areas; Purple = landfill mangrove areas.](image)

**Figure 4.2.** Google Maps satellite view of collection areas around Keys Marine Lab, Long Key Florida. Location markers: Blue = Keys Marine Lab; Red = developed mangrove areas; Green = protected mangrove areas within Long Key State Park; Yellow = exposed mangrove areas; Purple = landfill mangrove areas.

KML12-14MG-B2a is a *Penicillium* sp. isolated from the 2012 Keys Marine Lab trip. This organism was isolated from the stem tissue of a juvenile *Rhizophora mangle* tree in an exposed mangrove community on the west side of Long Key. This fungus was isolated on a solid water agar plate containing sub-lethal doses of both nystatin and cycloheximide. On SDA this organism has a typical *Penicillium* morphology, growing radially from the inoculated mycelia with a light green/white color and fluffy sporulating bodies.
4.3. Initial Bioactivities, Scale-up, Epigenetic Modification, and Identification

Like most of the fungal isolates in the Baker Lab microbial library, KML12-14MG-B2a was cultured and screened as a part of the screening program introduced in Chapter 3. This isolate emerged early as an important hit against the ESKAPE pathogens, hitting against both gram positive and gram negative pathogens in the Control and HDACi growth conditions (Table 4.1). In support of the design of the screening program, the HDACi culture produced a consistently active extract across replicate cultures, while repeated Control cultures produced inconsistent activity. Demonstrating the ability of these organisms to regulate their biosynthetic machinery and the effectiveness of the HDACi culture conditions, KML12-14MG-B2a became a model organism for scale-up and chemical investigation protocols.

**Table 4.1. Screening results for KML12-14MG-B2a.**

<table>
<thead>
<tr>
<th>Date</th>
<th>Loc</th>
<th>Extract ID</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sample Information</td>
<td>200 100 50 25 10</td>
</tr>
<tr>
<td>1/9/2014</td>
<td>F1</td>
<td>KML12-14MG-B2a (HIT) CONTROL</td>
<td>EKAP EKAP EKAP EAP EA</td>
</tr>
<tr>
<td>1/9/2014</td>
<td>F2</td>
<td>KML12-14MG-B2a (HIT) HDAC</td>
<td>EKAP EKAP - - -</td>
</tr>
<tr>
<td>1/9/2014</td>
<td>F3</td>
<td>KML12-14MG-B2a (HIT) DNMT</td>
<td>- - - - -</td>
</tr>
<tr>
<td>2/3/2014</td>
<td>P2</td>
<td>KML12-14MG-B2a (HIT) CONTROL</td>
<td>- - - - -</td>
</tr>
<tr>
<td>2/3/2014</td>
<td>P2</td>
<td>KML12-14MG-B2a (HIT) HDAC</td>
<td>ESKAP ESKAP ESKAP EKP EK</td>
</tr>
<tr>
<td>2/3/2014</td>
<td>P2</td>
<td>KML12-14MG-B2a (HIT) DNMT</td>
<td>- - - - -</td>
</tr>
</tbody>
</table>

This organism was screened multiple times as a part of a subset of isolates chosen to verify reproducibility in the screening protocol. Activity is indicated by the first letter of the pathogen the extract showed activity against. E = *Enterococcus faecium*, S = *Staphylococcus aureus*, K = *Klebsiella pneumoniae*, A = *Acinetobacter baumannii*, P = *Pseudomonas aeruginosa*. 

57
KML12-14MG-B2a was scaled up in control culture conditions on 700g of brown rice (autoclaved with 1.4L DI water) and inoculated in 100mL of SDB in 2 types of Unicorn brand mycobags and a 3L fernbach flask. After 21 days of incubation, both types of mycobag (Unicorn types 3T and 14A) were found to produce sterile, fully optimized growth (i.e. all rice material covered with growth) that resulted in extracts of similar mass. The fernbach flask culture proved significantly less desirable, with fungal growth only on the top of the solid rice due to an inability to agitate the culture throughout the culture process.

Epigenetically modified cultures on KML12-14MG-B2a were then scaled up using the same protocols (at this time, Unicorn bag types 3T and 14A were used interchangeably according to supply. Type 3T bags were later purchased in bulk and all further scale-ups done in those bags). Again, resulting growth was uncontaminated and represented complete media coverage. The cultures each resulted in approximately 70g of crude extract after a 3 day exhaustive extraction (day 1: 50mL MeOH, 750 mL EtOAc; days 2 and 3: 800 mL EtOAc). Extracts were filtered over celite and sent for bioassay. Unfortunately, the crude extracts of the scale-ups did not replicate the activity seen in the small scale cultures. A liquid: liquid partition between EtOAc and H$_2$O, followed by NP MPLC separation of the EtOAc partition was performed on each extract, and the resulting fractions sent for bioassay. Again, disappointingly, the fractions returned inactive. Scale-up optimization efforts were continued with other organisms in search of a scale up protocol that could be standardized and used in a scale-up screening program. Due to interesting NMR data, chemical investigation continued on the fractions of the HDACi extract of KML12-14MG-B2a.
KML12-14MG-B2a was identified using sanger sequencing of the 18S ribosomal spacer region (Appendix C). Agreeing with the previously observed morphology, the isolate was identified to the genus level as a *Penicillium* sp.

4.4. Compound Isolation and Structure Elucidation

The MPLC separation of KML12-14MG-B2a HDACi resulted in 10 fraction (Figure 4.3). With no bioactivity in any of the fractions, compound elucidation proceeded via NMR guided fractionation. NMR analysis of all 10 fractions identified fraction F as interesting for further investigation. HPLC separation of F was completed in normal phase (n-hexanes: ethyl acetate) on a silica column to yield 10 fractions (Figure 4.4). Fraction F-2 was discovered to contain the new meroterpenoid, citreohybriddione D (1).

**Figure 4.3.** The NP MPLC chromatogram of KML12-14MG-B2a HDACi.
Fraction F-2 was further purified on silica to yield compound 1. The $^1$H NMR spectrum of 1 was notable in that it contained 8 methyl signals (Table 4.2) and almost nothing in the olefin region. Investigation of the $^{13}$C data confirmed the presence of a large number of quaternary carbons, indicating a highly functionalized fused ring system such as a polyketide. The HRESIMS of 503.2640 [M + H]$^+$ resembled the terpenoid skeletons of the citreohybridones and citreohybriddiones, but did not match any of the previously reported compounds. $^{13}$C NMR signals of carbons 1-12 strongly matched citreohybriddone D, however carbons 13-18 more closely resembled citreohybriddione B.$^1$
Table 4.2. 1D and 2D data for citreohybriddione D (1) in CDCl₃.

<table>
<thead>
<tr>
<th>Pos</th>
<th>δCᵇ</th>
<th>δH (m, J(Hz))ᵃ</th>
<th>HMBCᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.8</td>
<td>1.03 (m, 1 H)</td>
<td>2, 9, 10, 22, 23, 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.38 (m, 1 H)</td>
<td>3, 5, 9, 10</td>
</tr>
<tr>
<td>2</td>
<td>23.3</td>
<td>1.6 (m, 2 H)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>76.8</td>
<td>4.68 (t, 2, 6, 1H)</td>
<td>1, 5, 26</td>
</tr>
<tr>
<td>4</td>
<td>36.9</td>
<td>2.38 (m, 1 H)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>47.7</td>
<td>1.83 (m, 1 H)</td>
<td>4, 6, 10, 23, 24</td>
</tr>
<tr>
<td>6</td>
<td>16.9</td>
<td>1.8 (m, 1 H)</td>
<td>4, 8, 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.01 (m, 1 H)</td>
<td>7, 10</td>
</tr>
<tr>
<td>7</td>
<td>30.8</td>
<td>2.83 (m, 1 H)</td>
<td>5, 6, 8, 22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.38 (m, 1 H)</td>
<td>5, 9, 10</td>
</tr>
<tr>
<td>8</td>
<td>38.6</td>
<td>8.10, 11, 12, 23</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>53.5</td>
<td>2.21 (m, 1 H)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>52.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>126.4</td>
<td>5.85 (s, 1 H)</td>
<td>8, 9, 10, 13, 21</td>
</tr>
<tr>
<td>12</td>
<td>133.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>60.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>70.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>210.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>72.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>206.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>19.8</td>
<td>1.4 (s, 3 H)</td>
<td>15, 16, 17</td>
</tr>
<tr>
<td>19</td>
<td>167.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>16.4</td>
<td>1.31 (s, 3 H)</td>
<td>12, 13, 14, 17</td>
</tr>
<tr>
<td>21</td>
<td>18.8</td>
<td>1.7 (s, 3 H)</td>
<td>11, 12, 13</td>
</tr>
<tr>
<td>22</td>
<td>19.5</td>
<td>1.17 (s, 3 H)</td>
<td>7, 8, 9, 14</td>
</tr>
<tr>
<td>23</td>
<td>204.4</td>
<td>10.14 (s, 1 H)</td>
<td>1, 10, 22</td>
</tr>
<tr>
<td>24</td>
<td>26.5</td>
<td>0.97 (s, 3 H)</td>
<td>2, 3, 4, 5, 25</td>
</tr>
<tr>
<td>25</td>
<td>21.3</td>
<td>0.9 (s, 3 H)</td>
<td>3, 4, 5, 24</td>
</tr>
<tr>
<td>3-OAc</td>
<td></td>
<td>170.6</td>
<td></td>
</tr>
<tr>
<td>3- OAc</td>
<td>21.3</td>
<td>2.12 (s, 3 H)</td>
<td>26</td>
</tr>
<tr>
<td>16- OH</td>
<td>2.16 (s, 1 H)</td>
<td>15, 16, 17</td>
<td></td>
</tr>
<tr>
<td>19- OMe</td>
<td>52.0</td>
<td>3.63 (s, 3 H)</td>
<td>19</td>
</tr>
</tbody>
</table>

ᵃ¹H NMR recorded at 500 MHz, reported in ppm (multiplicity, J-coupling in Hz, integration); ᵇ¹³C NMR recorded at 200 MHz; ᵇrecorded from a gHMBCAD experiment at 500 MHz and reported as positions of carbons.

With conformation from 2D NMR experiments (Figure 4.5), it was determined that 1 is, in fact a new citreohybriddione lacking any lactone or epoxide ring structures. 2D NOESY data confirmed that the absolute stereochemistry matches what has been previously reported for similar compounds (Figure 4.6).¹
Figure 4.5. Important COSY, HMBC, and NOESY correlations in Citreohybriddione D (1).

NOE correlations between the methoxy at C-19 (δ_H 3.63) and the methyl groups at C-18 (δ_H 1.40) and C-20 (δ_H 1.31) confirmed that the D-ring in 1 is up as in the other citreohybriddiones. The alcohol on C-16 of 1 has the same orientation as that in citreohybriddione B. Through-space correlations between C-22, 23, and 25 confirm that the stereochemistry of the A and B rings of 1 are as reported in citreohybriddione A.

Figure 4.6. A 3D depiction of 1 for stereochemical review.
4.5. Future Directions

Citreohybriddione D (1) was isolated from the HDACi treatment extract. Initial investigation via HPLC of similar fractions indicated that 1 may have also been present in the DNMTi extract as well, but appeared absent in the control treatment fractions. However, HRESIMS investigation of the ethyl acetate partitions of all three treatment extracts revealed that 1 could be found in all treatments. Nevertheless, HRESIMS and NMR analysis shows that there are notable chemical differences between the control and modified conditions, indicating that there may be many other new compounds to be found from this *Penicillium* sp. yet.

The culture optimization of KML12-14MG-B2a was not completed due to time restraints. This organism remains active in the small scale, and warrants further study to be able to replicate that activity on a culture scale that allows for chemical investigation of the active compound(s). The isolation of 1 confirms that this *Penicillium* sp. contains PKSs (polyketide synthases) that are capable of producing interesting secondary metabolites. Additionally, the small scale screening results confirm that this organism responds favorably to HDACi treatment, indicating that efforts in culture optimization would likely be rewarded with the production of many otherwise silenced natural products.

While there are no reported activities for any of the citreohybriddiones (including 1) against human disease, further testing of this new compound is warranted. Due to mass limitations, 1 was only screened against the ESKAPE pathogens and the *Leishmania donovani* parasite. Feeding studies and cytotoxicity profiling would be interesting to see how it compares to the rest of the compounds in this class.
4.6. References


Appendix A: Experimental and Supporting Data for Chapter 2

Fungal Strain Identification
MPLC Run Parameters and Chromatograms
HPLC Run Parameters and Chromatograms
Compound Data

Fungal Strain Identification

The fungal isolate “BB11-2” was sequenced using sanger sequencing of the 18S ribosomal spacer region. The forward and reverse primers used can be found in table A1. The PCR product was sent to europhins sequencing company which yielded the sequences that were blasted using NCBI nucleotide blast for sequence similarity.
Table A1. The forward and reverse primers used for the fungal strain identification of BB11-2.

<table>
<thead>
<tr>
<th>Primers Type</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S forward</td>
<td>NNNNNNNNNNNTTGGTTTCTAGGACCGCGTAAATGATTAAT AGGGACGATCGGGGCCCCTGATATATGAGTTTCAATCGTACAGAG</td>
</tr>
<tr>
<td></td>
<td>GTGAAATTCTGGATCGATTGAAAGACTAACTACTGCGAAAGCA TTTGCCAAGGATGTTTTCCATTAATCAGGAACGAAAGT</td>
</tr>
<tr>
<td></td>
<td>TAGGGGATCGAAAAACGATCAGATAACCGTTGAGTCTTTAAATCAT AAACATATGCCAATCAGGATCNGGCCGTGTTATTCTT</td>
</tr>
<tr>
<td>18S reverse</td>
<td>NNNNNNNNNNNCCGNNTCNCCCTTGGTGGTGCCCTTCCGT CAATTCTTTTAAGTTTGACGCTTACCTCC</td>
</tr>
<tr>
<td></td>
<td>CCCAGAAACCCAAAAACTTTACTTTTCTGTGTAAGGTTGCGAGCGG GTCAAGAATATAACACCGCCCCTAGTCGCATA</td>
</tr>
<tr>
<td></td>
<td>GTTTATGTTTAAGACTAAACCGGTATCTGATCGTTTTCCGATNCC CTAACCTTCTCGTTCTGATNANGANAAACATCCTTGG</td>
</tr>
<tr>
<td></td>
<td>GAAATGCTTTCCCNANTAAATNNNGNCTTCCNATCAAATCCTCA</td>
</tr>
</tbody>
</table>

Figure A1. NCBI nucleotide blast results for BB11-2 forward primers.
<table>
<thead>
<tr>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query cover</th>
<th>E value</th>
<th>Identity</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsheathed herpes virus for 18S RNA, partial sequence, clone: AK1545_42</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>LC212311.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. 32 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KU950744.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. SC1104 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KX824516.1</td>
</tr>
<tr>
<td>Fungal sp., voucher Robert L. Gilbertson: Mirological Herbarium 8702 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT289966.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. 115F1 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT190167.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. 15F1 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT190151.1</td>
</tr>
<tr>
<td>Endothia sp., N11U4 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT100146.1</td>
</tr>
<tr>
<td>Endothia sp., N11U4 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT100146.1</td>
</tr>
<tr>
<td>Endothia sp., N11U4 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT100146.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. SF62 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT100123.1</td>
</tr>
<tr>
<td>Diaporthe sp., SF64 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT100120.1</td>
</tr>
<tr>
<td>Diaporthe sp., SF62 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT100110.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. SF61 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT100109.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. SF62 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT100097.1</td>
</tr>
<tr>
<td>Diaporthe amygdali strain OA-63 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KR642445.1</td>
</tr>
<tr>
<td>Diaporthe sp., OA-63 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KR642445.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. OA-91 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT642445.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. OA-91 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KT642445.1</td>
</tr>
<tr>
<td>Stenocarpellidium maydis strain 6m A-1 18S ribosomal RNA intergenic spacer, partial sequence, 18S ribosomal RNA gene, internal transcribed spacer 1, 8.</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KP868731.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. SI-32 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KP868731.1</td>
</tr>
<tr>
<td>Prorhodosporidium sp. SI-32 18S ribosomal RNA gene, partial sequence</td>
<td>403</td>
<td>403</td>
<td>81%</td>
<td>1e-108</td>
<td>98%</td>
<td>KP868731.1</td>
</tr>
</tbody>
</table>

**Figure A2.** NCBI nucleotide blast results for BB11-2 reverse primers.
MPLC Run Parameters and Chromatograms

Sample: BB11_2CONTROLrun1b  
RediSep Column: Silica 120g  
SN: E04107CA794E8 Lot: 2317325010X  
Flow Rate: 85 ml/min  
Equilibration Volume: 0.0 CV  
Initial Waste: 0.0 CV  
Air Purge: 1.0 min  
Solvent: A1 hexane  
Solvent: A2 methanol  
Solvent: B1 ethyl acetate  
Rf 200 : Baker Lab MPLC-ELSD  
Monday 16 June 2014 04:08PM  
Peak Tube Volume: Max.  
Non-Peak Tube Volume: Max.  
Loading Type: Solid  
Wavelength 1 (red): 254nm  
Wavelength 2 (purple): 250nm  
Peak Width: 4 min  
Threshold: 0.20 AU  
All Wavelength (orange): 200nm - 780nm  
Peak Width: 4 min  
Threshold: 0.20 AU

Run Notes:

Figure A3. NP MPLC chromatogram of BB11-2 Control EtOAc partition.
Figure A4. A second NP MPLC of BB11-2 Control fraction D from the first MPLC separation. Fraction C (bottles 4 & 5) contained the phomopsichromins.
**Figure A5.** NP MPLC chromatogram of BB11-2 HDACi EtOAc partition.
Figure A6. NP MPLC chromatogram of BB11-2_DNMTi EtOAc partition.

HPLC Run Parameters and Chromatograms

Normal phase HPLC separation was completed with a normal phase gradient of hexanes to ethyl acetate over 35 minutes on a semi-preperative Phenominex® Cyano column (5µm, 100Å, 250 x 10mm) using UV and ELSD detection. Reverse phase HPLC separation was completed with a RP gradient of water to acetonitrile or methanol on a Phenominex® C18 column (5µm, 100Å, 250x 4.6mm) using UV and ELSD detection.
Figure A7. NP HPLC chromatogram (black) and ELSD trace (blue) of BB11-2_D_C.
**Compound Data**

Phomopsichromin A (1): C$_{23}$H$_{30}$O$_{5}$; HRESIMS $m/z$ 369.2034 [M + H – H$_2$O]$^+$ (C$_{23}$H$_{29}$O$_4$ calculated, 369.2066), $m/z$ 387.2137 [M + H]$^+$ (C$_{23}$H$_{31}$O$_5$ calculated, 387.2171), $m/z$ 409.1954 [M + Na]$^+$ (C$_{23}$H$_{30}$O$_5$Na calculated, 409.1991); UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 250 (4.97) nm; $[\alpha]_{D}^{20}$ $+1.2$ ($c$ 0.1, MeOH); IR (thin film) 3450, 2980, 2360, 1700, 1600, 1575, 1450, 1400, 1300, 1090 cm$^{-1}$; $^1$H NMR Data (500 MHz, CDCl$_3$) $\delta$ ppm 0.59 (s, 3 H), 0.89 (d, $J$=6.8 Hz, 3 H), 0.91 (d, $J$=6.8 Hz, 3 H), 1.41 (m, 1 H), 1.44 (s, 3 H), 1.48 (m, 1 H), 1.52 (m, 1 H), 1.64 (m, 1 H), 1.77 (m, 1 H), 1.85 (m, 1 H), 1.97 (m, 1 H), 2.33 (m, 1 H), 2.36 (m, 1 H), 2.41 (q, $J$=6.6 Hz, 1 H), 2.55 (s, 3 H), 5.46 (d, $J$=10.2 Hz, 1 H), 6.24 (s, 1 H), 6.76 (d, $J$=10.2 Hz, 1 H), 11.74 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ ppm 7.5 (CH$_3$, C-12), 14.9 (CH$_3$, C-13), 15.4 (CH$_3$, C-14), 30.6 (CH$_2$, C-7), 30.8 (CH$_2$, C-4), 34.3 (CH$_2$, C-8), 36.1 (CH, C-5), 41.5 (CH$_2$, C-3), 43.2 (C-6), 50.4 (CH, C-1), 79.8 (C-9), 103.6 (C-18), 106.8 (C-16), 111.9 (CH, C-20), 116.9 (CH, C-11), 125.8 (CH, C-10), 144.4 (C-19), 158.6 (C-21), 160.6 (C-17), 175.1 (C-23) 213.8 (C-2).
Figure A8. $^1$H NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin A (1).
Figure A9. $^{13}$C NMR Spectrum (125 MHz, CDCl$_3$) of Phomopsichromin A (1).

Figure A10. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin A (1).
Figure A11. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin A (1).

Figure A12. $^1$H-$^1$H gCOSY NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin A (1).
Figure A13. $^1$H-$^1$H NOESY NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin A (1).

Phomopsichromin B (2): C$_{23}$H$_{32}$O$_5$; HRMS $m/z$ 371.2222 [M + H – H$_2$O]$^+$ (C$_{23}$H$_{31}$O$_4$ calculated, 371.2222), $m/z$ 389.2322 [M + H]$^+$ (C$_{23}$H$_{33}$O$_5$ calculated, 389.2328), $m/z$ 411.2145 [M + Na]$^+$ (C$_{23}$H$_{32}$O$_5$Na calculated, 411.2147); UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 250 (4.08) nm; $[\alpha]^{20}_D$ -1.5 (c 0.1, MeOH); IR (thin film) 3450, 2930, 2380, 1675, 1625, 1600, 1575, 1460, 1400, 1300, 1090 cm$^{-1}$; $^1$H NMR Data (600 MHz, CDCl$_3$) $\delta$ ppm 0.82 (d, $J$=6.6 Hz, 3 H), 0.86 (s, 3 H), 0.95 (d, $J$=7.1 Hz, 3 H), 1.27 (m, 1 H), 1.35 (m, 2 H), 1.41 (s, 3 H), 1.43 (m, 1H), 1.45 (m, 1 H), 1.46 (m, 1H), 1.55 (m, 1 H), 1.63 (m, 2 H), 1.8 (m, 1 H), 2.53 (s, 3 H), 3.85 (q, 2.8, 1 H), 5.46 (d, $J$=10.2 Hz, 1 H), 6.22 (s, 1 H), 6.74 (s, $J$=10.2 Hz, 1 H), 11.80 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ ppm 12.3 (CH$_3$, C-12), 15.6 (CH$_3$, C-13), 17.3 (CH$_3$, C-14), 24.4 (CH$_3$, C-22), 25.4 (CH$_2$, C-4), 27.0 (CH$_3$, C-15), 30.9 (CH$_2$, C-7), 33.9 (CH$_2$, C-3), 34.4 (CH$_2$, C-8), 36.5 (CH, C-5), 38.2 (C-6), 39.4 (CH, C-1), 73.1 (CH, C-2), 80.1 (C-9), 103.4 (C-18), 106.9 (C-16), 111.9 (CH, C-20), 116.6 (CH, C-11), 126.2 (CH, C-10), 144.1 (C-19), 158.7 (C-21), 160.6 (C-17), 174.7 (C-23).
Figure A14. $^1$H NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin B (2).
Figure A15. $^{13}$C NMR Spectrum (125 MHz, CDCl$_3$) of Phomopsichromin B (2).

Figure A16. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin B (2).
Figure A17. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin B (2).

Figure A18. $^1$H-$^1$H gCOSY NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin B (2).
Figure A19. $^1$H-$^1$H NOESY NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin B (2).

Figure A20. $^1$H NMR Spectrum (400 MHz, CDCl$_3$) of methylated phomopsichromin B (7). A new methoxy signal can be seen at $\delta_H$ 3.92.
Figure A21. $^{13}$C NMR Spectrum (500 MHz, CHCl$_3$) of methylated phomopsichromin B (7). 24 $^{13}$C signals can be seen.

Phomopsichromin C (3): C$_{25}$H$_{34}$O$_6$; HRESIMS $m/z$ 413.2330 [M + H – H$_2$O]$^+$ (C$_{25}$H$_{33}$O$_5$ calculated, 413.2328), $m/z$ 431.2434 [M + H]$^+$ (C$_{25}$H$_{35}$O$_6$ calculated, 431.2434), $m/z$ 453.2256 [M + Na]$^+$ (C$_{25}$H$_{34}$O$_6$Na calculated, 453.2253); UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 255 (4.21) nm; [$\alpha$]$^{20}_D$ +1.3 (c 0.1, MeOH); IR (thin film) 2930, 1740, 1675, 1600, 1580, 1400, 1300, 1350, 1090 cm$^{-1}$; $^1$H NMR Data (600 MHz, CDCl$_3$) $\delta$ ppm 0.83 (2d, $J$=6.6 Hz, 6 H; s, 3 H), 1.29 (m, 1 H), 1.40 - 1.43 (m, 6 H), 1.47 (m, 1 H), 1.5 (m, 2 H), 1.55 (m, 1 H), 1.65 (m, 1 H), 1.81 (m, 1 H), 2.05 (s, 3 H), 2.53 (s, 3 H), 4.97 (q, 2.0, 1 H), 5.45 (d, $J$=10.2 Hz, 1 H), 6.22 (s, 1 H), 6.74 (d, $J$=10.0 Hz, 1 H), 11.82 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ ppm 12.0 (CH$_3$, C-12), 15.6 (CH$_3$, C-13), 16.7 (CH$_3$, C-14), 21.4 (CH$_3$, C-25), 24.4 (CH$_3$, C-22), 25.8 (CH$_2$, C-4), 27.1 (CH$_3$, C-15), 30.7 (CH$_2$, C-7), 30.9 (CH$_2$, C-3), 34.4 (CH$_2$, C-8), 36.2 (CH, C-5), 38.3 (C, C-6), 38.5 (CH, C-1), 75.1
(CH, C-2), 80.1 (C, C-9), 103.3 (C, C-18), 106.8 (C, C-16), 111.9 (CH, C-20), 116.7 (CH, C-11), 126.1 (CH, C-10), 144.1 (C, C-19), 158.7 (C, C-21), 160.6 (C, C-17), 170.8 (COCH3, C-24), 174.4 (COOH, C-23).

Figure A22. 1H NMR Spectrum (600 MHz, CDCl3) of Phomopsichromin C (3).
Figure A23. $^{13}$C NMR Spectrum (125 MHz, CDCl$_3$) of Phomopsichromin C ($3\text{)}$.

Figure A24. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin C ($3\text{)}$.
Figure A25. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, CDCl$_3$) of Phomopsichromin C (3).

Figure A26. $^1$H-$^1$H gCOSY NMR Spectrum (600 MHz, CDCl$_3$) of Phomopsichromin C (3).
Phomopsichromin D (4): C_{23}H_{34}O_5; HRESIMS m/z 373.2373 [M + H – H_2O]^+ (C_{23}H_{33}O_4 calculated, 373.2379), m/z 391.2473 [M + H]^+ (C_{23}H_{35}O_5 calculated, 391.2484), m/z 413.2298 [M + Na]^+ (C_{23}H_{34}O_5Na calculated, 413.2304); UV (MeOH) λmax (log ε) 225 (5.89) nm; [α]^{20}_D +0.2 (c 0.1, MeOH); IR (thin film) 3400, 2930, 1600, 1500, 1450, 1360, 1300, 1180, 1080 cm\(^{-1}\); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) δ ppm 0.74 (d, \(J=6.6\) Hz, 3 H), 0.75 (s, 3 H), 1.08 (m, 3 H), 1.13 (m, 1 H), 1.17 (m, 1 H), 1.23 (m, 1 H), 1.35 (m, 1 H), 1.4 (m, 2 H), 1.53 (m, 1H), 1.6 (m, 1 H), 1.69 (m, 1 H), 1.70 (s, 3 H), 1.72 (s, 1 H), 2.38 (s, 3 H), 3.09 (q, 2 H), 3.16 (br d, \(J=7.1\) Hz, 2 H), 3.62 (q, 2.4, 1 H), 5.12 (br t, \(J=6.9\) Hz, 1 H), 6.23 (s, 1 H), 10.02 (s, 1 H), 12.61 (s, 1 H); \(^1^3\)C NMR (125 MHz, DMSO-\(d_6\)) δ ppm 12.7 (CH_3, C - 12), 15.7 (CH_3, C-13), 16.1 (CH_3, C-15), 17.3 (CH_3, C-14), 21.5 (CH_2, C-11), 23.8 (CH_3, C-22), 25.5 (CH_2, C-4), 32.4 (CH_2, C-8), 34.1 (CH_2, C-3), 36.0 (CH, C-5), 36.1 (CH, C-7), 38.0 (C, C-6), 39.0 (CH, C-1), 70.4 (CH, C-2), 103.6 (C, C-18), 110.4 (CH, C-20), 112.0 (C, C-16), 121.8 (CH, C-10), 134.7 (C, C-9), 139.8 (C, C-19), 159.6 (C, C-17), 162.8 (C, C-21), 174.1 (C, C-23).

Figure A27. \(^1\)H NMR Spectrum (500 MHz, DMSO-\(d_6\)) of Phomopsichromin D (4).
Figure A28. $^{13}$C NMR Spectrum (125 MHz, DMSO-$d_6$) of Phomopsichromin D (4).
Figure A29. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin D (4).
Figure A30. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, DMSO-$d_6$) of Phomopsichromin D (4).
Figure A31. $^1$H-$^1$H gCOSY NMR Spectrum (500 MHz, DMSO-$d_6$) of Phomopsichromin D (4).
Phomopsichromin E (5): C\textsubscript{23}H\textsubscript{32}O\textsubscript{5}; HRESIMS m/z 371.2229 [M + H – H\textsubscript{2}O]\textsuperscript{+} (C\textsubscript{23}H\textsubscript{31}O\textsubscript{4} calculated 371.2222), 389.2363 [M + H]\textsuperscript{+} (C\textsubscript{23}H\textsubscript{35}O\textsubscript{5} calculated 389.2328), 411.2152 [M + Na]\textsuperscript{+} (C\textsubscript{23}H\textsubscript{32}O\textsubscript{5}Na calculated 411.2147); UV (MeOH) \(\lambda_{\text{max}}\) (log \(\varepsilon\)) 220 (4.14); \([\alpha]^{20}\text{D} -0.4\) (c 0.1, MeOH); IR (thin film) 3390, 2950, 1590, 1300, 1160, 1090, 1020 cm\(^{-1}\); \(^1\)H NMR Data (600 MHz, DMSO-\(d\textsubscript{6}\)) \(\delta\) ppm 0.46 (s, 3 H), 0.76 (d, \(J=6.6\) Hz, 3 H), 0.81 (d, \(J=6.6\) Hz, 3 H), 1.23 (m, 1 H), 1.32 (m, 1 H), 1.47 (m, 1 H), 1.73 (s, 3 H), 1.76 (m, 1 H), 1.80 (m, 1 H), 1.91 (m, 1 H), 1.99 (s, 1 H), 2.10 (m, 1 H), 2.39 (s, 3 H), 2.40 (m, 1 H), 2.53 (q, \(J=6.7\) Hz, 1 H), 3.17 (d, \(J=7.1\) Hz, 2 H), 5.18 (br t, \(J=6.7\) Hz, 1 H), 6.23 (s, 1 H), 10.04 (s, 1 H), 12.62 (s, 1 H); \(^{13}\)C NMR (125 MHz, DMSO-\(d\textsubscript{6}\)) \(\delta\) ppm 7.5 (CH\textsubscript{3}, C-12), 14.7 (CH\textsubscript{3}, C-13), 15.0 (CH\textsubscript{3}, C-14), 16.1 (CH\textsubscript{3}, C-15), 21.5 (CH\textsubscript{2}, C-11), 23.7 (CH\textsubscript{3}, C-22), 30.3 (CH\textsubscript{2}, C-4), 32.0 (CH\textsubscript{2}, C-8), 35.0 (CH, C-5), 35.4 (CH\textsubscript{2}, C-7), 40.7 (CH\textsubscript{2}, C-3), 42.7 (C, C-6), 49.2 (CH, C-1), 103.6 (C, C-18), 110.4 (CH, C-20), 111.9 (C, C-16), 122.2 (CH, C-10), 134.2 (C, C-9), 139.9 (C, C-19), 159.6 (C, C-17), 162.8 (C, C-21), 174.1 (COOH, C-23), 212.6 (C, C-2).
Figure A32. $^1$H NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin E (5).
Figure A33. $^{13}$C NMR Spectrum (201 MHz, DMSO-$d_6$) of Phomopsichromin E (5).

Figure A34. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin E (5).
Figure A35. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin E (5).

Figure A36. $^1$H-$^1$H gCOSY NMR Spectrum (600 MHz, DMSO-$d_6$) of Phomopsichromin E (5).

LL-Z1272ε (6): C$_{23}$H$_{32}$O$_4$; HRESIMS $m/z$ 373.2411 [M + H]$^+$ (C$_{23}$H$_{33}$O$_4$ calculated 373.2334);

UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$) 224 (4.03) nm; IR (thin film) 3400, 2950, 2360, 1600, 1460, 1380,
1300, 1080 cm$^{-1}$; $^1$H NMR Data (600 MHz, CDCl$_3$) $\delta$ ppm 0.59 (s, 3 H), 0.89 (d, $J$=6.6 Hz, 3 H),
0.92 (d, $J$=6.6 Hz, 3 H), 1.39 (m, 1 H), 1.45 (m, 1 H), 1.62 (m, 1 H), 1.85 (m, 1H), 1.85 (s, 3 H),
1.91 (m, 1 H), 2.00 (m, 1 H), 2.06 (m, 1 H), 2.34 (m, 1 H), 2.35 (m, 1 H), 2.47 (q, \( J=6.5 \) Hz, 1 H), 2.51 (s, 3 H), 3.41 (d, \( J=7.1 \) Hz, 2 H), 5.29 (br t, \( J=6.6 \) Hz, 1 H), 6.12 (br s, 1 H), 6.23 (s, 1 H), 10.09 (s, 1 H), 12.76 (s, 1 H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \) ppm 7.5 (CH\(_3\), C-12), 15.0 (CH\(_3\), C-13), 15.3 (CH\(_3\), C-14), 16.5 (CH\(_3\), C-15), 18.0 (CH\(_3\), C-22), 21.2 (CH\(_2\), C-11), 30.9 (CH\(_3\), C-14), 32.6 (CH\(_2\), C-8), 35.6 (CH\(_2\), C-7), 36.1 (CH, C-5), 41.5 (CH\(_2\), C-3), 43.4 (C-6), 50.4 (CH, C-1), 110.6 (CH, C-20), 111.5 (C-16), 113.2 (C-18), 121.1 (CH, C-10), 139.2 (C-9), 142.0 (C-19), 162.1 (C-21), 163.5 (C-17), 193.0 (CH, C-23), 213.9 (C-2).

![Figure A37. \(^1\)H NMR Spectrum (600 MHz, CDCl\(_3\)) of LL-Z1272ε (6).](image)
Figure A38. $^{13}$C NMR Spectrum (125 MHz, CDCl$_3$) of LL-Z1272c (6).

Table A2. Pure compound bioactivities of 1-6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRSA IC$_{50}$ (µM)</th>
<th>L. donovani IC$_{50}$ (µM)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>518</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>515</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>515</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

MRSA MBC$_{99}$: 47 µM
HepG4 LD$_{50}$: 37 µM
Appendix B: Experimental and Supporting Data for Ch. 3

Microbial Isolation Protocols
Screening Protocols
LC-QToF-MS Protocols
Metabolomic and Statistical Analysis Protocols
Scale-up Protocols

Microbial Isolation Protocols

Nutrient media components (SDB, PDB, TSA, Actinomycete Isolation Agar, Malt Extract Agar) and agar were produced by BD™ Difco™ and purchased through Fisher Scientific. Glycerol, nystatin, cycloheximide, and chloramphenicol were purchased from Sigma Aldrich®.

Solid media was mixed, heated, and autoclaved according to manufacturer’s instructions and poured to set in Fisherbrand™ petri dishes.

After collections, field plates were incubated at 20-25°C (room temperature), 4°C (refrigerated), or 26-30°C (heated) as source organism or microbial targets dictated. Generally, bacterial plates were heated and fungal plates were left at room temperature for collections in warm climates. Cold water microbial isolation generally took place with refrigeration. Field plates were incubated for 1-4 weeks, and disposed of after all colonies were isolated and/or after the whole plate was covered in microbial growth.

Isolated pure colonies were grown on SDA (fungi) and TSA (bacteria) for archiving as described above. Descriptions of each organism on this standard media were recorded.
**Screening Protocols**

Each fungal isolate was grown on SDA from either glycerol stocks or isolation plates, and after a colony was established, was subsampled for screening. 1cm cubes of fungal material and agar were inoculated in triplicate into 3 sterile Eppendorf™ tubes containing 1.25mL each: SDB (control), 100 µM sodium butyrate in SDB (HDACi), and 100µM 5-aza-cytidine in SDB (DNMTi). Sodium butyrate and 5-aza-cytidine were purchased from Sigma Aldrich®. The SDB/modifier/ fungal mixture was agitated, and then each poured over 1g rice media in a Fisherbrand™ 20mL glass scintillation vial. Rice media was made by autoclaving 1g (1/4 tsp) of brown rice with 4mL DI water. Once inoculated, rice vials were incubated at 28°C for 21 days. After 21 days, all contaminated/ non-growing culture sets were removed. Cultures were spritzed with ~500µL distilled MeOH. The fungal rice cake was broken apart with a clean spatula. 10mL distilled EtOAc was added using a glass 10mL pipette and allow to extract overnight on bench top. Extracts were carefully decanted into clean, pre-weighed scintillation vials after 24 hours. Extracts were dried under air for 24 hours, resuspended in DMSO at a concentration of 10mg/mL, and plated in 96-well format on a TECAN Freedom EVO 150 liquid handling automated workstation. Five replicate Corning™ clear polystyrene 96-well microplates were prepared with 150µL of extracts/ well for bioassay. Remaining extract material was stored in duplicate Fisherbrand™ 96-well DeepWell™ polypropylene microplates. All extract plates were stored at -20°C.
**LC-QToF-MS Protocols**

Extracts were dried of DMSO and suspended in MeOH at a concentration of 0.1mg/mL. The resulting solution was filtered over 0.2µm Phenomenex® RC syringe filters and were injected in triplicate on the LC-QToF-MS for analysis.

Analysis was completed on an Agilent 6540 LC/QTOF with Agilent Jet-stream Electrospray Ionization. A Kinetex C18 (5µm, 100 Å, 2.1mm ID, 50mm length) column was used with the following instrument parameters:

**Acquisition Method Report**

TOF/Q-TOF Mass Spectrometer Parameters:
Component Name  MS Q-TOF
Component Model  G6540A
Tune File        Autotune.tun
MS Abs. threshold 200
MS Rel. threshold (%) 0.010
Can wait for temp.  Enable
Fast Polarity  N/A
Ion Source        Dual AJS ESI
Stop Time (min)   No Limit/As Pump
Storage Mode      Both
Ion Mode          Dual AJS ESI
Acquisition Mode MS1:
Scan Rate (spectra/sec)  5.46
Max Range (m/z) 1000
Min Range (m/z) 50
Source Parameters:
Sheath Gas Flow 8
Sheath Gas Temp 300
Nebulizer (psig) 40
Gas Flow (l/min) 10
Gas Temp (°C) 300
Ion Polarity Positive
Scan Source Parameters:
Octopole RF Peak 750
Skimmer1 65
Fragmentor 125
Nozzle Voltage (V) 1000
VCap     3500
Chrom Type: TIC; Label: TIC; Offset: 15; Y-Range: 10000000

Auto Recalibration:
Min Height (counts)   1000
Detection Window (ppm)  100
Average Scans 1
Reference Masses:
Ref Nebulizer (psig) 0
Use Bottle A Ref Nebulizer True
Ref Mass Enabled Enabled
Reference Masses:

<table>
<thead>
<tr>
<th>Mass</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;Positive&gt;</td>
<td>922.00979800</td>
</tr>
<tr>
<td></td>
<td>121.05087300</td>
</tr>
</tbody>
</table>

HiP Sampler Parameters:
Component Name  HiP Sampler
Component Model  G1367E
Draw Speed  100µL/min
Eject Speed  100µL/min
Draw Position Offset 0.0 mm
Wait Time After Drawing 0.0 s
Sample Flush Out Factor 5.0
Vial/Well bottom sensing Yes
Injection Mode Injection with needle wash
Injection Volume 10.0µL/min
Needle Wash Location Flush Port
Wash Time 5.0 s
Automatic Delay Vol Red No
Enable Overlap. Inj. No
Valve Switching No
Stop Time As pump/ No limit
Post Time Mode Off

Pump Parameters:
Component Name  Binary Pump
Component Model  G1312B
Flow 0.600 mL/min
Use Solvent Types Yes
Low Pressure Limit 0.00 bar
High Pressure Limit 600.00 bar
Maximum Flow Gradient 100.000 mL/min²
Auto Stroke Calc. A Yes
Auto Stroke Calc. B Yes
Compressibility A 50 10e-6/bar
Compressibility B 115 10e-6/bar
Stoptime 9.00 min
Posttime 1.00 min
Timetable

<table>
<thead>
<tr>
<th>Time</th>
<th>Function</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 min</td>
<td>Change Flow</td>
<td>Flow 0.6 mL/min</td>
</tr>
<tr>
<td>0.00 min</td>
<td>Change Solvent Composition</td>
<td>Solvent Composition A: 75.0 % B:25.0 %</td>
</tr>
<tr>
<td>0.50 min</td>
<td>Change Solvent Composition</td>
<td>Solvent Composition A: 75.0 % B:25.0 %</td>
</tr>
<tr>
<td>3.00 min</td>
<td>Change Solvent Composition</td>
<td>Solvent Composition A: 5.0 % B:95.0 %</td>
</tr>
<tr>
<td>0.00 min</td>
<td>Change Solvent Composition</td>
<td>Solvent Composition A: 75.0 % B:25.0 %</td>
</tr>
<tr>
<td>7.00 min</td>
<td>Change Solvent Composition</td>
<td>Solvent Composition A: 75.0 % B:25.0 %</td>
</tr>
<tr>
<td>8.00 min</td>
<td>Change Solvent Composition</td>
<td>Solvent Composition A: 75.0 % B:25.0 %</td>
</tr>
</tbody>
</table>

Solvent Composition

<table>
<thead>
<tr>
<th>Channel</th>
<th>Solvent 1</th>
<th>Name 1 Used</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>H₂O</td>
<td>H₂O+0.1% Formic Acid</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>ACN</td>
<td>ACN+0.1% Formic Acid</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Column Comp. Parameters:

- Component Name: Column Comp
- Component Model: G1316A
- Left Temperature Control:
  - Temperature Control Mode: Temperature Set
  - Temperature: 35.00 °C
  - Enable Analys. Left Temp: No
- Right Temperature Control:
  - Right Temp Cont Mode: Combined
  - Enable Analys. Right Temp: No
- Stop Time mode: As pump/injector
- Post Time mode: Off

Metabolomic and Statistical Analysis Protocols

Each sample for metabolomics analysis was prepared as above and run in triplicate according to the run parameters above. Resulting chromatograms were subjected to processing by Agilent MassHunter Qualitative Analysis. First, a list of compounds was generated using the Find By Molecular Feature tool. A peak height cutoff was set at 100 counts and results were limited to the largest 5000 compounds. Results were exported to .cef files that were transferred to Agilent Mass Profiler Professional (MPP). In MPP replicates were averaged and blanks were subtracted. The resulting table of samples and chemical entities (mass @ retention time) was
exported via Microsoft Excel and opened in Primer 6 for statistical analysis (Clarke, K. R.; Gorley, R. N. 2006. PRIMER v6: User Manual/Tutorial. Primer-E, Plymouth). Abundances were square root normalized and factors such as culture conditions and bioactivities were added to each sample identity. A Bray-Curtis similarity matrix was constructed from which cluster (dendogram) and multidimensional scaling (MDS) plots could be created.

Scale-up Protocols

A scale-up protocol that mirrored the screening culture protocol was developed. The optimized procedure was enacted for all scale-up level screening. Rice media was prepared in a Type 3T Unicorn bag according to the following procedure: 300g of brown rice was mixed with 500mL DI water and a heat sealer was used to seal the bag. Rice was autoclaved on a liquid cycle for 30 minutes at 121°C. Each hit organism chosen for scale-up was grown on SDA from either glycerol stocks or isolation plates, and after a colony was established, was subsampled for screening. 1cm cubes of fungal material and agar were inoculated in triplicate into 3 sterile 50 mL Falcon™ tubes containing 50mL each: SDB (control), 100 µM sodium butyrate in SDB (HDACi), and 100µM 5-aza-cytidine in SDB (DNMTi). Rice bags were cut open in a sterile environment, the fungal/liquid media mixture poured in, and resealed. Rice was agitated once a week for a 21 day culture period. Extraction was completed by spritzing the culture with distilled MeOH to dampen the spores, transferring the material to a large beaker, and extracting overnight in a 1:3 MeOH: EtoAc mixture, followed by two 24-hour extractions in EtOAc. Extracts were collected, filtered, and dried down for chemical analysis.
Appendix C: Experimental and Supporting Data for Ch. 4

Fungal Strain Identification
MPLC Run Parameters and Chromatograms
HPLC Run Parameters and Chromatograms
Compound Data

Fungal Strain Identification

The fungal isolate KML12-14MG-B2a was sequenced using sanger sequencing of the 18S ribosomal spacer region. The resulting sequences were blasted using NCBI nucleotide blast for sequence similarity.

**Figure C1.** NCBI nucleotide blast results for KML12-14MG-B2a forward primers.

**Figure C2.** NCBI nucleotide blast results for KML12-14MG-B2a reverse primers.
**MPLC Run Parameters and Chromatograms**

Sample: KML12-14MG-B2a_HDAC_EtOAc  
RadiSap Column: Silica 126g  
SN: E04107CA791F Lot: 2217325010X  
Flow Rate: 55 ml/min  
Equilibration Volume: 2.0 CV  
Initial Waste: 0.0 CV  
Air Purge: 0.0 min  
Solvent: A1 hexane  
Solvent: A2 methanol  
Solvent: B1 ethyl acetate  
Peak Tube Volume: Max.  
Non-Peak Tube Volume: Max.  
Loading Type: Solid  
Wavelength 1 (red): 284nm  
Peak Width: 4 min  
Threshold: 0.20 AU  
Wavelength 2 (purple): 280nm  
Evaporative Light Scattering (green)  
Peak Width: 4 min  
Threshold: 0.05 v  
Spray Temperature: 30C  
Drift Temperature: 60C

Run Notes:

**Figure C3.** NP MPLC chromatogram of KML12-14MG-B2a_HDAC EtOAc partition. Control and DNMT EtOAc partitions were run with the same parameters.

**HPLC Run Parameters and Chromatograms**

Normal phase HPLC separation was completed with a normal phase gradient of hexanes to ethyl acetate over 35 minutes on a semi-preparative Phenominex® Luna Silica (2) column (5µm, 100Å, 250 x 10mm) using UV and ELSD detection.
**Figure C4.** NP HPLC ELSD trace of KML12-14MG-B2a_Control_D. This fraction from MPLC was most similar in elution time to fraction KML12-14MG-B2a_HDAC_F and was used for comparison.

**Figure C5.** NP HPLC ELSD trace KML12-14MG-B2a_HDAC_F. Citreohybriddione D was isolated from the most abundant peak, fraction 2.
Figure C6. NP HPLC ELSD trace of KML12-14MG-B2a_DNMT_C. This fraction from MPLC was most similar in elution time to fraction KML12-14MG-B2a_HDAC_F and was used for comparison.

Compound Data

Citreohybriddione D (1): C_{28}H_{38}O_{8}; HRESIMS m/z 443.2431 [M - OAc]^+ (C_{26}H_{35}O_{6} calculated, 443.2434), 503.2645 [M + H]^+ (C_{28}H_{39}O_{8} calculated, 503.2645), 525.2465 [M + Na]^+ (C_{28}H_{38}O_{8}Na calculated, 525.2464); [α]_{D}^{20} + 0.4 (c 0.1, MeOH); \(^1\)H NMR Data (500 MHz, CDCl\(_3\)) δ ppm 0.90 (s, 3 H), 0.97 (s, 3 H), 1.03 (m, 1 H), 1.17 (s, 3 H), 1.31 (s, 3 H), 1.40 (s, 3 H), 1.68 (m, 1 H), 1.70 (m, 3 H), 1.80 (m, 1 H), 1.83 (m, 1 H), 2.01 (m, 1 H), 2.12 (s, 3 H), 2.16 (s, 1 H), 2.21 (m, 1 H), 2.38 (m, 1 H), 2.83 (m, 1 H), 3.63 (s, 3 H), 4.68 (t, J=2.6 Hz, 1 H), 5.85 (s, 1 H), 10.14 (s, 1 H); \(^{13}\)C NMR (200 MHz, CDCl\(_3\)) δ ppm 16.4 (CH\(_3\), C-20), 16.9 (CH\(_2\), C-6), 18.8 (CH\(_3\), C-21), 19.5 (CH\(_3\), C-22), 19.8 (CH\(_3\), C-18), 21.3 (CH\(_3\), C-25), 21.3 (CH\(_3\), C-27), 23.3 (CH\(_2\), C-2), 26.5 (CH\(_3\), C-24), 27.8 (CH\(_2\), C-1), 30.8 (CH\(_2\), C-7), 36.9 (C, C-4), 38.6 (C, C-8), 47.7 (CH, C-5), 52.0 (CH\(_3\), C-28), 52.2 (C, C-10), 53.5 (CH, C-9), 60.8 (C, C-13), 70.4 (C, C-14), 72.1 (C, C-16), 76.8 (CH, C-3), 133.0 (C, C-12), 126.4 (CH, C-11), 167.3 (C, C-19), 170.6 (C, C-26), 204.4 (CH, C-23), 206.7 (C, C-17), 210.6 (C, C-15).
Figure C7. $^1$H NMR Spectrum (500 MHz, CDCl$_3$) of citreohybriddione D.
Figure C8. $^{13}$C NMR Spectrum (200 MHz, CDCl$_3$) of citreohybriddione D.
Figure C9. $^1$H-$^{13}$C gHSQCAD NMR Spectrum (800 MHz, CDCl$_3$) of citreohybriddione D.
Figure C10. $^1$H-$^{13}$C gHMBCAD NMR Spectrum (500 MHz, CDCl$_3$) of citreohybriddione D.

Figure C11. $^1$H-$^1$H gCOSY NMR Spectrum (600 MHz, CDCl$_3$) of citreohybriddione D.
Figure C12. $^1$H-1H NOESY NMR Spectrum (800 MHz, CDCl$_3$) of citreohybriddione D.