THE STRUCTURES, BIOLOGICAL ACTIVITY, AND MECHANISTIC STUDIES OF
NOVEL MARINE-DERIVED ANTIBACTERIAL COMPOUNDS

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By

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THE STRUCTURES, BIOLOGICAL ACTIVITY, AND MECHANISTIC STUDIES OF NOVEL MARINE-DERIVED ANTIBACTERIAL COMPOUNDS

Jessica Lee Keffer, B.S.

Thesis Advisor: Carole A. Bewley, Ph.D.

ABSTRACT

Due to the rise and global proliferation in drug-resistant bacterial infections, we face a continuing need to have a supply of novel antibacterial agents that are not just more potent or useful against a broader spectrum of organisms, but are structurally distinct from current compounds. One benefit of identifying antibacterial compounds with novel structures includes the potential to circumvent current resistance mechanisms by inhibiting new essential targets. Natural products have a long history of being useful as drugs and inhibitors, and marine natural products offer additional benefits because the unique compounds of the marine environment often have no terrestrial analogs; a higher percentage of bioactive compounds are found in marine organisms; and bioactive agents can be detected and isolated directly without the need for culture. The work presented in this dissertation describes the isolation and identification of two diverse structural classes of antibacterial agents from a marine sponge and a marine chrysophyte alga. Both classes of compounds inhibit drug-susceptible and drug-resistant Gram-positive organisms. The work with the first class of compounds, termed the motualevic acids, describes the biological activity of both natural product compounds and synthetic analogs. The work on the second class of compounds, named the chrysophaentins, describes the biological activity of the natural compounds and synthetic fragments, the geographic variability in the production of the natural chrysophaentins by the source alga, and the mechanism of action of the
chrysophaentins. This work shows that the chrysophaentins prevent bacterial cell division through inhibition of the prokaryotic cytoskeletal protein, FtsZ. The details of the mechanism and the mode of inhibition of the chrysophaentins are further described in the context of other commercially available FtsZ inhibitors. This body of work contributes to the field of marine natural products chemistry by disclosing novel antibacterial agents, and heightens our understanding of the broader picture of FtsZ inhibitors and the inhibition of FtsZ as an antimicrobial target.
First and foremost, this dissertation is dedicated to my mentor, Carole. Without her, none of this would be possible. She is a role model as an advisor, researcher, teacher, and a woman in science. I am extremely thankful for the opportunity to work with her and in her lab, and for the experiences I have gained while in her lab. I have matured as a scientist by becoming a better writer, speaker, and experimentalist under her guidance, and while having to read these pages some multiple times may not seem like much of a thank you…it is.

Second, my dissertation is dedicated to my family. My parents have continuously encouraged me and supported me to do anything I have chosen to do, and without them, I would not be the person that I am today. They were my first teachers, and have instilled in me a love of nature and science, which continues to this day.

To my godparents and my grandparents, some of whom are not around to see this day…

To my extended family and future in-laws…

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I am thankful for all the other teachers I have had along the way, several of whom played specific roles in shaping me into the scientist I am today.

I am grateful for the advice and suggestions provided to me by my committee: Dr. Banerjee, Dr. Brown, Dr. Cihlar, Dr. Roepe, and Dr. Vasudevan.

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And finally, I dedicate this to Cody. Now that we have collectively made it through med school and grad school, it will be smooth sailing from here on out.

You are the sand to my beach, the stars to my sky, and the H₂ my O.

Jessica
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1D</td>
<td>one dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>4T1</td>
<td>mouse mammary gland tumor cell line</td>
</tr>
<tr>
<td>A</td>
<td>absorbance</td>
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<tr>
<td>AA</td>
<td><em>Aquifex aeolicus</em></td>
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<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
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<tr>
<td>Abs</td>
<td>absorbance</td>
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<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
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<tr>
<td>AMP</td>
<td>ampicillin</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>enzyme that hydrolyzes ATP to adenosine diphosphate</td>
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<tr>
<td>BC</td>
<td>bactericidal</td>
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<tr>
<td>BS</td>
<td><em>Bacillus subtilis</em></td>
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<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSC-1</td>
<td>monkey kidney cell line</td>
</tr>
<tr>
<td>bis-ANS</td>
<td>4,4'-Bis(1-anilinonaphthalene-8-sulfonate)</td>
</tr>
<tr>
<td>CAD</td>
<td>collisionally activated dissociation</td>
</tr>
<tr>
<td>CAM</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>community-associated methicillin-resistant <em>S. aureus</em></td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>Chrys</td>
<td>chrysophenin</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
</tr>
<tr>
<td>cryo-EM</td>
<td>cryo-electron microscopy</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenyl-indole</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylethanolamine</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EcFtsZ</td>
<td><em>E. coli</em> derived FtsZ</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF</td>
<td><em>Enterococcus faecium</em></td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>E-MEM</td>
<td>Eagle’s minimal essential medium</td>
</tr>
<tr>
<td>envA1</td>
<td>mutant <em>E. coli</em> with increased permeability of outer membrane (<em>Escherichia coli</em> K12 C600 leu thr lac (thi) galK lpxC::Tn10)</td>
</tr>
<tr>
<td>ESBL</td>
<td>extended spectrum β-lactamase</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>ex.</td>
<td>excitation</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FM5-95</td>
<td>N-(3-trimethylammonium-propyl)-4-(6-(4-(diethylamino)phenyl)hexatrenyl)pyridinium dibromide</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>FtsZ</td>
<td>filamenting temperature sensitive mutant Z</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>γ-PO₄</td>
<td>gamma phosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Gram⁺</td>
<td>Gram-positive organisms (possess a thick peptidoglycan cell wall)</td>
</tr>
<tr>
<td>Gram⁻</td>
<td>Gram-negative organisms (possess an outer membrane)</td>
</tr>
<tr>
<td>GSP</td>
<td>GTPγS</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>enzyme that hydrolyzes guanosine triphosphate to guanosine diphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>non-hydrolyzable analog of GTP</td>
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</table>
HA-MRSA – hospital acquired methicillin-resistant *S. aureus*
HCT-116 – human colorectal cancer cell line
HIV-1 – human immunodeficiency virus type 1
HMBC – heteronuclear multiple-bond coherence
HOBt – hydroxybenzotriazole
HPK – histidine protein kinase
HPLC – high pressure liquid chromatography
HR-ESI-MS – high resolution electrospray ionization mass spectrometry
HR-MS – high resolution mass spectrometry
HSQC – heteronuclear single-quantum coherence
IC – inhibitory concentration
ICU – intensive care unit
IPTG – isopropyl β-D-1 thiogalactopyranoside
ITC – isothermal titration calorimetry
IV – intravenous
LAC – Los Angeles Country clone
LB – Luria-Bertani
LCMS – liquid chromatography coupled to mass spectrometry
M – mass
m/z – mass to charge ratio
mAU – milliabsorbance unit
MBC – minimum bactericidal concentration
MD – Maryland
MDR – multidrug-resistant
MDRSA – multidrug-resistant *S. aureus*
MDR-TB – multidrug-resistant tuberculosis
MES – 2-(N-morpholino)ethane sulfonic acid
MFS – major facilitator superfamily
MHII – Mueller Hinton II broth
MIC – minimum inhibitory concentration
MJ – *Methanocaldococcus jannaschii*
MP – *Marchantia polymorpha*
MRSA – methicillin-resistant

*Staphylococcus aureus*
MS – mass spectrometry
MS/MS – tandem mass spectrometry
MTB – *Mycobacterium tuberculosis*
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MurA – enolpyruvate transferase
NA – not active
NCBI – National Center for Biotechnology Information
NCI – National Cancer Institute
n.d. – not determined
NMR – nuclear magnetic resonance
NNIS – National Nosocomial Infection Surveillance
OD – optical density
OTBA – 3-(5-(4-oxo-2-thioxo-3-(3-trifluoromethyl-phenyl)-thiazolidin-5-ylidenemethyl)-furan-2-yl)-benzoic acid

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CHAPTER 1
INTRODUCTION

1.1 The first antibiotics

In 1945, the Nobel Prize in Physiology or Medicine was awarded jointly to Sir Alexander Fleming, Ernst Boris Chain and Sir Howard Walter Florey “for the discovery of penicillin and its curative effect in various infectious diseases” [1]. Prior to the serendipitous discovery of a penicillin-producing fungus in 1928, the world only had one true antimicrobial compound, Prontosil, a sulfonamide [2]. Other early antibiotics included streptomycin, an aminoglycoside produced by Streptomyces griseus (discovered in 1943) [3], the small molecule chloramphenicol (chloromycetin) isolated from cultures of Streptomyces venezulae (1947) [4], and tetracycline, a polyketide produced by Streptomyces sp. (discovered in 1945) [5] (Fig. 1).

These new antibacterial agents finally gave the world a way to treat the leading infections of the day, including syphilis, tuberculosis, pneumonia, and staphylococci and streptococci infections [6]. At penicillin’s first use in 1943, almost all infections caused by Gram-positive organisms were susceptible, but in 1946, just one year after the Nobel Prize was given for discovery and work on this antibiotic, bacteria capable of producing an enzyme inhibitor known as penicillinase were first observed in hospital settings, rendering penicillin ineffective against these organisms [7-9]. The resistance phenomenon is not unique to penicillin: the other four antibiotics in Fig. 1 subsequently developed clinical resistance by 1959 [7].

Each of the antibiotics in Fig. 1 has different structural features, and each has a correspondingly different mechanistic target. Sulfonamides competitively inhibit dihydropteroate synthetase, an enzyme necessary for folate synthesis in bacteria. Without the ability to synthesize dihydrofolic acid, bacteria cannot synthesize purines for nucleic acids [10].
Penicillin targets transpeptidases, known as penicillin-binding proteins, which are essential for cross-linking peptidylglycan within the bacterial cell wall. Penicillin binds covalently to these transpeptidases, and prevents formation of new cell wall [11]. Unlike sulfonamides and penicillin, streptomycin, chloramphenicol, and tetracycline are all protein synthesis inhibitors, but each has a different specific target. Streptomycin is bactericidal, and prevents protein synthesis by binding to the 30S ribosome subunit in a way that blocks formyl-methionyl-tRNA [12]. Chloramphenicol is bacteriostatic and binds to the 50S ribosomal subunit preventing protein chain elongation by inhibiting the peptidyl transferase step of protein synthesis [13]. Finally, bacteriostatic tetracycline blocks the binding of aminoacyl-tRNA to the acceptor site on the 30S ribosomal subunit[13,14].

1.2 Structure and function of antibiotics

There are approximately twenty structural classes of antibacterial compounds used in the clinic [15-17], including those mentioned in Section 1.1 (see Table 1 for full list). These compounds target four essential pathways in bacteria to exhibit their antimicrobial actions, namely folate synthesis, cell wall biosynthesis, protein synthesis, and DNA/RNA synthesis. The sulfonamides and trimethoprim are the only compounds that inhibit folate synthesis, by structurally mimicking para-aminobenzoic acid (PABA), an essential precursor in this pathway. There are several different “sulfa drugs” but all have the same mechanism of action because of their structural similarities [10].

Five classes of compounds interfere with the cell wall or cell membrane: the β-lactams, like penicillins, cephalosporins, monobactams, and carbapenems; the glycopeptides, such as
vancomycin and teicoplanin; the lipopeptides, like daptomycin; phosphonic acids (fosfomycin); and peptides. All types of β-lactams inhibit the penicillin-binding proteins, but only some are broad-spectrum and capable of inhibiting the growth of both Gram-positive and Gram-negative organisms. Conversely, the glycopeptides are only useful against Gram-positive organisms because the outer membrane of Gram-negative organisms offers an additional layer of protection to these bacteria and prevent the glycopeptides from reaching their target, the D-Ala-D-Ala units (part of Lipid II) on the outside of the cell wall. When glycopeptides bind to the Lipid II termini, additional building blocks cannot be added, preventing new cell wall synthesis [18]. Similarly, daptomycin, a first-in-class lipopeptide, is only useful for treatment of infections caused by Gram-positive organisms [19]. The lipophilic tail of daptomycin irreversibly inserts into the bacterial cell wall, which causes a series of reactions consisting of disruption of the membrane, potassium efflux, depolarization of the cell membrane, and final shut down of macromolecular synthesis [20-22]. Unlike the other cell wall/cell membrane inhibitors, peptides can have several mechanisms of action. Polymyxin inhibits Gram-negative organisms preferentially due to its specificity for anionic lipids, while bacitracin interrupts dephosphorylation reactions necessary for peptidoglycan transfer. Finally, the last member of the class of cell wall inhibitors, fosfomycin inhibits enolpyruvate transferase (MurA) because of its analogous structure to phosphoenolpyruvate [11].

The quinolones and coumarins inhibit DNA synthesis; specifically, they inhibit DNA gyrase (Gram-negative) or topoisomerase IV (Gram-positive), enzymes that are responsible for relieving strain while DNA is being unwound during replication [10]. The ansamycins inhibit RNA polymerase, the enzyme responsible for synthesizing RNA [23]. The remaining structural
classes of antibacterial agents inhibit protein synthesis by binding to different positions on the 30S or 50S bacterial ribosomal subunits, or to a particular tRNA synthetase [12,13,24]. While there are many other antibacterial agents that are being explored as therapies, notably antimicrobial peptides and assorted small molecules [15,25,26], the antibiotics that target cell wall, protein, DNA/RNA, or folate synthesis are the only classes in current clinical use. Knowing the core structure of any one of these agents can give an indication of its antimicrobial target and mechanism of action.

1.3 The increasing prevalence of antibacterial resistance

1.3.1 The rise of resistance

Today, penicillin is rarely used due to widespread resistance among bacteria. Methicillin, a dimethoxybenzoyl derivative of penicillin, was introduced into the clinic to combat penicillinase-producing strains, but not long after its introduction, the first case of methicillin-resistant Staphylococcus aureus (MRSA) was described [27,28]. In the late 1980s and 1990s, the proportion of methicillin resistant infections rose significantly compared to susceptible strains worldwide [29]. In 1999, more than 50% of the S. aureus isolates from ICU patients in the National Nosocomial Infection Surveillance (NNIS) system were methicillin-resistant [30]. By the early 2000’s, more patients in the U.S. succumbed to lethal MRSA infections than to AIDS, tuberculosis, and hepatitis B combined [31].

The concern over resistance is not limited to the β-lactams and S. aureus. Globally, we face an increase in both the number of bacterial species that are resistant to clinically used antibacterials as well as an increase in the percentage of bacteria within a species that are
resistant. Problem organisms (besides MRSA) include vancomycin-resistant *Enterococcus* species, extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella* sp., and fluoroquinolone- or carbapenem-resistant Enterobacteriaceae or *Pseudomonas aeruginosa* [32-35]. In most cases, the clinical manifestations of these infections are similar whether caused by drug-susceptible or resistant bacteria [36]. The difference lies in the available options for treatment, which are often limited, and may become even more limited with the increase in multidrug-resistant pathogens [37,38].

Antibiotic resistant bacteria are tracked by a number of different organizations, including The Surveillance Network (TSN), which provides data on five Gram-negative human pathogens (Fig. 2A) and five groups of Gram-positive organisms (Fig. 2B) and the percentage of resistant bacteria in each category over the course of twelve years (1999-2010). Figure 2A shows the data specifically from 1999 and 2010 to illustrate the trends occurring over this time frame. In some cases, there are high levels of resistance observed, such as the 39.9% (1999) to 44.1% (2010) aminoglycoside resistance in *Actinobacter baumannii*, or 39.1% (1999) to 46.1% (2010) aminopenicillin resistance in *E. coli*. The second trend is steep increases in carbapenem resistance in *A. baumannii* (5.21-40.8%) and fluoroquinolone resistance in *E. coli* (3.33-21.7%). Even more disturbing is the increasing percentage of multidrug resistance observed in all five species. Similar trends are evident in Gram-positive organisms (Fig. 2B) in 1999 and 2010. Of note is the 69-74% resistance in *Enterococcus faecium* to vancomycin, once considered a drug of last resort.
1.3.2 Intrinsic resistance

Many antibiotics are natural products, produced by fungi or bacteria to inhibit the growth of, or kill, other organisms (this topic will be dealt with more thoroughly in section 1.6). Antibiotics can have the same lethal effect on the producing organisms unless they employ self-protective mechanisms, commonly known as intrinsic or natural resistance. There are four mechanisms of intrinsic resistance: 1) enzymatic inactivation of the antibiotic; 2) efflux of the antibiotic; 3) modification of the target within the producing strain; and/or 4) decrease in permeability to the antibiotic [39]. These intrinsic resistance mechanisms within the producing organism can act as a reservoir of resistance genes. These genes can be transferred to susceptible organisms, as they are often encoded within mobile genetic elements [37,40-42]. The reservoir of resistance (the “resistome”) can be made of endemic antibiotic-producing organisms in the environment, or those that have acquired resistance genes through contact with human or farm animal effluent [43-47].

Under pressure from the presence of an antibiotic, there is selection for organisms that are intrinsically resistant or have the ability to acquire resistance, or the chance development of a beneficial (protecting) mutation. Several studies in hospital settings have shown a clear correlation between administration of an antibiotic and the development of resistant bacteria within the population [48-53]. The same four mechanisms that provide intrinsic resistance to the antibiotic-producing organisms can provide protection to other bacteria capable of acquiring and utilizing the resistance genes.
1.3.3 Mechanisms of resistance

Just as structure plays a role in determining the function of an antibiotic, structure also plays a role in determining susceptibility to resistance mechanisms (Table 1). Some of the resistance mechanisms, such as modification of the antibiotic, the action of some efflux pumps, and alteration of some targets, are specific for a particular antibiotic class. Other resistance mechanisms, such as changes in permeability, other efflux pumps, and changes to promiscuous targets, are more broad-spectrum, and can contribute to multidrug resistance.

Enzymatic inactivation of the antibiotic is one resistance mechanism that is usually specific for a particular structural class. The inactivation occurs when an enzyme makes an modification to the antibiotic that changes its function, rendering it inactive or ineffective. For example, enzymatic inactivation of the β-lactam antibiotics occurs through the production of β-lactamases that catalytically open the four member β-lactam ring. The many subclasses of β-lactams in use, like the cephalosporins, the monobactams, and the carbapenems, are a direct consequence of trying to outmaneuver β-lactamase specificity [54]. Each subclass was designed to subvert the enzymatic activity of known β-lactamases, but bacteria have continued to develop and/or acquire increasingly more broad-spectrum β-lactamases. For instance, an extended spectrum β-lactamase (ESBL) known as CTX-M-15 became the primary cause of acquired resistance in Enterobacteriaceae once the gene spread from its intrinsic Kluyver spp producer [42,55-57]. This β-lactamase provided resistance to the most potent cephalosporins at the time; however, the bacteria were still susceptible to carbapenems. The increasing use of carbapenems then led to the rise of a new type of resistance enzyme, known as New Delhi metallo-β-lactamase
1, which conferred resistance to carbapenems. The gene is carried on a cassette that encodes resistance mechanisms for several other antibiotic classes as well [58,59].

Other known mechanisms of enzymatic antibiotic inactivation through modification include the production of aminoglycoside-modifying enzymes that perform $N$-acetylation, $O$-phosphorylation, and $O$-adenylation reactions on the aminoglycoside core [60,61], and the inactivation of chloramphenicol by an acetyltransferase that attaches acetyl groups to the hydroxyls of the antibiotic, rendering it unable to bind to the ribosome [62]. These types of modifications are specific for only certain antibiotic classes.

The second major mechanism of resistance is efflux, where resistant bacteria are able to eliminate the presence of the antibiotic from their cytoplasms by acquiring or upregulating expression of efflux pumps [63,64]. Some transporters, such as those that efflux tetracycline, recognize only a few compounds from any particular structural class [65,66]. Others recognize more diverse targets. There are five major classes of efflux pumps that recognize either a narrow or broad spectrum of structural targets, and are driven by either proton motive force (PMF) or ATP hydrolysis [67-71]. For example, the multidrug transporter NorA was first discovered in a clinical methicillin-resistant *Staphylococcus aureus* isolate that was also resistant to quinolones [72]. NorA is a member of the major facilitator superfamily (MFS), has twelve transmembrane segments (12-TMS), and is capable of conferring resistance to a number of hydrophilic compounds [73-75]. Another example of an efflux pump is the broad-spectrum lactococcal multidrug transporter, LmrA, a member of the ATP-binding cassette (ABC) family, that confers resistance to eight classes of antibiotics, including aminoglycosides, lincosamides, macrolides, quinolones, streptogramins, tetracyclines, chloramphenicol, and surprisingly, some $\beta$-lactams [76].
The third mechanism of resistance is modification or replacement of the antibiotic target, such that the antibiotic can no longer interact with its binding partner. Methicillin-resistant *S. aureus*, rather than having a β-lactamase that can degrade methicillin, have acquired the *mecA* gene, which encodes a penicillin-binding protein (PBP2A), which has a low binding affinity for almost all β-lactams, rendering the antibiotic inert [77]. Methicillin sensitive strains of *S. aureus* usually have 1100 total copies of PBP1, PBP2, PBP3, and PBP4, and 45% of these encode PBP2. In contrast, methicillin-resistant *S. aureus* has almost twice as many copies of the four PBPs, and 40% of these are the low affinity PBP2A, while a further 25% are normal PBP2 [78].

Another example of a target alteration is modification of the ribosome. The macrolides and lincosamides target the same location on the 50S ribosomal subunit. Methylation at a specific site on the 23S rRNA not only provides resistance to both these classes, but also to streptogramins [79]. Finally, target modification is the mechanism by which organisms acquire resistance to glycopeptides. Vancomycin targets the peptide termini of peptidoglycan. In organisms that have acquired resistance, the presence of vancomycin induces expression of genes that reprogram D-Ala-D-Ala to D-Ala-D-Lac, which results in the loss of one hydrogen bond in the interaction between vancomycin and its modified target, and a thousand-fold loss in vancomycin affinity [80].

The final mechanism of resistance is the ability to alter permeability. Gram-negative bacteria and Gram-positive mycobacteria intrinsically have lower permeability to many antibiotics because of their outer membranes (Gram-negative) and specialized mycolic acid-rich cell walls (Mycobacteriaceae) [81-83]. Furthermore, β-barrel channels that are responsible for
controlling diffusion across the outer membrane, known as porins, can be lost or selectively downregulated, further decreasing permeability [63,84,85].

Finally, there are those bacteria that contain multiple resistance mechanisms, often referred to as multidrug-resistant (MDR) organisms, or “superbugs”. For instance, MRSA strain N315 and vancomycin-resistant *S. aureus* strain Mu50 were sequenced and found to contain eleven mechanisms of resistance, including five for modification of antibiotics, two for efflux, three for modification of antibacterial targets, and one encoding a protein that binds and sequesters the antibiotic [86]. In addition, Mu50 must contain at least one additional resistance cassette, for vancomycin resistance, as well as an unidentified gene or set of genes that contributes to a higher level of β-lactam resistance than seen in N315, but at the time of this study, the mechanisms were not known and could not be determined from the whole genome sequence alone. More recently, a strain of totally-drug resistant *M. tuberculosis* (TDR-TB) has been described from India [87], following reports of TDR-TB in Iran and Italy. The emergence of this strain illustrates the continual difficulty in treating *M. tuberculosis* that is multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB) [88].

While some of these resistance mechanisms are applicable to a variety of antibiotic structural classes, especially those involved in decreased permeability and efflux, many others are specific for certain antibiotic structural classes. This specificity suggests that one way to circumvent certain resistance mechanisms is to use antibiotics from new structural classes that will not be susceptible to these mechanisms.
1.4 The continuing need for new antibiotics

1.4.1 The economics of resistance

Even Fleming, the discoverer of penicillin, recognized early on that bacterial resistance was probably inevitable. In 1946, he was quoted as saying, “There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring ‘fastness’ [resistance]” [89]. The usefulness of antibiotics is undermined by the preferential selection of resistant mutants in the presence of the antibiotic. This is an unavoidable result of antibiotic use, and consequently, we are constantly selecting for new strains of antibiotic-resistant bacteria [90]. Drug-resistant bacteria were initially identified in hospital settings, where antibiotics were most often administered. However, the rise and spread of drug-resistant organisms has been such that community-associated resistant microbes now pose a health threat. In particular, MRSA infections have moved out of hospital settings and into the community. These community-associated MRSA (CA-MRSA) strains have become so prevalent in the United States that almost everyone can be considered at risk for these infections [91,92]. With the widespread prevalence of methicillin-resistant \textit{S. aureus}, vancomycin became the drug of choice for treatment of these infections, despite concerns over toxicity and limited tissue distribution. However, 1996 saw the first reported cases of vancomycin-intermediate-susceptible \textit{S. aureus} (VISA) and the first vancomycin-resistant \textit{S. aureus} (VRSA) [93,94]. With the emergence of VRSA and the decrease in the usefulness of vancomycin, other drugs with better profiles, activity, and no resistance were needed. However, the new drugs introduced this past decade, daptomycin, tigecycline, and linezolid, all have reported clinical resistance [95-97].
Treatment of antimicrobial resistant infections increases the cost of healthcare in a number of ways. Most often, alternative therapies are more expensive compared to generic antibiotic drugs. Patients with antibiotic resistant infections often require extended treatment and investigation to determine a course of action that is suitable. Over a decade ago, the American Society for Microbiology estimated in the United States, healthcare costs associated with treating resistant infections topped $4 billion annually [98]. A more recent estimate by the Institute of Medicine puts that number at over $30 billion [99]. Over-prescription of antimicrobials by physicians is a contributing problem; but antibiotic misuse is not limited to over-prescription. Unjustified prescription, under-prescription, under-dosing, and insufficient treatment durations can all play a role in development of antibiotic resistance [100-103]. Hospitals, in particular, are breeding grounds for antibiotic resistant microorganisms. Hospitals deal with large numbers of often immunosuppressed patients, and frequently treat patients with intensive and/or prophylactic antibiotic regimens [104,105].

1.4.2 The reality of resistance

There are currently two approaches being used in an effort to limit further antibiotic drug resistance: 1) decreasing the extent of antibiotic use and then using only when necessary in a more efficient manner, or 2) developing new antibiotics. Prudent use of antibiotics is difficult to enforce, and often needs to be complemented with other therapies and education as well [106-108]. Therefore, the onus lies on developing new medicines whose actions either block or bypass the current resistance mechanisms, or are developed against new targets entirely [37]. Unfortunately, antibiotic discovery programs are being eliminated from most large
pharmaceutical and biotechnology programs. The decline in antibacterial drug discovery programs worldwide has led to a concurrent decrease in the number of new antibacterial compounds [109]. This decline is apparent from the decrease in the number of antibacterial agents (not including vaccines) approved for use from 1981-2010. During this period, 56 drugs were approved from 1981-1990, 32 were approved in 1991-2000, and only 16 were approved from 2001-2010 [17] (Fig. 3). Of the 89 new drugs approved for use in 2002, none were antibiotics. Four new antibacterials were approved between 2003 and 2006, but three are no longer as useful since resistant organisms have already been detected in populations, and the fourth (gemifloxacin, a new generation fluoroquinolone) is a known substrate for certain efflux pumps [110-113].

The four antibacterials approved in 2008-2009 are later generations of antibiotic classes currently used [17]. In fact, most antibiotics used are iterations of previous structural scaffolds. Three classes (β-lactams, quinolones, and macrolides) account for >70% of the antibacterial “new” chemical entities filed between 1981 and 2005 [110]. The increasing prevalence of resistance, even to drugs of last resort such as vancomycin, indicates that new compounds are still needed [114,115]. Because incremental changes to lead compound scaffolds result in increased potency and spectrum but generally do not provide a permanent solution to combating resistance, these trends suggest that new antibiotic compounds should differ structurally from earlier generation antibacterials.

A compound with a new scaffold would be more likely to inhibit a novel bacterial target. There are many bacterial targets that can be exploited beyond the classically targeted pathways of cell wall biosynthesis, protein biosynthesis, DNA replication and repair, and folate coenzyme
1.5 New antibacterial targets

1.5.1 Discovery of new targets

Antibiotics are intended to stop the growth of an organism and/or kill it. This means that the process inhibited by the antibiotic is essential; without this process functioning, the bacteria cannot grow or survive. Synthesis of cell wall, protein, folate, and nucleotides are all essential processes, which is why current antibiotics work so well, but current commercial antibiotics only exploit less than thirty putative antimicrobial targets. In order to expand the number of useful antibiotics, we need to develop compounds that inhibit new targets. However, the targets chosen should still be essential, conserved, and either absent or different from human homologs [116]. There are several different ways of finding and identifying new targets. In particular, genomic methods are highly popular, and have had some success [117]. An antisense RNA method was used in 2001 to find and identify 150 essential genes in *Staphylococcus aureus* [118]. Other studies in *Bacillus subtilis* identified 271 genes that were predicted to be essential [119], and found 303 essential genes in *Escherichia coli* [120]. In a biocomputational study on *Pseudomonas aeruginosa*, 306 essential genes were found with no homologs in *Homo sapiens*. Of these, 9% encode for a protein involved in the transport of small molecules. These proteins are potentially efflux pumps, and can be targeted as a therapy for resistant organisms [121].
Current antibiotics only target about 15 of the hundreds of essential genes in bacterial genomes, indicating the potential for finding new antibacterial targets [122].

Identification of essential genes is not enough; these putative targets need to be validated. Some recent “first in class” examples of novel compounds that inhibit previously unused antibacterial targets include platensimycin acting on FabF, an enzyme involved in fatty acid synthesis [123]; small molecule inhibitors of RnpA, which are involved in mRNA turnover [124]; viriditoxin, which inhibits the bacterial cell division protein, FtsZ [125]; a small molecule, BB-83698, that inhibits peptide deformylase and is in clinical trials [126]; an acyldepsipeptide that activates the ClpP peptidase to destroy other essential proteins [127]; and substrate-inhibitors of SpsB, a signal peptidase [128]. Other ideas for new antibacterial compounds include targeting virulence pathways, quorum sensing, and efflux [129,130]

1.5.2 FtsZ and the bacterial cytoskeleton

One of the previously mentioned “new targets” is a member of the bacterial cytoskeleton, FtsZ (filamenting temperature sensitive mutant Z), which plays an essential role in bacterial cell division, and is a structural homolog to the eukaryotic tubulin. The bacterial cytoskeleton and its components are excellent candidates for antibiotic study. Finding and utilizing inhibitors of cytokinesis has several positive outcomes, including the ability to shut down bacterial cell division, which would prevent proliferation. Furthermore, proper functioning of the bacterial cytoskeleton is necessary to maintain viability, the cytoskeletal proteins are conserved across many bacterial pathogens, and they differ structurally from their eukaryotic counterparts, making them ideal antibacterial targets [131].
FtsZ is found in almost all known bacteria and archaebacteria, except for members of the Crenarchaea, such as *Aeropyrum pernix*, Chlamydiae [132,133], the Planctomycetes, Verrucomicrobia, and *Ureaplasma urealyticum* [134,135]. Interestingly, some members of the verrucomicrobia have a different tubulin-like protein, potentially acquired by horizontal gene transfer [136]. FtsZ has also been found to play a role in the division of chloroplasts and some mitochondria, although in the mitochondria of higher eukaryotes, FtsZ has largely been replaced by dynamin [137]. Several crystal structures of FtsZ have been solved, including seven of the FtsZ from the archaeon, *Methanocaldococcus jannaschii* [138-141], eleven of FtsZ from Gram-positive organisms *Bacillus subtilis* and *Mycobacterium tuberculosis* [139,142-145], and five of FtsZ from Gram-negative organisms *Pseudomonas aeruginosa*, *Thermatoga maritima*, and *Aquifex aeolicus* [139,146-148]. The FtsZ protein structure is remarkably well-conserved among these different species, as has been demonstrated from a structural alignment of several of these crystal structures (Fig 4.). However, there is limited sequence identity among them. In fact, in the sequences from *P. aeruginosa*, *B. subtilis*, and *M. jannaschii* pairwise sequence identity ranges between 37-50% (Fig. 4). The region responsible for ligand binding is highly conserved even in FtsZ identified from chloroplasts and in the eukaryotic tubulin (Fig. 5).

Prior to the 1990’s it was thought that only eukaryotes had a cytoskeleton. Since then much work has been done to elucidate the components of the prokaryotic cytoskeleton and their functions [149]. The bacterial cytoskeleton plays a role in division, determination of polarity, and maintenance of shape. FtsZ is not the only member of the bacterial cytoskeleton, but it is one of the most widely conserved. A bacterial actin homolog, MreB plays a role in maintaining cell shape, protein localization, and chromosome segregation, and is found exclusively in rod-
shaped bacteria. MinD, a polymerizing ATPase, and FtsA, another actin homolog, both play roles in division (see Section 1.5.3). Furthermore, there are other tubulin-like proteins, BtubA and BtubB, which have a high sequence similarity to eukaryotic tubulin, and TubZ, which plays a role in plasmid maintenance, as well as crescentin, a homolog of intermediate filaments. However, these cytoskeletal proteins, as well as approximately six others, are not as widely conserved, and found only in certain bacterial species [150].

1.5.3 Biochemical and physiological functions of FtsZ

FtsZ and many other fts genes were identified by the isolation of temperature-sensitive mutants that are blocked in septation, but proficient in DNA replication and chromosome segregation. FtsZ is a conserved cytoskeletal protein that is the prokaryotic structural homologue of the eukaryotic protein, tubulin. Similar to tubulin, FtsZ also binds and hydrolyzes a guanine nucleotide. FtsZ contains limited sequence homology to tubulin (<20% identity), but the crystal structures of FtsZ and the beta-subunit of tubulin bound to guanosine diphosphate (GDP) share structural similarities, and thus they form a distinct family of GTPases separate from others [151]. Despite some overall structural similarities, tubulin assembles as a dimer (alpha and beta subunits) into a multi-stranded microtubule, while FtsZ assembles as a monomer, and forms mostly single-stranded filaments. During division, FtsZ polymerizes and assembles into a dynamic structure called the Z-ring at the center of the bacterial cell. This Z-ring forms the plane of cell division and is the site where several proteins come together to form the divisome complex [152,153]. FtsZ is the first protein to localize to the future division site [152]. In E. coli, at least ten other proteins are recruited to mid-cell, the future site of division.
FtsZ has four main protein domains, a variable N-terminal segment, a highly conserved core region, a variable spacer, and a C-terminal conserved peptide [140]. Self-interaction occurs through the core region, and the C-terminal conserved peptide is essential for interaction with two other division proteins, FtsA and ZipA, where the presence of either is required for Z-ring stability [154,155]. There is approximately 5000-20,000 monomers of FtsZ in growing *E. coli* [156]. The estimated concentration of FtsZ in a bacterial cell is 10 µM, which is significantly higher than estimates for the critical concentration for FtsZ polymerization, indicating other assembly proteins must play a role in regulating FtsZ assembly to the Z-ring at the proper time during division [157]. Approximately 30% of the cellular FtsZ assembles to the Z-ring; the remaining 70% stays in the cytoplasm. The cytoplasmic pool is continually exchanging with the polymerized Z-ring pool, on a time scale that gives an individual FtsZ subunit in the ring a half-life of approximately 8-9 seconds in *E. coli* [158]. This time-scale is not coincidently similar to the time necessary to GTP hydrolysis [159].

The MinCDE system is responsible for ensuring proper positioning of the Z-ring [160,161]. These proteins oscillate from one cell pole to the other, inhibiting FtsZ assembly at the poles. SlmA, an *E. coli* protein involved in nucleoid occlusion, is also involved in preventing FtsZ assembly on top of chromosomal DNA. This adds another layer of regulation, as FtsZ cannot assemble at mid-cell until the chromosome has been replicated and segregated to the cell poles [162,163]. The Z-ring assembles at the same time as chromosomal replication terminates [164]. Other regulators of Z-ring assembly include the previously mentioned ZipA, which bundles FtsZ and is essential for division [165,166]. ZipA is found only in *E. coli* and a few other related bacteria. ErzA, a protein that is only present in Gram-positive organisms, is
topologically similar to ZipA, however, its function differs from ZipA in that it is a negative regulator of Z-ring assembly [167,168]. ZapA and ZapC, along with binding partner ZapB, promote bundling of FtsZ protofilaments [169-173]. SulA is an inhibitor of FtsZ assembly, which becomes active under conditions of DNA damage as part of the SOS response [146,174]. Its role is to prevent division until the damage can be repaired and the chromosomes segregated. SulA binds to the bottom face of a FtsZ monomer, interacting specifically with residues in the C-terminal domain, including the T7-loop, the loop connecting sheet 8 to helix 10, and the loop between sheet 9 and sheet 10 [146]. It functions by physically blocking the protofilament interface and preventing longitudinal association, without inducing an overt conformational change [139].

Following Z-ring formation at mid-cell, other proteins are recruited. In *E. coli*, this happens in an almost linear fashion. FtsA and ZipA are required to tether FtsZ to the membrane, and are the first to arrive at mid-cell to ensure proper location of the FtsZ Z-ring [166,175-177]. Following the arrival of these two proteins, the rest of the divisome complex assembles, all of which is dependent of FtsZ. The divisome complex consists of seven Fts proteins. FtsK is a DNA translocase that plays a role in chromosome segregation [178,179]. The complex of FtsQ, FtsB, and FtsL are involved in linking together the divisome complex and ensuring correct timing of division events [180-182]. The Lipid II flippase, FtsW [183], is essential for proper localization of FtsI, also known as penicillin-binding protein 3, which is a peptidoglycan synthase necessary for cell wall synthesis at the septum [184]. Finally, FtsN [185] arrives last [186].
There are specific differences in this process and in the order of arrival of divisome proteins in other bacteria. Particularly well-studied systems include that of *Bacillus subtilis* and *Caulobacter crescentus*. In *B. subtilis*, the divisome complex forms in a two-stage manner, where some of the proteins have been shown to be interdependent for localization [187], while in *C. crescentus*, the formation of the Z-ring and divisome complex occurs in seven stages [188]. In other bacteria, such as *S. aureus*, many homologs of the division proteins have been identified, FtsZ is known to be conserved and localizes first, but the cascade of events for the remaining proteins has not been fully identified [189].

The Z-ring is potentially made up of overlapping short (average 120-200 nm) single stranded filaments [190]. Cryo-EM tomography of Z-rings in *Caulobacter* have confirmed the Z-ring structure in that organism [191], and *in vitro* work with *E. coli* indicates perhaps a similar organization [192,193]. While it is not known how the filaments connect to one another, it has been shown FtsZ is tethered to the membrane by FtsA [175]. Studies have shown that FtsZ alone can provide the force necessary for constriction. Modified FtsZ, with an amphipathic tail allowing attachment to a membrane, was reconstituted in tubular liposomes, and at sites where Z-rings were present, constrictions were observed [194,195].

FtsZ is a GTPase [196,197] with a nucleotide binding domain similar to that of tubulin [151]. FtsZ binds and hydrolyzes GTP during its polymerization cycle, which has been studied most extensively in *E. coli*. Association of two FtsZ molecules induces hydrolysis of GTP at the interface of the two monomers [198]. The active site is formed by the insertion of the T7-loop into the nucleotide binding site of the next subunit, placing two highly conserved catalytic aspartate residues in the vicinity of the $\gamma$-PO4, and activating the GTPase activity of FtsZ by
polarizing an attacking water molecule. The aspartates coordinate a magnesium ion [199], and cannot be mutated to glutamates without the loss of hydrolysis. Assembly of FtsZ into polymers during formation of the Z-ring is dependent on GTP binding; hydrolysis occurs instantly and the hydrolyzed inorganic phosphate is released at some point later [200]. The nucleotide can be exchanged, and once the GTP supply is exhausted, FtsZ protofilaments disassemble [201]. The concentration of FtsZ does not vary much during the cell cycle, so cell division is regulated at the level of FtsZ assembly [202].

*In vivo*, FtsZ is regulated by a number of different proteins, both positive and negative regulators, as mentioned earlier. *In vitro*, however, FtsZ activity can be modulated by changes in pH, divalent metal cations, and molecular crowders. FtsZ polymerization was monitored by light scattering, TEM, and centrifugation and it was shown to polymerize best at slightly acidic pH (6.5 rather than 7.9) and millimolar magnesium concentrations (10 mM rather than 0 mM) [203]. While this study seems to indicate Mg\(^{2+}\) is necessary for GTP hydrolysis, but not polymerization, other studies have shown magnesium to be required for polymerization [204]. Monovalent cations also play a role; one potassium ion is located at a stable position between the γ-phosphate of GTP and the FtsZ monomer. This ion (potassium is preferable to sodium) holds a water molecule in position for the catalytic attack [205].

FtsZ polymerization has also been show to be influenced by the presence of DEAE-dextran [206,207], calcium [208], monosodium glutamate [209], and other macromolecular crowders [210]. While these agents have been shown to change the behavior of FtsZ, *in vitro*, they are not necessary additions. Only magnesium, a monovalent cation such as potassium, and
the nucleotide are essential. Many of these regulators are species specific, as significant changes in assembly dynamics have been seen for *Mycobacterium tuberculosis* FtsZ [211,212].

1.5.4 Targeting FtsZ for antimicrobial therapy

If proper FtsZ assembly does not occur, there is not a functional Z-ring, and division is inhibited. Compounds that can affect the assembly and function of FtsZ are potentially lethal for bacteria [213]. Cell division inhibitors have long been used to target the components of the eukaryotic cytoskeletal machinery to combat infection and uncontrolled cell proliferation. Many of these inhibitors target microtubules, and its subunit, tubulin, the eukaryotic structural homologues of FtsZ. Because FtsZ plays an essential role in prokaryotic cell division, is widely conserved among pathogenic bacteria and is absent in the mitochondria of higher eukaryotes, it is an attractive target for developing drugs to combat bacterial pathogens. FtsZ, with its GTP binding domain, is considered a druggable target, which is an attractive feature in novel antibacterials [214]. Antibacterial compounds can be screened for FtsZ-inhibitory activity by analyzing effects on the *in vitro* GTPase activity of FtsZ. There has been a steady increase in the number of publications describing small molecules as FtsZ inhibitors in recent years. No FtsZ inhibitors are currently used clinically, and the therapeutic potential to selectively shut down bacterial division warrants further study of the interaction between inhibitors and this protein. Furthermore, the knowledge that several inhibitors are active against both drug-susceptible and drug-resistant bacteria indicate that natural resistance to FtsZ inhibitors may not be widespread in bacterial populations, and that bacteria may not be able to easily acquire resistance to antibiotics operating by this mechanism [213]. A recent example of a small-molecule FtsZ
inhibitor is the benzamide derivative, PC190723. This compound is a potent anti-staphylococcal agent that was able to protect mice from a lethal dose of *S. aureus* when given subcutaneously or by IV 24 hours after infection, validating the therapeutic potential of inhibiting FtsZ *in vivo* [142].

### 1.6 The role of natural products in drug discovery

#### 1.6.1 Secondary metabolites as drugs

The identification of natural products, or chemical compounds produced by a living organism, has been a major contributor to the field of drug discovery. Besides their long history in ancient Chinese and Ayurvedic medicine, the first biologically active compound isolated from a plant was morphine, discovered in 1805 [215]. Most of the early natural products were isolated from plants, but the field has since expanded to include compounds produced by actinomycetes and other microorganisms, fungi, and marine invertebrates, among others [216]. A further characteristic of biologically active natural products is that they are often produced as secondary, rather than primary, metabolites. Primary metabolites do not differ much between organisms, and include molecules necessary for survival, growth, development, or reproduction. These include, but are not limited to amino acids, sterols, and important elements. On the other hand, secondary metabolites are produced sporadically within organisms, and play no role in basic metabolism or nutritional requirements. However, because energy is required to produce secondary metabolites, they often play some indirect role in increasing fitness potential, sexual attraction, preventing predation, or somehow promoting long-term survival of the organism [217].
Between 1981 and 2006, 60% of the small molecule chemical entities submitted as new drugs were either natural products or synthetic molecules inspired by natural products. While the majority of the new chemical entities introduced in this time frame are active as antibacterials or anticancer agents, natural products have provided us with drugs with many other functions, including analgesics, antidiabetics, antiparasitics, and immunomodulatory agents, among others [110]. The contribution of natural products to drug development peaked around 1990, when 80% of drugs were natural products or their synthetic analogues [216]. Natural products have been an inspiration for medicinal chemists because of the unique structures and accompanying biological activity found in nature that cannot be easily duplicated in the lab. One factor as to why natural products make such good drug leads is the idea that they comprise “privileged structures” [218], and the molecules contain inherent biological relevance since they are made by Nature [219]. These structures are molecular scaffolds with binding properties that make them widely applicable as potential drug leads. For instance, a privileged structure would be one that is able to provide potent and selective ligands for different biological targets through modification of functional groups on a core structure [219,220].

Pharmacognosy (Greek – “drug knowledge”) is the study of the physical, chemical, biochemical, and biological properties of potential drugs of natural origin as well as the search for new drugs from natural sources. Natural products have been identified with many structural motifs and just as many biological targets. The list of natural product drugs is extensive. A few well-known examples of different structural classes with different targets include the analgesic morphine [215], the antitumor microtubule stabilizer paclitaxel [221], the antibacterials penicillin [222,223] and erythromycin [224], and the multiapplicable acetylsalicylic acid (aspirin) [225]
Bacteria, particularly those of the order Actinomycetales, have yielded a large number of antibiotics. From 1942-2001, it was estimated ~3000 known antibiotics came from this Order, and over 90% from the genus *Streptomyces* [226]. These numbers are thought to represent only 1-3% of the total number of antibiotics potentially produced by all actinomycete strains [227,228].

1.6.2 Marine natural products

Marine organisms in particular have been the focus of drug discovery efforts by many research groups, because many of the compounds isolated from these organisms do not have structural similarities to any compounds found in the terrestrial environment [229]. The chemically variable and physically harsh conditions in the marine environment increase the chances that an organism will produce a variety of molecules with unique structural features and interesting biological activity to cope and thrive under these conditions. The majority of these bioactive compounds have been found in sponges and other marine invertebrates which must rely on chemical defenses to protect themselves [230]. Many pharmacologically active marine compounds have no terrestrial analog, contain halides that are rare outside the marine environment, and/or represent entirely new classes of drug leads [231]. Marine natural products include not only those produced by macroorganisms, such as algae, sponges, bryozoans, and tunicates, but also microorganisms, such as marine bacteria, cyanobacteria, fungi, diatoms, and dinoflagellates.

There is often much ambiguity surrounding the producing organism for many marine natural products because many marine microorganisms cannot be stably cultured, and many
Macroorganisms live in obligate symbiosis with other macroorganisms and microorganisms [232]. Furthermore, 34 of the 36 animal phyla are represented in the ocean, and there is intense competition for space and much overlap in environments, occasionally making it difficult to pinpoint producing organisms [233]. However, certain types or classes of compounds are known to be produced by one type of organism or another, and often, active compounds can be classified in this way [234,235]. Inroads have been made into determining the biosynthetic potential, and signature, of producing organisms, and genomic mining has provided access to cryptic biosynthetic gene clusters and previously unknown natural products [236-243]. While the potential compound-producing microorganisms are often considered unculturable, in many cases, the active compounds can be directly detected in the host, without the need for culture. For instance, dolastatin 10 was originally isolated from *Dolabella auricularia*, a marine sea hare, but the compounds are actually produced by cyanobacteria, obtained by the sea hare through its diet [244].

Bioassays have shown that more than 10 percent of marine organisms contain cytotoxic compounds, compared to just 2-3% of terrestrial organisms [231]. The field of marine natural product chemistry started in 1951 when the isolation of unusual nucleosides spongouridin and spongothymidin was reported from the marine sponge, *Cryptotethya crypta* [245]. Prior to this, marine compounds were used in the ancient medicine of China, India, the Near East, and Europe. Records of Hippocrates indicated extracts from marine mollusks contained compounds useful as laxatives [231]. An extensive review of marine natural products published between Jan-Dec 2010 list 1003 new compounds from 352 peer-reviewed articles. This number is similar to those published in 2009 and illustrate the contribution of marine natural products to drug discovery and
the structure elucidation field [246]. Since the 1960’s, more than 20,000 compounds have been reported in marine organisms. The most commonly used marine antibiotic was cephalosporin C, isolated from the marine fungus *Cephalosporium* [231]. Furthermore, a statistical analysis of marine natural product trends from 1985-2008 summarized the novel compounds from marine invertebrates (~75%), marine algae (15%), marine microorganisms (10%), and the distribution of novel compounds from different structural classes (predominately terpenes and alkaloids) [247] (Fig. 7).

1.6.3 Drugs from the sea

A number of marine natural products have been successfully developed into drugs, with many others having been submitted to preclinical and clinical trials. These compounds are collectively known as “Drugs from the Sea”, and as of Jan. 2012, there have been seven marine pharmaceuticals approved by the FDA. The first two approved drugs were spongothymidine and virarabine, originally isolated from the Caribbean sponge, *Tethya crypta* [245,248,249]. Spongothymidine is manufactured under the name Cytosar-U, and inhibits DNA polymerase and DNA synthesis, and is used to treat cancer. Virarabine (ara-A) Vira-A is used to inhibit viral DNA polymerase, but is no longer used due to relatively low potency. The cone snail-produced ziconotide, known as Prialt, is a synthetic version of a 25 amino acid peptide produced as part of the venom of *Conus magus* [250,251]. These peptides have novel analgesic mechanisms of action, by blocking Ca$^{2+}$ channels [252]. The sponge-produced eribulin mesylate E7389 (Halaven), a simplified version of halichondrin B, is a macrolide inhibitor of microtubules [253-256]. Trabectadin ET-743 (Yondelis) is a tunicate alkaloid that binds to the minor groove of
DNA and is used to treat cancer. Recent metagenomic analysis has suggested that the producing organism of ET-743 is actually a gammaproteobacteria, rather than the tunicate, *Ecteinascidia turbinata* [257-261]. Brentuximab vedotin SGN-35 (Adcetris) is an antibody conjugate to a mollusk produced compound, auristatin E, used to treat cancer [262]. Finally, there is the well-known omega-3-acid ethyl ester, Lovaza, from fish oil, used to treat hypertriglyceridemia. Besides these seven, there are ten additional marine compounds in phase I, II, and III clinical trials, and numerous others in preclinical testing [263,264].

1.6.4 *Our lab’s contributions to the field*

The focus of our laboratory research is the isolation, structure elucidation, and biological activities of natural products isolated from marine organisms. Utilizing a robust technique involving bio-assay and LC-MS guided fractionation, a variety of biologically active compounds can be isolated from an extract, and then their structures determined by NMR and MS analysis [265-267]. This research has focused on lithistid sponges, which are well known for their bioactive compounds, perhaps due to the contribution of symbiotic microorganisms hosted by sponges of this Order [268-270]. We have been able to screen a number of samples both from the NCI Open Repository and organisms we have collected ourselves for biological activity including inhibition of HIV-1 entry, antitumoral and antimicrobial activities. The MarinLit database shows 408 unique compounds from the Order Lithistida, and 321 from family Theonellidae [271]. Initial screening efforts identified a marine sponge, *Siliquariaspongia mirabilis*, from Chuuk, with an outstanding level of chemical diversity. This single sponge contained several classes of bioactive compounds, including the mirabamides, a family of
glycosylated depsipeptides with anti-HIV activity [266], mirabalin, a 35-member macrolide with anti-cancer activity [267], a small cyclic peptide with potent inhibition of carboxypeptidase A [272], and the known antifungals, aurantosides A and B [273] (Fig 8).

1.7 Statement of research interest:

Nature provides us with many bioactive compounds. These “privileged structures” are molecularly compatible with binding to druggable targets, and can provide medicinal chemists with logical starting points for optimization and inspiration. Many of the antibiotics currently used were originally produced by living organisms. While there are twenty structural scaffolds in use, these compounds inhibit a limited number of biological targets in bacteria, and as such, widespread antibacterial resistance has become a global problem. Therefore, we need new antibacterials that are structurally distinct from previously used compounds. Finding a compound that has a new structural scaffold increases the odds that it will affect a novel target or pathway. Inhibiting a novel target, such as the bacterial cell division protein FtsZ, which is widely conserved and essential, will allow novel antibacterial compounds to potently work against current drug-resistant bacterial strains.

Our lab has used bioassay-guided fractionation to find a number of biologically active compounds from marine organisms. These compounds have many different structures and corresponding biological activities. These same methods will allow us to find new compounds through screening for activity against a panel of human pathogenic bacteria. Furthermore, the logical extension of drug discovery efforts of any new compound encompasses an examination
of its mechanism of action. The structures of the isolated compounds can give us an idea of possible mechanisms based on similarities to other known agents.

Hypothesis: The chemical structures of antibacterial compounds can give insight into their antibacterial targets. We need new antibiotics to combat rising resistance to available antibacterials, and compounds with unique structures that do not fall into known classes of antibiotics may have different targets. Marine organisms produce bioactive natural products that can be detected directly, and may provide a source of new antibacterial compounds with novel mechanisms of action.
1.8 Figures and figure legends

**Figure 1.** Structures of the first five antibiotics. Pictured here are the first five antibiotics used to treat patients. Counter-clockwise from the top: 1) sulfonamide, a sulfa-drug; 2) penicillin, an example of the beta-lactam drugs; 3) streptomycin, an aminoglycoside; 4) tetracycline, a polyketide; and 5) chloramphenicol, an example of the phenylpropanoids.
Figure 2. Antibiotic resistance trends in the United States. The x-axis lists organism name (horizontal) and antibiotic name (vertical). Top graph – The percentage of drug-resistant Gram-negative organisms in 1999 (dark red bars) and 2010 (light red bars). Resistant organisms include Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Proteus mirabilis. Bottom graph – The percentage of drug-resistant Gram-positive organisms in 1999 (dark blue bars) and 2010 (light blue bars). Resistant organisms include Enterococcus faecium, E. faecalis, Staphylococcus aureus, Streptococcus pneumoniae, and a grouping of coagulase-negative staphylococci such as S. epidermidis and S. saprophyticus. All data obtained from [274].
Figure 3. The number of new antibacterial compounds approved between 1981 and 2010. During the first fifteen years, new antibacterial drugs averaged five per year. During the recent fifteen years, new antibacterials decreased to less than two per year. All data are available from [17].
Figure 4. Structural similarities of three diverse FtsZ monomer crystal structures. (A) The crystal structure of Gram-negative *Pseudomonas aeruginosa* FtsZ (PDB ID 2VAW; amino acids 2-316 shown) in blue, GDP in black [139]. (B) The crystal structure of archaea *Methanocaldococcus jannaschii* FtsZ (1FSZ; amino acids 23-356) in pink, GDP in black [141]. (C) The crystal structure of Gram-positive *Bacillus subtilis* (2VXY; amino acids 11-316) in purple, citric acid in black [142]. Crystal structure coordinates were downloaded from Protein Data Bank (www.pdb.org), and structures were aligned using the software package Chimera (www.cgl.ucsf.edu/chimera). Pairwise rmsd values are 1FSZ/2VAW 0.998 Å; 1FSZ/2VXY 1.042 Å; and 2VXY/2VAW 1.054 Å. After alignment, structures were separated to create this figure.
**Figure 5.** Sequence alignment of FtsZ amino acids of seven bacteria (Bacillus subtilis [BS], Staphylococcus aureus [SA], Mycobacterium tuberculosis [MTB], Pseudomonas aeruginosa [PA], Escherichia coli [EC], Thermatoga maritima [TM], and Aquifex aeolicus [AA]; one FtsZ sequence from archaea (Methanocaldococcus jannaschii [MJ]); two chloroplast FtsZ sequences from plant (Marchantia polymorpha, [MP1 and MP2]); and sequences of alpha and beta tubulin from humans, TA and TB, respectively. All sequences were obtained from NCBI Protein database (http://www.ncbi.nlm.nih.gov/protein), and alignments created using the software program ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2). Fully conserved amino acids are marked with an [*], strongly similar groups of amino acids are marked with a [;], and weakly similar amino acids are marked with a [.]. Sequence identity between any two given sequences is less than 70% (between BS and SA is 68%; and between PA and EC is 60%). FtsZ has two main glycine-rich regions that are conserved (marked in yellow), but only one of those regions is shared with tubulin (similar region marked in green). The basic phylogram below the sequences was created by ClustalW2 and models the substitutions that have occurred over time and represents the evolutionary relationship between sequences. The branch lengths are proportional to the amount of inferred evolutionary change, and the distance value at the end of each branch is the number of substitutions as a proportion of the length of the aligned residues.
**Figure 5.** Sequence alignment of FtsZ amino acid sequences (continued).

| BS              | ESKEQIBEALKGAD----MVFTAGMGQTTGQTG-AAPVIAQIAKDGL--ALTGVVTPRF 136 |
| SA              | ESREQIDAIQGAD----MVFTSMGQGTTGQTG-AAPVIAQIAKDGL--ALTGVVTPRF 136 |
| MTB             | DAKDEEELLGRAD----MVFTAGEGGTGTG-GAPVIAQIAKDLG--ALTGVVTPRF 133 |
| MP2             | ESALVEALGRAD----MVFTMGGQTTGQTG-GAPVIAQIAKDLG--ALTGVVTPRF 136 |
| MP1             | ESLREIAEVDASD----LVFITAGGGQTTG-GAPVIAQIAKDLG--ALTGVVTPRF 293 |
| PA              | EDRERISEVLGQ--MVFTAGGGQTTG-GAPVIAQIAKDLG--ALTGVVTPRF 136 |
| EC              | EDRAALRALLEGQ--MVFTAGGGQTTG-GAPVIAQIAKDLG--ALTGVVTPRF 136 |
| TA              | SVLDFVRKEAESDCLOQFGQITHSGLGHTSGQMTLIIKESRIEYDPDRIMNTFVPSFK 174 |
| BS              | TFEGRKQQLQAAAGGISAMKEAVDTLIVIPNDRILIEVDKNTPMLEA-FREDNVLQVQV 195 |
| SA              | SFEGRKRQQLQAAAGGISAMKEAVDTLIVIPNDRILIEVDKNTPMLEA-FREDNVLQVQV 195 |
| MTB             | SFEGRKRQQLQAAAGGISAMKEAVDTLIVIPNDRILIEVDKNTPMLEA-FREDNVLQVQV 195 |
| MP2             | SFEGRKRQQLQAAAGGISAMKEAVDTLIVIPNDRILIEVDKNTPMLEA-FREDNVLQVQV 195 |
| MP1             | SFEGRKRQQLQAAAGGISAMKEAVDTLIVIPNDRILIEVDKNTPMLEA-FREDNVLQVQV 195 |
| PA              | SFEGRKRQQLQAAAGGISAMKEAVDTLIVIPNDRILIEVDKNTPMLEA-FREDNVLQVQV 195 |
| EC              | SFEGRKRQQLQAAAGGISAMKEAVDTLIVIPNDRILIEVDKNTPMLEA-FREDNVLQVQV 195 |
| TA              | SVLDFVRKEAESDCLOQFGQITHSGLGHTSGQMTLIIKESRIEYDPDRIMNTFVPSFK 174 |
| BS              | GISDLIATPGLINLDFADVKTIMSNKNGALGIGATAGENRAAEEAKKAIISSPLLEAA-I 254 |
| SA              | GISDLIAVSEGNLDFADVKTIMSNKNGALGIGATAGENRAAEEAKKAIISSPLLEAA-I 254 |
| MTB             | GISDLIATPGLINLDFADVKTIMSNKNGALGIGATAGENRAAEEAKKAIISSPLLEAA-I 254 |
| MP2             | GISDLIATPGLINLDFADVKTIMSNKNGALGIGATAGENRAAEEAKKAIISSPLLEAA-I 254 |
| MP1             | GISDLIATPGLINLDFADVKTIMSNKNGALGIGATAGENRAAEEAKKAIISSPLLEAA-I 254 |
| PA              | GISDLIATPGLINLDFADVKTIMSNKNGALGIGATAGENRAAEEAKKAIISSPLLEAA-I 254 |
| EC              | GISDLIATPGLINLDFADVKTIMSNKNGALGIGATAGENRAAEEAKKAIISSPLLEAA-I 254 |
| TA              | SVLDFVRKEAESDCLOQFGQITHSGLGHTSGQMTLIIKESRIEYDPDRIMNTFVPSFK 174 |
| BS              | DGAQGVLMN-ITGGTNLSLYEVQEAADIVASASDQDVNMIGSVINENL-KDEIIVVTVIA 312 |
| SA              | DGAQGVLMN-ITGGESSLFPEAQEAADIVQDAEDEVDNMIFGTVINEPL-QDEIIVVTVIA 312 |
| MTB             | DGAQGVLMN-ITGGESSLFPEAQEAADIVQDAEDEVDNMIFGTVINEPL-QDEIIVVTVIA 312 |
| MP2             | DGAQGVLMN-ITGGESSLFPEAQEAADIVQDAEDEVDNMIFGTVINEPL-QDEIIVVTVIA 312 |
| MP1             | DGAQGVLMN-ITGGESSLFPEAQEAADIVQDAEDEVDNMIFGTVINEPL-QDEIIVVTVIA 312 |
| PA              | DGAQGVLMN-ITGGESSLFPEAQEAADIVQDAEDEVDNMIFGTVINEPL-QDEIIVVTVIA 312 |
| EC              | DGAQGVLMN-ITGGESSLFPEAQEAADIVQDAEDEVDNMIFGTVINEPL-QDEIIVVTVIA 312 |
| TA              | SVLDFVRKEAESDCLOQFGQITHSGLGHTSGQMTLIIKESRIEYDPDRIMNTFVPSFK 174 |
Figure 5. Sequence alignment of FtsZ amino acid sequences (continued).

BS   TGFI EQ-EK DVT K V PQR S P SLNQS I KTHNQS VPKRD--AKREE PQQ QNT VS R HT SQ PADDT 368
SA   TGFDD KPT SHG RKSG STGF TS NT S N A TSV T SRTH T K E- 371
MTB  AGFDV SGP GRKPV VMGETGQA H RIESAK A GKL TSLTFEPVD AVSP VLHT NGATLS IG D D 369
MP2  TGFRGQD S ELR SVQ QTGR SMDGDHG RR-- P S G V PL SGS NGSTV-- 512
MP1  TGFSQT F KTLIDP-KVARQEQ QDSPK G--VDSPWKRPA VSSRFPQG-- 439
PA   TGLGARLEK PVKVVDNT V Q SAA AQ AP AQ RQSVNSY R DPT VM RNQ SHGSAATAAK 373
EC   TGIGMDKRPEITLVTN---KQVQQPV MDRYQ HMAPLTQ EQKP VAKVNVNDAP QTAKE 368
TM   TRFPDE-----------------------------------------------DKILFPEG-- 337
AA   TDFPEEK FQGKEVKF KV KKEE KEP------------------KEEPKLPSDT TYLEEE-- 353
MJ   TGVQSR----------------------------------------------IEFDTGLKRRK LE L T-- 359
TA   YQPTTVP GD LAKVQR A MVCML S N TT AIEA ANARDL HKDFL M YAKRAFVHW Y GEMEG 416
TB   DIPP--------RGLKMAVTFIGNS TAIQELFKRISEQFTAMFRK KLAFLHWYT GEMDE 406

BS   -----------L D IPT F L R N R KRG--------------- 382
SA   ------------DIPS F IRNRE SRR RRTR---------------- 390
MTB  D----------------VDVPFFMER--------------- 379
MP2  -----------DIPSFLKRGRSRYPRVG------------------ 530
MP1  ----------------L GSKGFL------------------ 446
PA   LNPQD DLDYLDIP A FL RRQAD------------------ 394
EC   P----------------DYLDIP AFL RRQAD--------------- 383
TM   ----------------DIPA IY R GLE G L---------------- 351
AA   ----------------EIPAVIRK RN K L R L---------------- 367
MJ   ----------------GIPKI------------------ 364
TA   E-FSEAREDMAALEKDYEVGVDSVE GE- GSEE GGEY 451
TB   E-FTEAESNMNDLSEYQQYQDATA EEEEDFGEAE AA 444
Figure 6. Structures of diverse natural products used in medicine. Counter-clockwise from the top: 1) morphine, a plant natural product with analgesic properties; 2) taxol, a microtubule inhibitor from isolated from the bark of the Pacific yew; 3) acetylsalicylic acid (aspirin) from plant extracts; 4) erythromycin from the actinomycete, *Saccharopolyspora erythaea*; and 5) penicillin, isolated from the *Penicillium* fungus.
**Figure 7.** The contribution of marine organisms to natural product discovery between 1985 and 2008. Top graph – The number of novel compounds isolated from marine organisms, categorized by producer type (invertebrates, algae, microorganisms). Bottom graph – The distribution of compounds from different structural classes colored by producer type (invertebrates – blue; algae – red; microorganisms – green). Data is available from [247].
Figure 8. Diverse compounds isolated from one collection of *Siliquariaspongia mirabilis*. Counter-clockwise from top: 1) mirabamide A, a glycosylated cyclic peptide that inhibits HIV-1; 2) the cytotoxic macrolide, mirabalin; 3) the antifungal aurantoside A; and 4) the small cyclic peptide, namalide, that inhibits carboxypeptidase A.
1.9 Tables and table legends

**Table 1.** Summary of clinical antibiotics. Examples of an antibiotic from each structural class are listed, as well as the decade when the antibiotic was introduced, its molecular target (if known), the synthesis pathway affected by the drug, and the mechanisms of resistance the drug class is sensitive to. Mechanisms of resistance are numbered as follows: 1) modification of the antibiotic; 2) efflux; 3) modification of the target; 4) change in permeability; 5) other (usually overproduction of a part of the pathway to circumvent shortages/inhibitions). References for this data [10-13,23,24].

<table>
<thead>
<tr>
<th>Structural class</th>
<th>Example</th>
<th>Target</th>
<th>Pathway</th>
<th>Resistance mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonamides</td>
<td>Sulfacytine</td>
<td>Dihydropteroate synthetase</td>
<td>Folate</td>
<td>3, 4, 5</td>
</tr>
<tr>
<td>β-lactams</td>
<td>Penicillin</td>
<td>Penicillin binding protein (PBP)</td>
<td>Cell wall</td>
<td>1,2,3,4</td>
</tr>
<tr>
<td>Phenylpropanoids</td>
<td>Chloramphenicol</td>
<td>30S ribosomal subunit</td>
<td>Protein</td>
<td>1,4</td>
</tr>
<tr>
<td>Polyketides</td>
<td>Tetracycline</td>
<td>30S ribosomal subunit</td>
<td>Protein</td>
<td>1,2,3</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Streptomycin</td>
<td>30S ribosomal subunit</td>
<td>Protein</td>
<td>1,3,4</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin</td>
<td>50S ribosomal subunit</td>
<td>Protein</td>
<td>1,2,3,4</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Vancomycin</td>
<td>d-Ala-d-Ala of Lipid II</td>
<td>Cell wall</td>
<td>3</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Ciprofloxacin</td>
<td>DNA gyrase/topoisomerase IV</td>
<td>DNA/RNA</td>
<td>3,4</td>
</tr>
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<td>Streptogramins</td>
<td>Quinupristin-dalfopristin</td>
<td>50S ribosomal subunit</td>
<td>Protein</td>
<td>1,2,3</td>
</tr>
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<td>Phosphonic acid</td>
<td>Fosfomycin</td>
<td>Enolpyruvate transferase (MurA)</td>
<td>Cell wall</td>
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</tr>
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<td>Pseudomonic acid</td>
<td>Mupirocin</td>
<td>Isoleucine tRNA synthetase</td>
<td>Protein</td>
<td>3</td>
</tr>
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</table>

References [10-13,23,24].
Table 1. Summary of clinical antibiotics (continued).

<table>
<thead>
<tr>
<th>Structural class</th>
<th>Example</th>
<th>Target</th>
<th>Pathway</th>
<th>Resistance mechanism</th>
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<td>Linezolid</td>
<td>23S rRNA (50S ribosomal subunit)</td>
<td>Protein</td>
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<td>Lipopeptides</td>
<td>Daptomycin</td>
<td>Cell wall/membrane</td>
<td>Cell wall</td>
<td>1,4</td>
</tr>
<tr>
<td>Mutilins</td>
<td>Retapamulin</td>
<td>50S ribosomal subunit</td>
<td>Protein</td>
<td>3</td>
</tr>
<tr>
<td>Pyrimidines</td>
<td>Trimethoprim</td>
<td>Dihydrofollic acid reductase</td>
<td>Folate</td>
<td>3, 4, 5</td>
</tr>
<tr>
<td>Lincosamide</td>
<td>Clindamycin</td>
<td>50S ribosomal subunit</td>
<td>Protein</td>
<td>1,3</td>
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<td>Ansamycin</td>
<td>Rifampin</td>
<td>RNA polymerase</td>
<td>DNA/RNA</td>
<td>3</td>
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<td>Novobiocin</td>
<td>DNA gyrase</td>
<td>DNA/RNA</td>
<td>3</td>
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<tr>
<td>Nitrofuran</td>
<td>Nitrofurantoin</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
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<td>Polymyxins, bacitracin</td>
<td>Membranes/Cell wall</td>
<td>Cell wall</td>
<td>2, 3, 4, 5</td>
</tr>
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Chapter 2
The research presented here was published as follows:


**Bioactive compounds from marine sponges: The antibacterial motualevic acids, synthetic analogs, and a structure-activity relationship analysis**

**2.1 Abstract**

The steady increase in numbers of drug-resistant bacterial infections is a pressing issue in global health. Decades of antibiotic use combined with the multiple challenges of, and limited efforts toward, the antibiotic drug discovery process have in part led to the existence of multiple groups of drug resistant microorganisms. Programs tackling discovery and development of new antibiotics continue to be necessary, and the value of natural products in such efforts is by now well-appreciated. Recently, we have described a number of natural products obtained from lithistid sponge collections, each of which exhibits anticancer, antifungal, or HIV-inhibitory activity. Although studied for many years and by several groups, lithistid demosponges continue
to be extraordinarily rich sources of new marine natural products, many of which feature hallmarks of prokaryotic biosynthetic origins. Here, we have identified several new classes of bioactive marine natural products from several collections of *Theonella* and *Siliquariaspongia* lithistid sponges. Furthermore, we have found a suite of seven new antibacterials, which have been rarely identified from these types of sponges. These new compounds, named motualevic acids A-F and (4E)-(R)-antazirine, whose structures have been determined by spectroscopic methods, are reported here. Motualevic acids A-D are the first glycyll conjugates of the ω-brominated lipid (E)-14,14-dibromotetradeca-2,13-dienoic acid, and motualevic acid F is the first long-chain 2H-azirine 2-carboxylic acid to be found in nature. Carboxylic acid-containing compounds inhibit the growth of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), and the combinatorial suite of the natural products provide a clear structure-activity relationship (SAR) for MRSA activity. Synthesis of the natural products and amino acid conjugate analogs allowed the SAR to be further explored.

2.2 Background

Lithistid demosponges have been studied for their chemistry for many years. In particular, of the fourteen recognized families within this Order [275], sponges of the family Theonellidae have yielded many types of compounds, including the macrolide swinholide A [276], sterols [277], sesquiterpenoids [278], depsipeptides [279] and peptides [280-282], pyridine alkaoids [283], tetramic acid glycosides [284], and polyketides, such as onnamide [285], among others. All of these compounds have interesting chemistries, many of which feature hallmarks of prokaryotic biosynthetic origins [268,270,286]. There are five recognized genera within
Theonellidae – *Discodermia, Manihinea, Racodiscula, Siliquariaspongia*, and *Theonella* [287]. Three of them, *Discodermia* (62 known compounds, including the discodermolides [288], the calyculins [289], and the discodermins [290]), *Siliquariaspongia* (31 known compounds), and *Theonella* (257 known compounds) are responsible for most of the chemistry reported [271].

As part of a research project looking for novel chemistry from marine organisms, we identified a sponge collected in 1994 from the open reef off Nama Island, southeast of Chuuk Lagoon, in the Federated States of Micronesia, at a depth of 50 m. The sponge was thickly encrusting with rounded conical projections and apical oscules, and slightly compressible. It was slightly fuzzy to the touch, with a reddish brown exterior and orange interior. The sponge was identified as *Siliquariaspongia mirabilis* (de Laubenfels, 1954) by Michelle Kelly at the National Centre for Aquatic Biodiversity and Biosecurity, New Zealand, but it lacked the non-articulated tetacladine desmas that normally characterize the genus [291]. Because of this, it was originally misclassified as *Plakinalopha mirabilis*. Reanalysis of *P. mirabilis* sponges as well as *Theonella conica* (Kieschnick, 1896) showed that all three Indo-Pacific species should be considered to be congeneric with *Siliquariaspongia japonica* (Hoshino, 1981) [292].

The *S. mirabilis* from Chuuk was analyzed for the presence of bioactive components and five classes were found, including: 1) four glycosylated depsipeptides, the mirabamides, which exhibited antifungal and antibacterial activities, as well as potent HIV-1 inhibition [266]; 2) a cytotoxic macrolide, mirabalin [267]; 3) a cyclic tetrapeptide, namalide, that potently inhibited carboxypeptidase A [272]; 4) the known antifungals aurantosides A and B [284]; and 5) a series of peptides that have not been fully characterized. This analysis suggested that *Siliquariaspongia* is chemically rich, but understudied, as previous work with the type species
Siliquariaspongia japonica yielded only several more tetramic acid glycosides [293,294]. We felt the taxonomy could therefore help identify other samples from different geographic locations that potentially contained similar chemical diversity to the S. mirabilis from Chuuk.

Chromatographic methods (LC-MS) were used to profile the compounds present in each Siliquariaspongia and Theonella extract in our collection (Fig. 9). Dereplication techniques, or the process of identifying previously isolated compounds based on mass, activity, or other unique features, were used to eliminate known compounds. All extracts were tested for antimicrobial activity against a panel of microorganisms (including Bacillus subtilis, Candida albicans, amphotericin-B-resistant C. albicans, Escherichia coli, Enterococcus faecium, vancomycin-resistant E. faecium, Pseudomonas aeruginosa, Staphylococcus aureus, and methicillin-resistant S. aureus), in cytotoxicity assays (against a human colo-rectal cancer cell line HCT-116, and monkey kidney cell line BSC-1), and in HIV-1 neutralization assays [295] (Table 2).

2.3 Novel bioactive compounds

Using the bioassay-guided fractionation and biological screening approach, we were able to find novel chemistries in many of the sponge extracts. In a Siliquariaspongia sp. from Indonesia, we identified three new cyclic depsipeptides with a polyketide moiety, termed celebesides A-C. These peptides contained five unnatural amino acids – isoserine, β-methyl asparagine, N-methyl valine, 3-carbamoyl threonine, and phosphoserine. Celebeside A inhibited HIV-1 entry in a neutralization assay with an IC₅₀ of 2.1±0.5 µM, while the unphosphorylated analog, celebeside C, was inactive up to 56 µM [296]. This sponge also contained three new
members of the theopapuamide family, originally isolated from *Theonella swinhoei* collected in Papua New Guinea [297]. The theopapuamides are undecapeptides with an N-terminal fatty acid. The new members differed from theopapuamide A in certain constituent amino acids, as the new theopapuamides contained 3-acetamido-2-aminopropanoic acid and 4-amino-2,3-dihydroxy-5-methylhexanoic acid. The theopapuamides were cytotoxic (IC$_{50}$s of 1.3-2.5 µM) to the HCT-116 cancer cell line, and antifungal (0.6-3.1 nmol/disk in a disk diffusion assay) to both *Candida albicans*, and amphotericin-B-resistant *C. albicans* [296]. Previously identified compounds keramamide A [282] and the aurantosides A and B [284] were also found in this extract. The profiling of *Siliquariaspongia* extracts allowed us to identify the celebesides in one other collection of *Siliquariaspongia* from Papua New Guinea (Fig. 9).

From a selection of deep-water *Siliquariaspongia* and *Theonella* from Palau, specimens that were completely white and lacked the red outer coloring common to many *Theonella* sponges, we found mutremdamide A, a sulfated cyclic depsipeptide containing an unusual $N^\delta$-carbamoyl-$\beta$-sulfated asparagine [298], and several new members of the highly $N$-methylated and N-terminal 2-methoxyacetic acid-containing koshikamide family [299-301]. These included the linear peptides, koshikamides C-E, and koshikamides F-H, seventeen-residue cyclic peptides that contain a ten residue macrolactone. Also present in the extract were known compounds koshikamide B and theonellamide A. The biological activity detected in the extract could be traced to certain compounds. Both koshikamide F and H inhibited HIV-1 entry with IC$_{50}$s of 2.3 and 5.5 µM, respectively. Koshikamide F was also cytotoxic to a number of cell lines, including HCT-116 (IC$_{50}$ of 10 µM), and the antifungal activity was traced to theonellamide A [298].
From a deep-water Palauan *Theonella swinhoei* of the more traditional coloring, we found a suite of new anabaenapeptins. The three new compounds, termed the paltolides, are ureido-containing hexapeptides with a five residue lactam ring, made from the carboxy-terminal acid connected to the N-terminal lysine [302]. Three other known anabaenapeptins, previously found in an Australian sponge, *Melophlus* sp. [33], were also present in this *T. swinhoei* from Palau. An interesting feature of the paltolides, the *Melophlus* anabaenapeptins, and psymbamide A from *Psammocinia*, was the presence of a D-lysine residue. While a lysine was present at the N-terminal location in all anabaenapeptins, sponge-derived compounds normally contain L-Lys; D-Lys is a feature previously found only in anabaenapeptins from cyanobacteria [303,304]. The HIV-1 inhibition activity of this extract was traced to the known compound theopalauamide A [286], a glycosylated peptide previously known to have antifungal activities only [305].

We had hoped to find an identifying chemotype to unify the members of *Siliquariaspongia*; however, that was not the case. The lack of defining chemotype is not surprising considering marine sponges are known hosts for biosynthetically-capable bacteria. While many of the compounds found in this selection of *Siliquariaspongia* and *Theonella* are new, the structural classes they represent are by no means unique to just one group of sponges. Mirabamides have recently been found in *Stelletta clavosa*, a demosponge from the order Astrophorida, quite distant in relatedness from *Siliquariaspongia* [306]. *Melophlus* sp. is also a sponge from the order Astrophorida, but *Psammocinia* is from the order Dictyoceratida [307]. Furthermore, theopalauamide, swinholide, onnamide, discodermin, and theonellamide are all putatively produced by bacteria, and these compounds have been found in multiple organisms [268,308].
2.4 A *Siliquariaspongia* sp. extract with antibacterial activity

Several of the extracts I analyzed exhibited some antibacterial activity despite there being very few reports of antibacterial compounds from Theonellidae sponges. We found this to be unexpected, given that the majority of antibacterial compounds are known to be produced by terrestrial bacteria, the Theonellidae are such prolific producers of bioactive compounds, and are known hosts for symbiotic marine bacteria. There are a few examples in the literature, including nagahamide [309], lysoplasmananylinositols [310], and discodermins [290], as well as the mirabamides, which inhibited the growth of *Bacillus subtilis* in a disk diffusion assay, but had no activity against any pathogenic bacterial strain [266]. However, the extract from one *Siliquariaspongia* sp. collected in Fiji tested positive for antibacterial activity against not only *Staphylococcus aureus* but methicillin-resistant *S. aureus* (MRSA) as well. This biological activity was unique relative to the other *Siliquariaspongia* species we had studied, and indicated that this sponge may contain novel chemistry. Thus, we used antimicrobial assays to guide the isolation of the antibacterial compounds and spectroscopic methods to determine their structures.

2.5 Discovery and structure elucidation of the motualevic acids

The marine sponge, *Siliquariaspongia* sp., was collected around Motualevu reef in Fiji at a depth of -40 meters, and was provided by the NCI Open Repository. Using methods that were previously developed in our lab to isolate bioactive compounds, the freeze-dried sponge (8 g) was extracted sequentially with H$_2$O and MeOH:CH$_2$Cl$_2$ (1:1) to provide crude aqueous and organic extracts, both of which inhibited the growth of *S. aureus* and MRSA in a disk diffusion assay. The $n$-butanol–soluble material from the aqueous extract and CHCl$_3$–soluble material
from the organic extract were chromatographed on Sephadex LH-20, a resin that separates compounds predominantly on the basis of size. The column was eluted with 3:1 MeOH:H2O to provide several active fractions that were combined and further purified by reverse-phase HPLC (65-85% MeOH in 0.05% TFA on a Synergi C18 column) to give pure compounds 1-7 (Fig. 10) [311].

We used a combination of NMR and HR-MS to determine the structures of 1-7. Motualevic acid 1 was obtained as a colorless solid (18.6 mg) that gave an [M – H] ion at \( m/z \) 436.0126 (calculated for \( \text{C}_{16}\text{H}_{24}\text{Br}_{2}\text{NO}_{3} \), 436.0123). The 1:2:1 isotopic distribution at \( m/z \) 436, 438, and 440 secured the presence of two bromine atoms and was consistent with the molecular formula \( \text{C}_{16}\text{H}_{25}\text{Br}_{2}\text{NO}_{3} \), requiring four sites of unsaturation. The downfield region of the \(^1\text{H}\) NMR spectrum (Table 4) contained signals for three olefinic protons at \( \delta_{\text{H}} \) 6.84 (1H, dt, \( J = 15.3, 7.3 \) Hz), 6.50 (1H, t, \( J = 7.3 \) Hz), and 6.00 (1H, d, \( J = 15.3 \) Hz) and a two-proton singlet at \( \delta_{\text{H}} \) 3.98 (2H, s). The presence of two methylene signals at \( \delta_{\text{H}} \) 2.13 (2H, dd, \( J = 7.3, 14.6 \)) and 2.24 (2H, dd, \( J = 7.3, 14.4 \)) and a methylene envelope from \( \delta_{\text{H}} \) 1.35–1.50 (14H) in the upfield portion of the spectrum indicated compound 1 contained an unsaturated fatty acid (Fig. 11). Similarly, the \(^{13}\text{C}\) NMR spectrum (Table 3) contained three olefinic carbons at \( \delta_{\text{C}} \) 124.4, 146.6, and 140.5, nine methylenes at \( \delta_{\text{C}} \) 28.9–34.1, a carbonyl at \( \delta_{\text{C}} \) 168.6, and a quaternary carbon at \( \delta_{\text{C}} \) 88.6 characteristic of a terminal dibromovinyl moiety [312,313] (Fig. 11).

COSY and TOCSY correlations revealed spin systems corresponding to C-2 to C-5 and C-11 to C-13, both of which were correlated to the methylenes at \( \delta_{\text{H}} \) 1.35; while the methylene at \( \delta_{\text{H}} \) 3.98 (2H, s) represented a separate spin system (Fig. 12). HMBC correlations (Fig. 13) from the olefinic proton at \( \delta_{\text{H}} \) 6.50 to the dibromovinyl carbon at \( \delta_{\text{C}} \) 88.6, and the olefinic protons at
\( \delta_H \) 6.84 and 6.00 to the carbonyl at \( \delta_C \) 168.6 established the structure of the 14-carbon chain (Fig. 14). The requirement of a nitrogen atom together with the HMBC correlations from the methylene singlet at \( \delta_H \) 3.98 to the carbonyls at \( \delta_C \) 168.6 (C-1) and 172.7 (C-1') revealed the presence of a glycine residue coupled to the acid to give compound 1. The \( E \) geometry of the double bond at C-2/C-3 was apparent from the large \( ^2J_{H2-H3} \) value of 15.3 Hz. To our knowledge, this is the first example of a glycylic lipid occurring in a natural product.

Motualevic acid B (2), one of the five minor components in the extract, was shown by HR-ESIMS to have the same molecular weight and formula as 1. The major differences in the \( ^1H \) NMR spectra of 1 and 2 were found in the chemical shifts and coupling constants of the olefinic protons at \( \delta_H \) 5.88 (1H, d, \( J = 11.6 \) Hz) and 6.05 (1H, dt, \( J = 11.6 \) and 7.5 Hz) indicating compound 2 to be the \( Z \) isomer of 1 (Fig. 15) (Table 4). On the basis of HR-ESIMS, the molecular formulae of motualevic acids C (3) and D (4) were established as C\(_{16}\)H\(_{25}\)Br\(_2\)N\(_2\)O\(_2\) (\( m/z \) 437.0446 [M + H]\(^+\)) and C\(_{18}\)H\(_{30}\)Br\(_2\)N\(_2\)O\(_2\) (\( m/z \) 465.0746 [M + H]\(^+\)), respectively, suggesting replacement of the hydroxyl by a primary amine in 3 and by \( N,N \)-dimethyl amine in 4. Consistent with the mass spectral data, the only changes observed in the \( ^1H \) NMR spectra traced to the glycine moiety. In particular, the \( ^1H \) NMR spectrum of 3 in CDCl\(_3\) showed the presence of an amide signal at \( \delta_H \) 6.08 (2H) (Fig. 15), while the spectrum of 4 (CD\(_3\)OD) showed the presence of two \( N \)-methyls at \( \delta_H \) 2.98 and 3.08 (Fig. 16), both of which showed HMBC correlations to C-1' at \( \delta_C \) 170.2. Thus 3 and 4 are the glycinamide and \( N,N \)-dimethylglycinamide derivatives of 1. Both glycinamide and \( N,N \)-dimethylglycinamide are new residues in natural products.

Motualevic acid E (5), present in the aqueous extract only, was shown by HR-ESIMS to have the molecular formula C\(_{14}\)H\(_{22}\)Br\(_2\)O\(_2\) (\( m/z \) 378.9908), indicating a loss of C\(_2\)H\(_3\)NO relative to
motualevic acid A. Similarly, $^1$H and $^{13}$C NMR spectra for 5 were nearly identical to those of 1, except for the absence of signals corresponding to the glycine residue (Fig. 16). Thus, the structure of 5 must be the free (E)-14,14-dibromotetradeca-2,13-dienoic acid unit.

Two additional compounds 6 and 7 were also found exclusively in the aqueous extract. The HR-ESIMS of 6 again showed the characteristic isotopic pattern of two bromines, at $m/z$ 418.0009, 419.9980, and 421.9957 [M - H], giving a molecular formula C$_{16}$H$_{23}$Br$_2$NO$_2$. Similarities between the NMR data of 1 and 6 suggested the presence of the same $\omega$-brominated tetradecadienoic acid present in 1-5; however, the glycine signals were conspicuously absent. Instead, a proton signal at $\delta_H$ 2.55 (1H, s), showing an HMBC correlation to the quaternary carbon at $\delta_C$ 156.1, along with a band in the FT-IR spectrum at $\nu$ 1770 cm$^{-1}$, suggested the presence of an azirine ring [312-314]. HMBC correlations from $\delta_H$ 2.55, the 2$H$ proton of the azirine ring, to the olefinic carbon at $\delta_C$ 112.7 established the position of the azirine ring in place of the glycine moiety, while the remainder of the molecule was unchanged relative to motualevic acid A, establishing the structure of 6, or motualevic F, as (E)-3-(13,13-dibromotrideca-1,12-dienyl)-2$H$-azirine-2-carboxylic acid (Table 5). In addition, azirine 6 was strongly levorotatory ($[\alpha]_D$ -75.0), indicating a 2$R$ configuration [313,315]. Finally, spectroscopic data showed compound 7 to be the methyl ester of azirine 6, and an $[\alpha]_D$ of -7.3 indicated a 2$R$ configuration, confirming 7 to be (4E)-R-antazirine, a new enantiomer of the antazirine series (Table 5).

Long chain azirine-2-carboxy methyl esters, exemplified by 7 and the non-halogenated lipid (4E)-R-dysidazirine [314], have been isolated previously from collections of the marine sponge *Dysidea fragilis*, and are known to exist as mixtures of enantiomers [312-314]. Using
chiral HPLC and polarimetry, Skepper and Molinski recently demonstrated that dysidazirines and antazirines can racemize spontaneously, and that their (4Z)-isomers occur with higher optical purity than the corresponding (E)-isomers [313]. In our case, chiral HPLC (Fig. 17) showed an 80% enantiomeric excess for (4E)-2H-azirine 2-carboxylic acid 6, significantly higher than optical purities reported for other (4E)-isomers, while the optical rotation of methyl ester 7 indicates lower optical purity. These measurements suggest factors in addition to configuration of the C-4/C-5 olefin contribute to rate of racemization.

2.6 Antibacterial activity and structure-activity relationship of the motualevic acids

Antimicrobial disk diffusion assays performed with pure motualevic acids A-F (1-6) and (4E)-R-antazirine (7) traced the MRSA-inhibitory activity to acids 1 and 6, which inhibited the growth of MRSA at loadings of 23 and 12 nmol/disk, respectively (Table 6). The same assay performed with S. aureus showed compounds 1, 2, 5, and 6 to be active at respective loadings of 23, 23, 130, and 4.8 nmol/disk. Activity in this assay was determined by the presence of a zone of inhibition around a 6 mm paper disk impregnated with the respective concentrations of the compound. For pure compounds, concentrations tested usually ranged from 2-150 nmol/disk, and the activity was reported as the lowest concentration that gave rise to a clear zone between 8-11 mm in size. The observations that compound 2 inhibited the growth of S. aureus but not MRSA (at concentrations up to 57 nmol/disk), that amides 3 and 4 were inactive toward both strains, and that tetradecadienoic acid 5 weakly inhibited the growth of S. aureus suggest antimicrobial activity toward MRSA is dependent on the presence of a carboxylic acid (in the form of glycine or 2-carboxylate) and 4E geometry.
A more quantitative measure of antibacterial activity can be obtained from microbroth dilution assays. I developed this assay for use in the lab following CLSI standards. Because the most potent compounds were acidic, it was necessary to test these compounds in the liquid assay to rule out the role of charge in influencing diffusion. Compounds for which sufficient material was available were further evaluated in liquid culture, which gave results similar to those of the disk diffusion assay (Fig. 18 and Table 6). Motualevic acid A (1) inhibited the growth of *S. aureus* and MRSA with MIC$_{50}$s of 25±9 µM and 21±6 µM, respectively, while the glycinamide-derivative, motualevic acid C, had MIC$_{50}$s 16-44 times higher, indicating the essential role of the carboxylic acid. Motualevic acid F (6) was again the most potent compound, with MIC$_{50}$s of 2.9±0.7 µM and 9.3±2 µM against *S. aureus* and MRSA, respectively. The similarity of results between the agar disk diffusion assay and the microbroth dilution assay indicate that the charge and diffusion properties of these compounds are not influencing the observed activity.

Compounds 1 and 6 were further evaluated for cytotoxicity toward a kidney cell line (BSC-1) and HCT-116. Motualevic acid A (1) showed no cytotoxicity up to 230 µM, but motualevic acid F (6) inhibited the growth of these two cells lines in an MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cytotoxicity assay with IC$_{50}$s of 48 and 36 µM, respectively. Compound 6 represents the first example of a long chain 2H-azirine 2-carboxylic acid and the second example of a 2H-azirine 2-carboxylic acid, with the first being the 3-methyl azirine, azirinomycin [316]. It is interesting to note that compound 6 was the most potent antibacterial agent, inhibiting the growth of *S. aureus* and MRSA at concentrations as low as 4.8 and 12 nmol/disk, while its methyl ester 7 was inactive at concentrations as high as 120 nmol/disk, consistent with a lack of antibacterial activity reported for other antazirines, all of
which contain methyl esters [312,313]. Similarly, azirinomycin was reported to show broad spectrum antimicrobial activity in its naturally occurring acid form, while that of its synthetic methyl ester derivative was greatly diminished [317]. Compounds 1-7 were not active against Candida albicans, Escherichia coli, Bacillus subtilis, nor Pseudomonas aeruginosa.

2.7 Summary of natural motualevic acids

Here we have described six new antibacterial, ω-terminus dibrominated acids that incorporate glycine, glycinamide, or N,N-dimethylglycinamide, or a rare 2-carboxy-2H-azirine ring. While ω-dibrominated unsaturated fatty acids such as 5 have been isolated from several species of Xestospongia [318-322], this is the first report of this type of compound occurring in a lithistid sponge. Likewise, prior to this report, long chain azirines have been found exclusively in the marine sponge Dysidea fragilis, unrelated to either Xestospongia or Siliquariaspongia. Finally, motualevic acid F (6) provides the first example of a long-chain 2H-azirine containing a C-2 carboxylic acid that inhibits the growth of MRSA. We have found that 2-carboxy-2H-azirine 6 co-occurs with the glycine adduct of 14,14-dibromotetradeca-2,13-dienoic acid (1), providing a possible precursor to and nitrogen atom source for the biosynthesis of azirine 6 proceeding through base-induced cyclization without the need for halogenation. Together with the antimicrobial results for glycyl lipid 1, these studies suggest that the presence of a free acid is necessary for inhibition of MRSA in both compound types. The structures reported here provide a starting point for optimization of new MRSA-inhibitory antibiotics as well as a probable precursor in the biosynthesis of 2H-azirine 2-carboxylates.
2.8 Structure investigation – effect of chain length and bromines

Initial work with the motualevic acids suggested the carboxylic acid was important for antibacterial activity. Furthermore, the decreased activity of motualevic acid E was attributed to lack of \(4E\) geometry, but chain length could also play a role. However, the suite of natural compounds provided no way to further test this or to investigate the importance of the dibromovinyl group. In order to explore these effects, a synthetic scheme was assembled to give access to glycyl or caprylic acid conjugates of \(\alpha,\beta\)-unsaturated C6, C7, and C12 acids, with and without the terminal dibromovinyl olefin [323] (Fig. 19). The five synthetic compounds were tested in the agar disk diffusion assay to determine their activities relative to motualevic acid A (Table 7). The length of the acid moiety and the presence of the bromines proved to be important to the antimicrobial activity. Glycyl conjugates 8a and 8b, which both lack the terminal bromines and possessed shorter alkyl chains (C12 and C6, respectively) were inactive. Compound 9, which differs from motualevic acid A by a single ethylene, had five-fold diminished antibacterial activity. Compound 10a, with its significantly diminished chain length was inactive, and compound 10b, with a full chain length, but different position of the amide, retained some activity. These constructs and results demonstrated that modification of the motualevic acid structure within the fatty acid portion of the molecule was detrimental to activity, and all further attempts at modification started with motualevic acid E.

2.9 Further investigation of the antibacterial activity of motualevic acid A

Subsequent to our original report describing the antimicrobial activities of motualevic acids A-E towards \(S.\ aureus\) and MRSA, we obtained several additional \(Staphylococcus\) strains,
including a multidrug-resistant *S. aureus* (MDRSA), a clinical osteomyelitis isolate (UAMS-1), and a community-associated MRSA strain (USA300). Additional testing showed motualemic acid A to have efficacy against all three strains. The \( \text{MIC}_{50} \) against MDRSA was \( 18 \pm 4 \mu M \), the \( \text{MIC}_{50} \) against UAMS-1 was \( 24 \pm 7 \mu M \), and the \( \text{MIC}_{50} \) against USA300 was \( 37 \pm 10 \mu M \) (Fig. 21). I developed a liquid assay for testing *Enterococcus faecium* and vancomycin-resistant *E. faecium* (VREF), and found both natural and synthetic motualemic acid A (see section 2.9) to have similar antibacterial effects against these strains in liquid culture. The \( \text{MIC}_{50} \) for natural motualemic acid A against *E. faecium* and VREF is \( 36 \pm 10 \mu M \) and \( 23 \pm 7 \mu M \), respectively, while the \( \text{MIC}_{50} \) for synthetic motualemic acid A against *E. faecium* and VREF is \( 22 \pm 4 \mu M \), and \( 23 \pm 6 \mu M \), respectively (Fig. 20).

The \( \text{MIC}_{90} \) of motualemic acid A, which is the lowest concentration where there is no visible growth of the bacteria, for *S. aureus* and MRSA is \( 72 \mu M \). Plating an aliquot of bacteria from this concentration showed no viable bacteria remained, indicating this concentration is bactericidal. Treating a population of growing bacteria with this concentration of motualemic acid A led to an immediate decrease in viable MRSA bacteria, and a slower but concurrent decrease in viable *S. aureus* bacteria over seven hours (Fig. 20).

### 2.10 Structure optimization of motualemic acid A – effects of charge, hydrophobicity, flexibility, and functionalization

Synthesis of motualemic acid A began with commercially available 1,9-nonanediol, preceded through several rounds of homologation, and motualemic acid E was created by an oxidation-*gem*-dibromoolefination hydrolysis sequence. Standard coupling reagents were used
to couple glycine methyl ester to motualevic acid E, followed by saponification of the methyl ester to complete the total synthesis of motualevic acid A. In order to synthesize the analogs, synthetic motualevic acid E was activated with HOBt and coupled to respective amino acid methyl esters followed by hydrolysis, or coupled to other functional groups to give the analogs 1b-1s, where the glycine has been replaced by the respective R group (Table 8) [323].

Analogs 1b and 1c were designed to continue testing the effects of chain length. Leaving the fatty acid portion and bromines intact, the chain length was increased by either one (1b) or two (1c) methylenes via the conjugation of β-alanine or 3-aminopropanoic acid, respectively. 1b and 1c showed a 3-5 fold increase in potency against S. aureus and MRSA, but no significant difference in activity against the Enterococci strains (Table 8). Three analogs were designed to test hydrophobicity. 1d was created by coupling to phenylglycine, 1e entailed phenylalanine, and 1f used leucine. 1d performed the worst, with MIC₅₀s between 32-48 µM against all four strains, perhaps due to its poor solubility. 1e and 1f showed increased potencies relative to motualevic acid A, and in fact, the leucine derivative (1f) was the most potent analog overall, with an MIC₅₀ against S. aureus of 4.7 µM.

The effects of rigidity were tested by incorporating proline analogs. The l-proline conjugate (1g) was similarly active to motualevic acid A, while, its enantiomer, d-proline (1h), was reduced in potency against all strains, except VREF. The hydroxyproline derivative (1i) had an MIC₅₀ against S. aureus of 86 µM, and was not tested further.

Based on work with the natural motualevic acids, we concluded a free carboxylic acid was essential for antibacterial activity. Therefore, we synthesized derivatives incorporating aspartic acid (1j) and diacetic acid (1k), both of which have an overall charge of -2. Somewhat
surprisingly, both analogs showed no antibacterial activity up to 200 µM. To probe this further, two additional analogs were synthesized. One (1m) contained the methyl ester of aspartic acid, so that the overall charge of the molecule was -1, but was otherwise similar to 1j; and the second (1n) incorporated N-methylglycine to test the effect of a tertiary nitrogen. While about twofold less potent than motualevic acid A, 1m regained some antibacterial activity relative to 1j and 1k, and 1n was similarly active to motualevic acid A. To explain the decreased potencies of the -2 charged analogs, we hypothesized that perhaps 1j and 1k were forming an intramolecular association between the two free acids, which was not possible in 1m. The alkyl substitution pattern on the nitrogen does not appear to play a role, as evident by the similar activities of 1a and 1n against S. aureus.

Quaternary ammonium compounds are often used as antibacterial agents because of their antiseptic and cationic detergent-like activities [24]. Thus, we incorporated the 2-amino-\(N,N,N\)-trimethylethanaminium conjugate as derivative 1l. This derivative showed improved potency against all four strains with MIC\(_{50}\)s between 5.2-11 µM. Unfortunately, in terms of specificity, 1l was not only antibacterial, this compound was also cytotoxic to eukaryotic cells, and inhibited the growth of HCT-116 with an IC\(_{50}\) of 39±10 µM. Analogs 1a-1h and 1j-1l were tested for bactericidal or bacteriostatic effects against S. aureus and MRSA. All were similarly bactericidal at their respective MIC\(_{90}\)s, except for the proline derivatives, which were bacteriostatic, indicating these analogs may have a somewhat different mode of action.

Finally analogs 1o-1s were created as motualevic acid probes that could be used for target identification. Two of the analogs, 1o and 1p, retained and even showed improved activity
against *S. aureus* and MRSA, but the remaining three showed decreased potency, and in the cases of the lysine and biotin derivatives, effectively no potency.

### 2.11 Discussion

Many marine natural products contain halogens, including bromines and iodine, which are potentially incorporated because of their abundance in the marine environment. Sponges are known to be resistant to bacterial decomposition, which suggests they contain potent protective antibiotic compounds, many of which have been found to contain bromines and other halogens. It is speculated that bromines may be essential to antibacterial activity since they could function in a similar capacity to iodine in the antiseptic tincture of iodine [231]. Even though few antibacterial compounds have been identified from sponges of the family Theonellidae, it is expected that these sponges could contain antibacterial agents. This work has identified a suite of long chain brominated acids from a Fijian specimen of the family Theonellidae, *Siliquariaspongia* sp., with antibacterial activity against drug-susceptible and drug-resistant *Staphylococcus aureus* and *Enterococcus faecium* that we have named motualevic acids A-F. Interestingly, we have in our collection another *Siliquariaspongia* from Fiji, collected at a shallower depth (-20 meters). When its chemistry was profiled by LCMS, the only detectable compounds were aurantosides, and although there was some antibacterial activity in the extract, initial fractionation did not reveal the presence of any of the motualevic acids or related long chain halogenated acids.

The suite of natural motualevic acids allowed us to determine an initial structure-activity relationship. We deduced that the free carboxylic acid and (4E) geometry were essential for
activity. To further test this, we synthesized analogs of motualevic acid A in order to test the
effects of chain length, the presence of bromines, charge, hydrophobicity, and flexibility. Even
though motualevic acid F was more potent, we decided to optimize motualevic acid A because of
its better stability and specificity (no cytotoxicity).

We found that the $\omega$-brominated lipid ($E$)-14,14-dibromotetra-deca-2,13-dienoic acid
present in all of the natural products is required for low micromolar antibacterial activity. All
antibacterial activity was originally evaluated using the agar disk diffusion assay. I developed
the microbroth dilution assay in accordance with the CLSI standards for use in our lab. This
assay provided a method that was quantitative and could be used for evaluating and comparing
antibacterial activities of many different types of compounds. All further analogs were tested
using this assay. This study allowed us to discover several additional antibacterial agents, with
improved or comparable potencies to the natural motualevic acid A. In general, more
hydrophobic amino acid conjugates (compounds 1b-1f with the exception of 1d) were found to
be more potent antibiotics, inhibiting the growth of $S. aureus$ at 4.7-9.4 µM. On the other hand,
Attempts to introduce polar amino acids or more than one negative charge decreased antibacterial
activity.

Potent analogs 1e, 1f, and 1l were tested for cytotoxicity against HCT-116 and BSC-1. 1e and 1f were not cytotoxic up to 200 µM, but 1l inhibited the growth of eukaryotic cell lines.

Another difference was found using a viability assay to determine bacteriostatic or bactericidal
effects. Most of the motualevic acid analogs were bactericidal, like the natural motualevic acid
A, but the proline analogs were bacteriostatic. These results indicate that motualevic acid
analogs with improved antibacterial potency can be constructed, and that their mode of action can be altered with composition.

Analogs 1o-1s were designed to serve as probes for studying the mechanism of action and biological target of the motualevic acids. The functional groups incorporated into these analogs could be used to site-specifically label potential binding partners within S. aureus through bioorthogonal "click" chemistry, or to attempt target pull-down by utilizing the interaction between the biotin on 1s and streptavidin [324]. A future project could entail determining the mechanism of action of these compounds, and/or a finding the biological target using the probes and tools synthesized here.

It is tempting to speculate on potential targets by analyzing the structure-activity relationship in comparison with examples from the literature. An eleven carbon unsaturated acid with a monobromovinyl group was described as the product of a specific ring cleavage reaction at the C1-C2 bond of zerumbone, an eleven-membered cyclic sesquiterpene that is the major component of the essential oil of the wild ginger Zingiber zerumbet [325]. This compound did not have detectable antibacterial activity, but was found to be a potent inhibitor of autophosphorylation of an essential histidine protein kinase (HPK), YycG [326]. HPKs are one of the major components in the two-component regulatory systems, which are involved in prokaryotic signal transduction in response to various environmental stressors. One of these two-component systems are the histidine kinase YycG and its partner response regulator YycF, which is conserved in many Gram-positive organisms including S. aureus [327]. In a recent study, a series of analogs of the eleven-carbon acid were constructed incorporating different amino acids on the free acid end of the unsaturated acid. One, the tryptophan analog, showed
inhibitory activity against MRSA and VREF, and retained activity against the HPK, while its
cognate methyl ester had no antibacterial activity. The authors concluded that the potency was
due to the properties of the tryptophan, including its hydrophobicity and negative charge (free
carboxylic acid) [328], while a previous study had determined that the (4E) geometry of the long
chain acid was also important [329].

These results have obvious parallels to the motualetic acids, and provide a potential
target for study. The YycG/YycF (also known as WalK/WalR) is essential in S. aureus and
represents an antibacterial target that plays a role in controlling cell wall metabolism and biofilm
formation [330,331]. The putative target of the motualetic acids can be investigated using the
probes designed in this study, while also investigating the potential inhibition of auto-
phosphorylation by bacterial protein histidine kinases.

2.12 Materials and methods

2.12.1 General experimental procedures

Optical rotations were measured with a Jasco P-2000 polarimeter and UV spectra were
recorded on an Agilent 8453 spectrophotometer. NMR spectra were recorded on a Bruker
Avance DRX-500 spectrometer equipped with a cryogenically cooled probe and z-shielded
gradients. DQFCOSY, 2D-HOHAHA, HSQC, HMBC, and ROESY experiments were recorded
using standard pulse programs. Delays in HSQC and HMBC experiments were set for $^{1}J_{C-H} =
145$ Hz and $^{2,3}J_{C-H} = 8$ and $5$ Hz, respectively. The accurate mass electrospray ionization (ESI)
mass spectra were measured on a Waters LCT Premier time-of-flight (TOF) mass spectrometer.
The instrument was operated in W-mode at a nominal resolution of 10,000. The electrospray
capillary voltage was set at 2kV and the sample cone voltage at 60 volts. The desolvation
temperature was set to 275 °C and nitrogen was used as the desolvation gas with a flow rate of
300 L/hr. Accurate masses were obtained using the internal reference standard method.

2.12.2 Sponge material, extraction and isolation

Samples of *S. mirabilis* (de Laubenfels, 1954) (lithistid Demospongiae: family
Theonellidae) were collected around Sulawesi Island, Indonesia, at a depth of 43 m in 1994. The
sample was identified as described previously, and a voucher sample has been deposited at the
Natural History Museum, London (BMNH 2007.7.9.1). Samples were frozen immediately after
collection and shipped frozen to Frederick, MD, where they were freeze-dried and extracted with
H₂O. A 6 g portion of the extract was partitioned between *n*-BuOH-H₂O (1:1) to afford a dried
*n*-BuOH extract (0.7 g) that was fractionated on a Sephadex LH-20 column (50 × 2.5 cm) eluting
with MeOH/H₂O (7:3). Fractions containing peptides were combined and dried *in vacuo* to give
130 mg that were subsequently purified by reverse-phase HPLC (Jupiter Proteo C12, 250 × 10
mm, 4 μm, DAD at 220 and 280 nm) eluting with a linear gradient of 50-80% MeOH in 0.05%
TFA in 50 min to afford compounds celebeside A (7.2 mg, *t*ₚ=43.9 min), celebeside B (2.4 mg,
*t*ₚ=34.9 min), celebeside C (1.1 mg, *t*ₚ=40.0 min), theopapuamide B (2.5 mg, *t*ₚ=32.9 min),
theopapuamide C (1.8 mg, *t*ₚ=33.5 min), theopapuamide D (0.8 mg, *t*ₚ=36.8 min), and
theopapuamide A (3.4 mg, *t*ₚ=24.3 min).

Samples of *T. swinhoei* subspecies *swinhoei* (Gray, 1869), and *T. swinhoei* subspecies
*verrucosa* (Wilson, 1925) (lithistid Demospongiae: family Theonellidae) were collected at
Mutremdiu Reef, Palau, at a depth of 100-120 m in June 2008, by scuba using mixed gases. *T. cupola* was collected on the same reef in 1997 at a depth of 90 m. All samples were frozen after collection and stored at -40 °C prior to extraction. A 6 g portion of an aqueous extract of *T. swinhoei* (PA08-05) was partitioned between *n*-BuOH-H2O (1:1) to afford a dried *n*-BuOH extract (0.2 g). A part of this extract (120 mg) was purified by reversed-phase HPLC (Jupiter Proteo C12, 250 x 10 mm, 4 μm, DAD at 220 and 280 nm) eluting with a linear gradient of 65-80% MeOH in 0.05% TFA in 40 min to afford mutremdamide A (10.7 mg, *t*<sub>R</sub>=27.3 min), koshikamide C (9.6 mg, *t*<sub>R</sub>=22.7 min), koshikamide D (3.7 mg, *t*<sub>R</sub>=26.9 min), koshikamide E (0.7 mg, *t*<sub>R</sub>=20.0 min), koshikamide F (4.0 mg, *t*<sub>R</sub>=40.7 min), koshikamide G (0.5 mg, *t*<sub>R</sub>=42.4 min), koshikamide H (6.6 mg, *t*<sub>R</sub>=63.5 min), and koshikamide B (20.0 mg, *t*<sub>R</sub>=55.4 min). Screening by LC-MS of aqueous extracts of *T. cupola* and *T. swinhoei* subspecies *verrucosa* (Wilson, 1925), was used to establish the presence of mutremdamide and koshikamides in aqueous extracts of these sponges.

The marine sponge *Theonella swinhoei* (lithistid Demospongiae: family Theonellidae) was collected by hand using scuba on Uchelbeluu Reef in Palau at a depth of 330 feet in June 2008 and frozen within 2 hours. Samples were flown to Maryland frozen and stored at -80 °C until freeze-drying prior to extraction. The lyophilized sponge (30 g) was sequentially extracted with hexanes, CHCl3, and MeOH. The MeOH extract (4 g) was partitioned between *n*-BuOH-H2O (1:1), and the organic layer (1 g) was fractionated on Sephadex LH-20. Fractions containing peptides (95 mg) were purified by reversed-phase HPLC (Jupiter Proteo C12, 250 x 10 mm, 4 μm, DAD at 220 and 280 nm) eluting with a linear gradient of 50-80% MeOH in 0.05% TFA in 50 min to afford paltolide A (0.5 mg, *t*<sub>R</sub>=19.0 min), paltolide B (1.5 mg, *t*<sub>R</sub>=20.7
min), paltolide C (0.6 mg, \( t_R=36.5 \) min), anabaenapeptins (6.8 mg, \( t_R=25.2 \) min, 0.3 mg, \( t_R=35.3 \) min, and 24.8 mg, \( t_R=26.3 \) min), and theopalauamide (0.9 mg, \( t_R=39.8 \) min).

Other samples were prepared for LC-MS analysis by dissolving some of the lyophilized sponge in methanol, and analyzed by reversed-phase HPLC (Jupiter Proteo C12 or Synergi Fusion C18, 250 x 10 mm, 4 \( \mu m \), DAD at 220) eluting with a linear gradient of 50-80\% MeOH in 0.05\% TFA in 50 min.

Samples of *Siliquariaspongia* sp. (deLaubenfels, 1954) (lithistid Demospongiae: family Theonellidae) were collected in Fiji at a depth of 40 m in 2000. A voucher specimen has been deposited at the Natural History Museum, London, United Kingdom. Samples were frozen immediately after collection, and shipped frozen to Frederick, MD, where they were freeze-dried and extracted first with \( H_2O \) and subsequently with \( MeOH:CH_2Cl_2 \) (1:1) to give crude aqueous and organic extracts, respectively. Isolation and purification were guided by antibacterial activity at each step. The aqueous extract (6 g) was partitioned between \( n\)-BuOH-\( H_2O \) (1:1) and the \( n\)-BuOH removed *in vacuo* from the active fraction to give 0.6 g of material. The organic extract (2 g) was partitioned sequentially with hexanes:MeOH:\( H_2O \) (10:9:1) and \( CHCl_3:MeOH:H_2O \) (10:6:4), and the solvent removed *in vacuo* to give 0.4 g of a dried chloroform extract. Less than 10 mg were obtained from the other inactive layers, neither of which were analyzed further. Both extracts were fractionated on a Sephadex LH-20 column (50 x 2.5 cm) using MeOH:\( H_2O \) (3:1) as the mobile phase. Fractions containing the fatty acids were combined and the solvent removed *in vacuo* to give 73 mg from the chloroform extract and 108 mg from the \( n\)-butanol extract. These samples were chromatographed by reverse-phase HPLC (Jupiter Fusion, 250 x 10 mm, 4 \( \mu m \), detection at 220 and 254 nm) eluting with a linear gradient
of 65–85% MeOH in 0.05% TFA in 42 minutes to afford motualevic acids A (1, 18.6 mg, $t_R=45.2$ min), B (2, 1.5 mg, $t_R=46.2$ min), C (3, 0.7 mg, $t_R=42.7$ min), D (4, 1.3 mg, $t_R=47.8$ min), E (5, 1.1 mg, $t_R=53.7$ min), and F (6, 27.2 mg, $t_R=48.9$ min), and (4E)-R-antazirine (7, 0.6 mg, $t_R=53.9$ min).

### 2.12.3 Chiral HPLC analysis

Motualevic acid F (6) was chromatographed by chiral HPLC (YMC Chiral NEA (1-naphthylethylamine) (R), 250 x 4.6 mm, 5 µm, detection at 220 nm) eluting with an isocratic gradient of 0.01% TFA in 70:30 hexanes:isopropanol at a flowrate of 0.5 mL/min. Peaks were integrated within the Agilent Chemstation to give $(R)$-6 = 83641.9 and $(S)$-6 = 7055.82. Calculated as a percent, $(R)$-6 = 92.2%, and $(S)$-6 = 7.8%. Enantiomeric excess was calculated using $%ee = ((R-S)/(R+S))\times100$ where $R$ and $S$ are the respective fractions of each enantiomer, and $R+S=1$. $%ee = ((0.922-0.078)/(0.922+0.078))\times100=84.4\%$.

### 2.12.4 Biological assays

Compounds 1-10 were tested for antimicrobial activity against *Staphylococcus aureus* (ATCC 25923), and MRSA (ATCC BAA-41) using a modified disk diffusion assay. Agar plates seeded with suspensions of bacteria were prepared by adding 500 µL of a 24 hr culture of bacteria to 100 mL of autoclaved Mueller Hinton II agar and cooled to 55 °C. This seeded liquid agar (10 mL) was transferred immediately to square Petri dishes and allowed to cool for 1 hr. Control drugs used for each microorganism included kanamycin (50 µg) for S.
*Staphylococcus aureus*, and nitrofurantoin (25 μg) for MRSA. Following incubation at 37 °C for 18 hr, zones of inhibition were measured.

MIC<sub>50</sub> values for compounds 1, 3, and 6 and synthetic analogs 1b-1s were determined using a microbroth dilution assay outlined in the Clinical and Laboratory Standards Institute methods for susceptibility tests for bacteria that grow aerobically. Antimicrobial assays were performed using *S. aureus* (ATCC 25923), methicillin-resistant *S. aureus* (ATCC BAA-41), multidrug-resistant *S. aureus* (ATCC BAA-44), *S. aureus* (UAMS-1), community-associated MRSA (USA300-LAC), *E. faecium* (ATCC 49032), and vancomycin-resistant *E. faecium* (ATCC 70022). Briefly, single bacterial colonies were used to inoculate 4 mL of BBL Trypticase Soy Broth (*Staphylococcus*) or BBL Brain-Heart Infusion (*Enterococcus*) for overnight growth and inoculum for each were diluted to 5 x 10<sup>5</sup> colony forming units (CFU) per milliliter using the 0.5 McFarland standard. In a 96 well plate (Costar, round bottom), control antibiotics (oxacillin for *S. aureus*, MRSA, MDRSA, USA300, and UAMS-1 and vancomycin for *E. faecium* and VREF) or motualevic acids were added to Muller Hinton II (MHII) broth (*Staphylococcus*) or 10% Brain-Heart Infusion in MHII broth (for *E. faecium* and VRE) in the first column of the well plates, and serially diluted across the plate, reserving wells for a positive growth control (no treatment). 10 μL of diluted bacteria were added to all wells with the exception of those kept for broth blanks. Plates were incubated at 37 °C overnight and read at absorbance 600 nm on a Molecular Devices plate reader. Growth curves were plotted and MIC<sub>50</sub> values obtained using Kalidagraph software. Curves were fit to a one-site model with Kaleidagraph 4.0 using the equation y=100/[1+(concentration/MIC<sub>50</sub>)], where MIC<sub>50</sub> is the concentration at which the growth of bacterial cultures are reduced by 50%.
MIC₉₀ values were visually calculated by determining the lowest concentration of test compound that allowed no visible growth (no difference of absorption between treated samples and blank controls).

Bacteriostatic or bactericidal properties were determined by plating onto a fresh trypticase soy agar (TSA) plate an aliquot from each well that showed no visible *S. aureus* or MRSA growth starting at the MIC₉₀. Plates were incubated for 24 hours at 37 °C, and colonies were counted. Bactericidal concentrations were defined as those that resulted in three logarithmic decrease in the number of viable bacteria relative to the starting inoculum.

A viability assay was performed by treating a growing population of *S. aureus* or MRSA with a concentration of motualevic acid A equal to the MIC₉₀. At increasing time points, 25 µL samples were removed and plated onto a fresh TSA plate. Plates were incubated for 24 hours at 37 °C, and colonies were counted as above. Log₁₀ viable bacteria was determined by taking the mathematical logarithm of the number of bacteria in CFU/mL, which was calculated by multiplying the number of colonies present on a plate by the dilution factor used and dividing by the volume (in milliliters) added to the plate.

Cytotoxicity assays using vertebrate kidney and colon tumor cell lines (BSC-1 and HCT-116, respectively) were carried out using an MTT cell proliferation assay kit (American Type Culture Collection) according to the instructions provided. Briefly, cells were seeded in 96-well tissue culture plates at a density of 2 x 10⁴ cells/well in 50 µL of Eagle’s Essential Minimal Media (E-MEM) and allowed to adhere for 18 hr at 37 °C, 5% CO₂. Attached cells were incubated with inhibitors for 24 hr, after which the media was diluted 3-fold with fresh growth
media. Following an additional 48 hr incubation period, cell viability was assessed upon
treatment with MTT (A570, Molecular Devices 96-well absorbance plate reader).

2.12.5 Physical Data

Motualevic acid A (1): colorless, amorphous solid; UV (MeOH) λ max (log ε) 204 (4.89),
226 (4.08), 250 (3.69) nm; IR (film) ν 2921, 2849, 1698, 1669, 1601, 1551, 1233 cm⁻¹; ¹H and
¹³C NMR data, see Table 3 (CD₃OD); HR-ESI-MS m/z 436.0126 [M - H]⁻ (calculated for
C₁₆H₂₄Br₂NO₃, 436.0123).

Motualevic acid B (2): colorless, amorphous solid; UV (MeOH) λ max (log ε) 204
(4.95), 226 (4.12), 250 (3.72) nm; IR (film) ν 2923, 2844, 1699, 1664, 1603, 1553, 1230 cm⁻¹; ¹H
and ¹³C NMR data, CD₃OD, see Table 4; HR-ESI-MS m/z 438.0275 [M + H]⁺ (calculated for
C₁₆H₂₆Br₂NO₃, 438.0276).

Motualevic acid C (3): colorless, amorphous solid; UV (MeOH) λ max (log ε) 206 (4.90),
226 (3.98), 256 (4.05) nm; IR (film) ν 2940, 2825, 1680, 1664, 1600, 1451, 1207 cm⁻¹; ¹H and
¹³C NMR data (CDCl₃), see Table 4; HR-ESI-MS m/z 437.0446 [M + H]⁺ (calculated for
C₁₆H₂₇Br₂N₂O₂, 437.0439).

Motualevic acid D (4): colorless, amorphous solid; UV (MeOH) λ max (log ε) 208 (4.93),
226 (4.01), 256 (4.09) nm; IR (film) ν 2945, 2820, 1678, 1674, 1620, 1448, 1200 cm⁻¹; ¹H and
¹³C NMR data (CD₃OD), see Table 4; HR-ESI-MS m/z 465.0746 [M + H]⁺ (calculated for
C₁₈H₃₁Br₂N₂O₂, 465.0752).

Motualevic acid E (5): colorless, amorphous solid; UV (MeOH) λ max (log ε) 210 (3.37),
228 (3.20), 254 (3.10) nm; IR (film) ν 2900, 2854, 1650, 1600, 1201 cm⁻¹; ¹H NMR (CDCl₃) δₜ
5.82 (1H, d, J=15.6 Hz, H-2), 7.06 (1H, dt, J=15.6, 6.95 Hz, H-3), 2.22 (2H, dd, J=6.95, 14.5 Hz, H-4), 1.45 (2H, m, H-5), 1.27 (10H, m, H-6 – H-10), 1.40 (2H, m, H-11), 2.07 (2H, dd, J=14.6, 7.3 Hz, H-12), 6.37 (1H, t, J=7.3 Hz, H-13); 13C NMR (CDCl₃) δC 169.0 (C-1), 119.9 (C-2), 152.9 (C-3), 32.6 (C-4), 28.0 (C-5), 29.4 (C-6 – C-10), 27.9 (C-11), 33.3 (C-12), 139.0 (C-13), 88.7 (C-14).; HR-ESI-MS m/z 378.9908 [M – H]⁻ (calculated for C₁₄H₂₁Br₂O₂, 378.9908).

Motualevic acid F (6): pale yellow, amorphous solid; [α]D  -74.0 (c 0.1, MeOH); UV (MeOH) λ max (log ε) 204 (4.69), 230 (4.11), 250 (3.80) nm; IR (film) ν 2925, 2853, 1770, 1700, 1458, 1429, 1230, 1203, 1141 cm⁻¹; ¹H and ¹³C NMR data (CDCl₃), see Table 5; HR-ESI-MS m/z 418.0009 [M - H]⁻ (calculated for C₁₆H₂₂Br₂NO₂, 418.0017).

(4E)-R-antazirine (7): pale yellow, amorphous solid; [α]D  -7.3 (c 0.1, MeOH); ¹H and ¹³C NMR data (CDCl₃), see Table 5; HR-ESI-MS m/z 434.0310 [M + H]⁺ (calculated for C₁₇H₂₆Br₂NO₂, 434.0330).

2.13 Acknowledgements

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2.14 Figures and figure legends

Figure 9. Chromatographic analysis of *Siliquariaspongia* and *Theonella* extracts from different geographic locations. Methanol and *n*-butanol extracts were prepared as described in Section 2.11.2. Samples were run on Phenomenex C12 or C18 columns, eluting with a linear gradient of methanol in water. Each trace is labeled with the species name and country of collection. Novel compounds were identified and are described in more detail in Sections 2.2 and 2.4, while known compounds were identified by comparing their molecular weight from ESI-MS data to a mass database available in the marine natural products database MarinLit [271]. Data were obtained and interpreted jointly by Jessica Keffer and Alberto Plaza.
Figure 10. Structures of motualevic acids A-F and \((4E)-R\)-antazirine. These seven natural compounds were isolated from the marine sponge, *Siliquariaspongia* sp. The structures were determined by NMR and HR-MS techniques. The motualevic acids are characterized by a fourteen carbon unsaturated acid chain, terminating in a dibromo vinyl moiety. Differences between them arise from the conjugation of a glycine, glycinamide, \(N,N\)-dimethylglycinamide, or an azirine ring.
Figure 11. $^1$H and $^{13}$C spectra of motualevic acid A (1) in CD$_3$OD.
Figure 12. 2D homonuclear NMR spectra for motualevic acid A (1) in CD$_3$OD. (A) TOCSY (TOtal Correlation SpectroscopY) spectra which shows all protons in a mutually coupled spin system. (B) COSY (COrrrelation SpectroscopY) spectra indicates which protons are spin-spin coupled.
Figure 13. 2D heteronuclear NMR spectra for mutuavlic acid A (1) in CD$_3$OD. (A) $^1$H-$^{13}$C HSQC (Heteronuclear Single Quantum Coherence spectroscopy) spectra giving one unique peak for each proton attached to a carbon. Red peaks indicate methylenes. (B) HMBC (Heteronuclear Multiple-Bond Coherence spectroscopy) detects heteronuclear correlations over 2-3 bonds.
Figure 14. COSY and selected HMBC correlations that were used to assign the structure of motualevic acid A (1).
Figure 15. $^1$H spectra showing defining features of (A) motualevic acid B (2) in CD$_3$OD, and (B) motualevic acid C (3) in CDCl$_3$. 

(A) 

$^1$H (ppm)

(B) 

$^1$H (ppm)
Figure 16. $^1$H spectra showing defining features of (A) motualevic acid D (4) in CD$_3$OD, and (B) motualevic acid E (5) in CDCl$_3$, with arrows indicating chemical shifts of glycine residue not present in this compound.
Figure 17. Chiral HPLC analysis of motualevic acid F (6). Compound 6 was injected onto a YMC Chiral NEA (1-naphthylethylamine) column, and eluted with 0.01% TFA in 70:30 hexanes:isopropanol. Peak integration determined 80% enantiomeric excess for the $R$ isomer.
Figure 18. Microbroth dilution assay curves for (A) *Staphylococcus aureus*, and (B) methicillin-resistant S. *aureus*. The lines indicate the inhibition curves used to calculate the minimum inhibitory concentration (MIC). Motualevic acid A (I), graphed in black circles, inhibited the growth of *S. aureus* and MRSA with MIC$_{50}$s of 25±9 µM and 21±6 µM, respectively. Motualevic acid C (3), graphed in blue, inhibited the growth of *S. aureus* and MRSA with MIC$_{50}$s of ~400 µM and ~900 µM, respectively. Motualevic acid F (6), graphed in red, inhibited the growth of *S. aureus* with an MIC$_{50}$ of 2.9±0.7 µM, and MRSA with an MIC$_{50}$ of 9.3±2 µM.
Figure 19. The structures of synthetic analogs of the motualevic acids, compounds 8-10. Compounds 8a and 8b are characterized by the lack of bromines and a truncation of the extended aliphatic chain. Compound 9 is only two carbons shorted than the natural motualevic acid A. Compounds 10a and 10b both contain a seven carbon unsaturated acid chain with the dibromo group, but compound 10b differs from the other analogs by the chain extension occurring on the free acid side of the molecule, giving rise to an analog with the one less carbon overall and the amide residue in the center. These compounds were synthesized by Dr. Cajetan Dogo-Isonagie and Dr. Pradeep Cheruku, my postdoctoral collaborators in the Natural Products Chemistry Section, LBC, NIDDK.
Figure 20. Exploration of the antibacterial effects with motualevic acid A (1). Microbroth dilution assay curves for (A) three *Staphylococci* strains, and (B) *Enterococcus faecium* and vancomycin-resistant *E. faecium* (VREF). The lines indicate the inhibition curves used to calculate the minimum inhibitory concentration (MIC). (A) Motualevic acid A inhibited the growth of multidrug-resistant *S. aureus* (MDRSA) with an MIC$_{50}$ of 18±4 µM, graphed in black circles. 1 also inhibited the growth of clinical osteomyelitis *S. aureus* (UAMS-1) with an MIC$_{50}$ of 24±7 µM, graphed in blue circles, and community-associated MRSA (USA300) with an MIC$_{50}$ of 37±10 µM, graphed in red circles. (B) Natural motualevic acid (circles) and synthetic motualevic acid A (squares) inhibited the growth of *E. faecium* (black) and VREF (red) with similar MIC$_{50}$s in a microbroth dilution assay (22-36 µM). (C) Viability assay showing motualevic acid A is bactericidal within two hours after addition to a growing population of bacteria. *S. aureus* (red) and MRSA (black) were grown in the presence (squares) or absence (circles) of 1. The definition of bactericidal activity is graphed as the blue line, and is a three-log decrease in number of the starting population.
2.15 Tables and table legends

**Table 2.** Chemistry and biological activity of *Siliquariaspongeia* and *Theonella* extracts. Twenty-four extracts were prepared from sponges collected in eight countries, including Chuuk, Fiji, Indonesia, Malaysia, Palau, Philippines, Papua New Guinea (PNG), and Vanuatu. The chemistry was determined by dereplication and LC-MS chromatographic analysis, and biological activity was determined by disk diffusion assay (antibacterial and antifungal), cell cytotoxicity assays, and neutralization (HIV-1) assays. Genera are **S** – *Siliquariaspongeia* and **T** - *Theonella*

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Table 3. NMR data for motuaevic acid A (1) in CD$_3$OD.

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$^a$Recorded at 125 MHz, reference to residual CD$_3$OD at $\delta_c$ 39.15 ppm. $^b$Recorded at 500 MHz, referenced to residual CD$_3$OD at $\delta_H$ 3.33 ppm. $^c$HMBC correlations from indicated proton, $^{2,3}J_{C-H}=6$ Hz.
Table 4. NMR data for motualevic acid B (2) in CD$_3$OD, motualevic acid C (3) in CDCl$_3$, and motualevic acid D (4) in CD$_3$OD.

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$^a$Recorded at 125 MHz, referenced to residual CD$_3$OD at $\delta$C 39.15 ppm. $^b$Recorded at 500 MHz, referenced to residual CD$_3$OD at $\delta$H 3.33 ppm. $^c$Recorded at 150 MHz, referenced to residual CDCl$_3$ at $\delta$C 77.23 ppm. $^d$Recorded at 500 MHz, referenced to residual CDCl$_3$ at $\delta$H 7.24 ppm.
Table 5. NMR spectroscopic data for motualevic acid F (6) and (4E)-R-antazirine (7) in CDCl₃.

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aRecorded at 125 MHz, referenced to residual CDCl₃ at $\delta^c_{C}$ 77.2 ppm. bReferenced to residual CDCl₃ at $\delta^f_{H}$ 7.24 ppm. cProton showing HMBC correlation to indicated carbon. d$\delta^a_{C}$ values for motualevic acid F (6). e$\delta^b_{C}$ values for (4E)-R-antazirine (7). f$\delta^c_{H}$ values for motualevic acid F (6); g$\delta^d_{H}$ values for (4E)-R-antazirine (7).
Table 6. Antimicrobial screening results for natural motualevic acids 1-7.

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<th>S. aureus</th>
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<td>25 ± 9</td>
<td>21 ± 6</td>
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<tr>
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<td>23</td>
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<td>—</td>
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<td>3</td>
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<td>NA</td>
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<td>&gt;900</td>
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<td>—</td>
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<td>12</td>
<td>2.9 ± 0.7</td>
<td>9.3 ± 2</td>
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<tr>
<td>7</td>
<td>NA</td>
<td>NA</td>
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\(^{a}\)Values in nmol/disk where values reported gave rise to zones of inhibition of 8-11 mm. \(^{b}\)MIC\(_{50}\) values reported in µM. NA (not active at loads as high as 54 nmol/disk for compounds 2-4 and 120 nmol/disk for compounds 5 and 7); —, not tested.
Table 7. Disk diffusion assay results for truncated synthetic motualevic acid analogs 8-10.

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\textsuperscript{a}Values in nmol/disk where values reported gave rise to zones of inhibition of 8-11 mm.
\textsuperscript{b}not active at loads as high as 200 nmol/disk.
\textsuperscript{c}not active at loads as high as 300 nmol/disk.
Table 8. Composition and antibacterial activity of synthetic motualevic acid A derivatives. Activity was determined against *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *E. faecium*, and vancomycin-resistant *E. faecium* (VREF), and the MIC<sub>50</sub> is reported in µM. nt – not tested.

These data are the result of a collaborative projection between Dr. Pradeep Cheruku and Jessica Keffer, where compound design was performed by the two of use, chemical synthesis by Dr. Cheruku, and all biological characterization and assay design by Jessica Keffer.

<table>
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<tr>
<th>Compound</th>
<th>R-group (replacing the glycine)</th>
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<th><em>E. faecium</em></th>
<th>VREF</th>
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Table 8. Composition and activity of synthetic motualevic acid A derivatives (continued).

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<td>MRSA</td>
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Chapter 3
The research presented here was published as follows:


**Novel antibacterial compounds from marine algae: Their spectrum of biological activity, geographic variability in production, synthetic fragments, and structure-activity relationship**

3.1 Abstract

Ten new antibacterial natural products, named chrysophaentins A-H, belonging to a new structural class have been identified from the marine chrysophyte algae *Chrysophaeum taylori*. Their structures were determined by 2D NMR and MS techniques and are characterized by the presence of two polyhalogenated, polyoxygenated ω,ω′-diarylbutene units connected by ether bonds. We have analyzed the geographic variability of chrysophaentin production in *C. taylori* located at different sites on the island of St. John, U.S. Virgin Islands, and we have synthetic fragments comprising conserved portions of the chrysophaentins. We have determined the antimicrobial activity of natural chrysophaentins and their synthetic analogs against five diverse *Staphylococcus aureus* strains. We find that the chrysophaentins show similar activity against all *S. aureus* strains, regardless of their drug sensitivity profiles. The synthetic chrysophaentin fragments mimic the natural compounds in their spectrum of antibacterial activity, and therefore
represent logical starting points for future medicinal chemistry efforts of the natural products and their analogs. Chrysophaentin A, the most potent of these antibiotics inhibited the growth of clinically relevant drug-resistant Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA), multidrug-resistant *S. aureus*, and vancomycin-resistant *Enterococcus faecium* (VREF).

### 3.2 Background

Marine invertebrates are not the only source of bioactive natural products in the marine environment. After sponges and coral, red algae have provided the greatest number of novel compounds in the years between 1985 and 2008 (Figure 7) [247]. Many of these alga-derived compounds possess interesting biological activities, exemplified by the bromophenols [332]. Several bromophenols isolated from the marine alga, *Rhodomela confervoides*, inhibited the growth of *S. aureus* and several other pathogenic bacterial strains [333,334]. Our previous work with the motualevic acids indicated that the bromine atoms of the dibromovinyl termini were essential for antibacterial activity [323], and halogens have been shown to be essential in other antibacterials [24]. From our collection of marine organisms harvested for analysis, we identified a rare marine alga collected in St. John, U.S. Virgin Islands, with antibacterial activity against Gram-positive organisms. The total ion chromatogram of the extract clearly indicated the presence of halogenated compounds based on observed isotopic patterns. These properties warranted further investigation of the active compounds from this alga.
3.3 Isolation and structure elucidation of the chrysopaentins

The alga was identified as the marine chrysophyte, *Chrysophaeum taylori*, on the basis of its morphology, cellular structure, and unusual color. The alga was found growing as sulfur yellow-colored, fluffy colonies on course sand or coral rubble substrate in the bays of St. John, U.S. Virgin Islands. The multi-cellular structure of *C. taylori* was extremely fragile, but on samples that were freshly collected or preserved, stalk-like structures made up of branching mucilaginous ‘streamers’ of pear shaped, invaginated cells were visible by light microscopy (Figure 21).

*C. taylori* was originally described by Lewis and Bryan in 1928 from samples found in the Dry Tortugas [335]. The taxonomy is often difficult to elucidate due to its rare occurrence and fragile cellular structures. However, *C. taylori* colonies have been found in Puerto Rico [336,337], Guam [338,339], Okinawa, Japan [339], the Great Barrier Reef [340], and recently has been identified as one of several mucilaginous algae responsible for blooms in the Mediterranean [341-345].

The *C. taylori* alga from St. John was lyophilized (200 g) and sequentially extracted with hexanes, chloroform, and methanol. The methanol extract (13 g) was partitioned between *n*-BuOH-H$_2$O (1:1) and the organic layer (1.1 g) was fractionated on Sephadex LH-20. All fractions were subjected to antibacterial testing, and fractions containing the halogenated compounds and antibacterial activity were purified by reverse-phase HPLC. This led to the isolation of ten new polyhalogenated, polyoxygenated bisdiarylbutene ether macrocycles termed chrysopaentins A-H (11-20) (Fig. 22) [346]. Their planar structures were determined by
extensive spectroscopic methods including 1D and 2D homonuclear and heteronuclear NMR, high resolution mass spectrometry, and MS in-source fragmentation.

High-resolution ESI-MS of chrysophaentin A (11) gave a molecular ion at \( m/z \) 675.0154 [M - H]⁻, consistent with a molecular formula of C\(_{32}\)H\(_{24}\)Cl\(_4\)O\(_8\), requiring 19 degrees of unsaturation. The presence of four chlorine atoms was confirmed by MS in-source fragmentation experiments. Fragment ions and their respective isotopic patterns clearly indicated the loss of four consecutive chlorines, indicating chrysophaentin A was one of the halogenated compounds present in the extract. The downfield region of the \(^1\)H NMR spectrum of 11 in CD\(_3\)OD contained signals for eight aromatic protons as well as signals for two olefinic protons. Thirty-two resonances were observed in the \(^{13}\)C NMR spectrum of 11, and the HSQC spectrum contained cross peaks ascribable to eight aromatic methine carbons, four benzyl methylene signals, and two olefinic methines. Analysis of the 2D NMR data of 11 indicated two main fragments (I and II) (Fig. 23). The geometry of the C8/C9 double bond was established as E on the basis of strong ROEs between the methylene protons H-7 and H-10. Thus, the partial structure I was characterized by the presence of two aryl rings \( \omega,\omega' \)-linked to an (E)-2-chlorobut-2-ene moiety.

2D NMR data indicated fragment II closely resembled I and also contained a \( \omega,\omega' \)-diarylalkene unit with E geometry at the C-8'/C-9' double bond. Rings A and C were virtually identical, while the only difference between rings B and D was the benzene substitution patterns. Partial fragments I and II together contained 18 of the required 19 degrees of unsaturation, and only 18 of the 24 hydrogens were attached to carbons. Consequently, there had to be six hydroxyl groups, whose presence was confirmed by a \(^1\)H NMR spectrum of 11 in DMF-\(d_7\), and
fragments I and II had to be connected through two ether linkages to satisfy the unsaturation index and molecular formula of 11. HMBC and ROESY correlations from these hydroxyl protons allowed unambiguous assignment of the positions of the hydroxyl groups and ether linkages connecting fragments I and II. The ether bonds must occur at C-1 and C-1’ in rings A and C, respectively, at C-16 in ring B, and at C-14 in ring D. Finally, connectivities between fragments I and II were assigned from a ROESY spectrum. Through space ROEs between the two fragments were observed between OH-15’ and H-6 which required C-14’ of ring D to be connected to C-1 of ring A via an ether bond, while an ROE between H-6’ and methylene protons H-10 suggested rings B and C were linked by the second ether bond at C-16 and C-1’, respectively. Therefore the structure of chrysophaentin A was established as a macrocyclic dimer composed of two ω,ω’-diaryl-2-chlorobut-2-ene moieties linked through two ether bonds in an asymmetrical fashion.

High resolution MS and MS in-source fragmentation showed chrysophaentin B (12) and C (13) to have the same molecular formula, C_{32}H_{24}BrCl_{3}O_{8} (m/z 718.9655 [M - H]^− and 718.9650 [M - H]^-), while the molecular formula of chrysophaentin D (14) was assigned as C_{32}H_{24}Br_{2}Cl_{2}O_{8} (m/z 762.9168 [M - H]^−). The 2D NMR data for these three compounds were very similar to 11. The only differences were in the respective carbon chemical shifts at C-2' in 12, C-2 in 13, and both C-2' and C-2 in 14, showing that 12 and 13 were the monobrominated analogs of 11, and 14 was the dibrominated analog.

The HR-ESI-MS of chrysophaentin E (15) showed a pseudomolecular ion peak at m/z 677.0317 [M - H]^− corresponding to a molecular formula of C_{32}H_{26}Cl_{4}O_{8} (calcd for C_{32}H_{25}Cl_{4}O_{8},
that differed from that of 11 by addition of two hydrogen atoms and one less degree of unsaturation. Furthermore, there were some significant differences in the chemical shifts of the protons and carbons of rings B and C, the most notable of which was the difference at C-16 (ring B) where the oxygenated substitution in 11 was absent in 15. Moreover, the $^1$H NMR spectrum of 15 in DMF-$d_7$ showed signals corresponding to seven hydroxyl protons (one more than 11).

Together these data suggested the two diarylalkene moieties in 15 were linked by only one ether bond. HMBC correlations confirmed the locations of the hydroxylated protons, showing the new hydroxyl to be located at C-1′. An ether bond remained between C-1 in ring A and C-14′ in ring D, completing the structure of chrysophaentin E as an acyclic bisdiarylbutene (15).

Two additional compounds, chrysophaentins E2 (16) and E3 (17) were obtained in insufficient quantities for full NMR characterization. However, through analysis of the respective fragmentation patterns observed in the HR-ESI-MS data, we were able to assign structures for each of these new linear chrysophaentins. HR-ESI-MS showed chrysophaentin E2 possessed a molecular formula of C$_{32}$H$_{26}$BrCl$_3$O$_8$ ($m/z$ 720.9786 [M – H$^-$], calcd for C$_{32}$H$_{25}$BrCl$_3$O$_8$, 720.9798), and chrysophaentin E3 possessed a molecular formula of C$_{32}$H$_{26}$Br$_2$Cl$_2$O$_8$ ($m/z$ 764.9282 [M – H$^-$], calcd for C$_{32}$H$_{25}$Br$_2$Cl$_2$O$_8$, 764.9293), indicating that a bromine replaced a chlorine in E2 (16), and two bromines replaced two chlorines in E3 (17), when compared to chrysophaentin E (15). Since positions C-2 and C-2′ were the only sites of halogen variability in chrysophaentins A-D, we reasoned that the dibrominated compound 17 likely contained a bromine at both of these carbons. On the other hand, chrysophaentin E2 must contain bromine at only one of these sites, with chlorine at the other. To identify the respective locations of chlorine and bromine in 16, we compared the mass fragmentation patterns of the
three linear analogs (Fig. 24). As seen in Fig. 24A, the fragmentation pattern for chrysopaentin E showed the successive loss of four chlorine atoms, giving fragment ions with clear isotopic signatures at \( m/z \) 641 [M – H – HCl], 605 [M – H – 2HCl], 569 [M – H – 3HCl], and 533 [M – H – 4HCl]. The fragmentation pattern and peak intensities for chrysopaentin E2 (16) was very similar to 15 (Fig. 24A and 24B), where successive loss of three chlorines and one bromine gave rise to fragment ions at \( m/z \) 685 [M – H – HCl], 649 [M – H – 2HCl], 613 [M – H – 3HCl], and 533 [M – H – HBr – 3HCl]. In contrast, although the loss of two bromines and two chlorines was apparent in the mass spectrum for chrysopaentin E3, the major ion for 17 appeared at \( m/z \) 395 [M – H – HBr – HCl – C\(_{16}\)H\(_{14}\)O\(_3\)], which corresponded to a fragment generated from cleavage at the ether bond (Fig. 24C). Although MS data on model halogenated biaryl ethers was not available, our data suggested that the presence of bromine ortho to the ether bond was destabilizing when compared to chlorine in the same position, and facilitated cleavage at the ether bond in compound 17. Thus, the nearly identical mass spectral patterns observed for 15 and 16 and the unique fragmentation at the ether bond in the spectrum for 17 allowed assignments of the structures of the linear chrysopaentins E2 and E3, as shown in Figs. 21 and 24.

The rationale behind investigating this extract was to identify the halogenated compounds which may be responsible for the observed antibacterial activity. The total ion chromatogram obtained from the LCMS contained three additional halogenated compounds not accounted for by compounds 11-17 that eluted at considerably longer retention times. Among this group, chrysopaentin F (18) eluted from the C12 HPLC column after chrysopaentin A (11 \( t_R = 28.0 \) min; 18 \( t_R = 41.3 \) min) and its molecular formula was determined to be identical to 11, indicating
18 to be an isomer of 11. However, the $^1$H and $^{13}$C NMR spectra showed only half as many signals as that of 11, suggesting that chrysopaentin F was symmetrical, comprising two identical diarylalkenes linked through two ether bonds. Chrysopaentin F differed from 11 in the location of the ether bond in ring B (para versus ortho). Chrysopaentin G (19) displayed a major ion peak identical to 12 and 13, and comparison of the NMR data indicated the bromine atom was located at the C-2' position, while the ether linkage was para in ring B.

Chrysopaentin H (20) was the most hydrophobic compound of this group, eluting from a C12 HPLC column at even longer retention times than 18 ($t_R = 41.3$ min; 20 $t_R = 50.5$ min). Its molecular formula was assigned as $C_{32}H_{23}BrCl_4O_8$ (HR-ESI-MS $m/z$ 752.9255 [M - H]−), which was 78 mu higher than 18. The difference was attributed to an additional bromine, making chrysopaentin H a pentahalogenated analog, and the carbon chemical shift data indicated the bromine to be present at C-12'.

Despite their differences, the ten chrysopaentins were remarkably similar to one another. All possessed an ether linkage between C-1 and C-14'. If present, the second ether linkage was located between either C-14 or C-16 and C-1', and halogen variability only occurred at C-2 and C-2'. Chrysopaentin H additionally differed in its pentahalogenation, with replacement of the hydrogen at C-12' with a bromine.

3.4 Geographic variability of chrysopaentin production

The original collection of the C. taylori sample described here was made in Round Bay, located on the southern side of St. John, U.S. Virgin Islands, in July 2007. We returned to St. John in June 2009 and made additional collections at Round Bay, Long Bay, and Hawk’s Nest.
(Fig. 25). These four collections allowed us to compare the chrysophaentin production in each location. Each methanolic extract from the three new collections in 2009 plus the original collection from 2007 were chromatographed by reverse-phase HPLC on a Jupiter Proteo C12 column with a linear gradient of 50-80% methanol in 0.05% TFA-water in fifty minutes. This afforded a chromatographic picture of the chrysophaentins present in each extract (Fig. 26). The two collections from Round Bay (Fig. 26A and 26C), separated by two years, were remarkably similar, as was the collection from Long Bay (Fig. 26B). However, it was immediately obvious that the methanol extract from Hawk’s Nest (Fig. 26D) was different; in fact, it lacked the asymmetrically-linked chrysophaentins A-D. Instead, the linear chrysophaentins E, E2 and E3 were the most abundant. Algae collected from all locations contained the symmetrical chrysophaentins, albeit at much lower abundance relative to compounds A-E. Because of its increased retention time and low abundance, chrysophaentin H was not visible under the conditions used to create Fig. 26.

3.5 Chrysophaentin fragment synthesis

While the extracts from Long Bay and Round Bay afforded more of the asymmetrical chrysophaentins, it became apparent that a synthetic approach to chrysophaentin production was necessary. A synthetic strategy was devised by our collaborators at the University of Pittsburgh to allow for a total synthesis of the chrysophaentins as well as the investigation of the structure-activity relationships. A retrosynthetic analysis showed fragment 22 (Fig. 27), which could be used twice for the assembly of chrysophaentin A, could be constructed by a Negishi cross-coupling between vinyliodide and benzylic zinc [347]. Microwave heating in the presence of
palladium acetate gave rise to chloroalkenes 21 and the Z-diastereomer in a 2:1 ratio after methanolysis. Compound 21 was purified by supercritical fluid chromatography (SFC), and boron tribromide was used to cleave the two methyl esters on 21 to give tetraphenol 22, which was obtained as the (E)-isomer and purified by SFC. The E/Z-configurations of these compounds were assigned on the basis of literature data [348,349].

3.6 Antibacterial activity and structure-activity relationship of the natural and synthetic chrysophaentins

Antimicrobial-assay guided fractionation indicated that the halogenated chrysophaentins were the source of the antibacterial activity, and potently inhibited the growth of Gram-positive organisms including *S. aureus*, *E. faecium*, and *Bacillus subtilis*. A microbroth dilution assay was used to evaluate the anti-staphylococcal activity of compounds 11, 14-15, and 18-22, and the minimum inhibitory concentration that led to a 50% decrease in growth (MIC$_{50}$) was determined using a model curve fit to the data. The MIC$_{90}$ was determined as the lowest concentration where there was no visible growth, and the minimum bactericidal concentration (MBC) was determined as the lowest concentration where a plated aliquot of treated bacteria led to a three logarithmic decrease in colony forming units (CFU) per milliliter relative to the starting inoculum. Compounds were evaluated against three different *S. aureus* strains from ATCC and two diverse clinical isolates. The strains used in this study included: *S. aureus* ATCC 25923, a clinical isolate from Seattle, 1945; methicillin-resistant *S. aureus* ATCC BAA-41, a hospital-acquired strain isolated in New York City in 1994; multidrug-resistant *S. aureus* (MDRSA) ATCC BAA-44, a Lisbon, Portugal hospital-acquired strain known as the Iberian clone, with
resistance toward ampicillin, methicillin, oxacillin, penicillin, erythromycin, gentamicin, tetracycline, azithromycin, amikacin, clindamycin, cephalothin, ceftriaxone, imipenem, lincomycin, streptomycin, perfloxacin, rifampin, and neomycin [29,350]; *S. aureus* UAMS-1, a clinical osteomyelitis isolate [351]; and a community-associated methicillin-resistant *S. aureus* (CA-MRSA), USA300-LAC [352,353].

Chrysophaentin A (11) inhibited the growth of *S. aureus* 25923 with an MIC<sub>50</sub> of 2.7±0.9 µM (Table 9) (Fig. 28A). It exhibited similar effects on MRSA BAA-41 and MDRSA BAA-44 with MIC<sub>50</sub>s of 2.3±1.0 and 1.8±0.5 µM (Fig. 28B), respectively, while the MIC<sub>90</sub> was 4.6-9.2 µM against all three strains. Compound 11 was bacteriostatic at its MIC<sub>90</sub>, becoming bactericidal only at concentrations four to eight times higher than the MIC<sub>90</sub>. The bacteriostatic effects of 11 were evident by 2.5 hours after treatment and were maintained over the course of 24 hours (Fig. 28C). Chrysophaentin A was the most potent antibacterial compound, but chrysophaentins F (18) and H (20) also inhibited *S. aureus* 25923 and MRSA BAA-41 with single digit micromolar concentrations. Their MIC<sub>90</sub>s were higher, 17-19 µM, but the MBC for 18 was still four-fold higher than the MIC<sub>90</sub>. The acyclic natural compound, chrysophaentin E (15), was even less active, with MIC<sub>50</sub>s of 16±5 and 14±4 µM against *S. aureus* 25923 and MRSA BAA-41, respectively. Compound 15 was also bacteriostatic at its MIC<sub>90</sub> of 37 µM, and its MBC was 74 µM for both strains. Chrysophaentins D (14) and G (19) were the least active natural compounds, with MIC<sub>50</sub>s of 17-37 µM against *S. aureus* 25923 and MRSA BAA-41, and MIC<sub>90</sub>s of 65-69 µM.

Synthetic compound 21 inhibited the growth of *S. aureus* 25923 with an MIC<sub>50</sub> of 12±4 µM (Table 9) and an MIC<sub>90</sub> of 34 µM. At the MIC<sub>90</sub>, compound 21 was bacteriostatic; its MBC
was 68 µM. Compound 21 also inhibited the growth of MRSA BAA-41 with an MIC$_{50}$ of 11±5 µM, MDRSA BAA-44 with an MIC$_{50}$ of 13±5, and an MIC$_{90}$ of 34 µM for both strains. Compound 22 was slightly less active with MIC$_{50}$s of 20±5, 23±10, 27±9 µM against *S. aureus* 25923, MRSA BAA-41, and MDRSA BAA-44, respectively. The MIC$_{90}$ was 74 µM against all three strains, and compound 22 was bacteriostatic at this concentration; its MBC was ~150 µM.

Chrysophaentins A (11), D (14), and F (18), as well as compounds 21 and 22, were tested against *S. aureus* UAMS-1 and CA-MRSA USA300-LAC to determine the MIC$_{50}$, MIC$_{90}$ and MBC for each compound against these clinical isolates. Chrysophaentin A was again the most potent, with MIC$_{50}$s of 5.1±2 and 5.0±2 µM against *S. aureus* UAMS-1 and CA-MRSA USA300-LAC, respectively (Table 9). The MIC$_{90}$ was 9.2 µM for both strains, and while 11 was still bacteriostatic at this concentration, the MBC was only 2 times higher at 19 µM. The symmetrical compound 18 was slightly less active with an MIC$_{50}$ of 12 µM and an MIC$_{90}$ of 37 µM, and it was also bacteriostatic at the MIC$_{90}$. Compounds 21 and 22 had MIC$_{50}$s between 18 and 31 µM. The MIC$_{90}$ was 34 µM for compound 21 and 74 µM for compound 22. For both synthetic compounds, the MBC was two-fold higher. Finally, chrysophaentin D (14) was the least active with MIC$_{50}$s between 41 and 49 µM, and MIC$_{90}$s at 65 µM.

Growth of *S. aureus* in the presence of chrysophaentins A (11), D (14), and F (18), and compound 22 were graphed as percentage growth of untreated bacteria for *S. aureus* UAMS-1 (Fig. 29A) and CA-MRSA USA300-LAC (Fig. 29B). These curves clearly demonstrate the relationship between the structure of the chrysophaentins and analogs, halogen composition, and anti-staphylococcal activity.
Natural compounds for which sufficient material was available (11, 14-15, 18-20) were evaluated in microbroth dilution assays against *E. faecium* and VREF and 11 was tested against *B. subtilis*. Chrysophaentin A (11) was the most potent antibiotic giving minimum inhibitory concentrations (MIC$_{50}$) of 5.6±3 µM and 4.2±1 µM toward *E. faecium* and VREF (Fig. 30), and 11±5 µM against *B. subtilis* (Fig. 28A). Chrysophaentins F and H (18 and 20) were the next most potent compounds with MIC$_{50}$ values of 12.3-14 µM against VREF. The remaining chrysophaentins were significantly less active against *E. faecium* and VREF with MIC$_{50}$ values of 35 µM and higher.

The chrysophaentins were tested against Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, but had no inhibitory activity against these pathogens. However, a permeable *E. coli* BL21(DE3) strain (*envA1*) with a defect in its outer membrane [125,354] was utilized to test whether the outer membrane of Gram-negative organisms was playing a role in the apparent inactivity. Compound 11, 14, 18, 21, and 22 were tested against *envA1 E. coli*. These compounds were able to inhibit the growth of *envA1 E. coli*, indicating that perhaps the chrysophaentins have a conserved intracellular target since they are able to inhibit the growth of Gram-negative *E. coli* when the outer membrane does not restrict access to the cytoplasm.

Chrysophaentin A remained the most potent compound against the permeable *E. coli*, with an MIC$_{50}$ of 27 µM. Compounds 21 and 22 had MIC$_{50}$s of 120 µM and 72 µM, respectively, while compound 18 and 14 were even less active, inhibiting the growth of *envA1 E. coli* with MIC$_{50}$s of 176 µM and >250 µM, respectively.

These results provided insight into structure-activity relationships for the chrysophaentins (Fig. 31). The tetrachlorinated macrocycle 11 with the asymmetrical linkage between C-16 and
C-1’ was the most potent antibacterial agent against all bacterial strains tested. When the ether linkage occurred between C-14 and C-1’, creating a symmetrical macrocycle (18), there was a decrease in overall potency, indicating the position of the ether linkage relative to the 2-butene unit affects activity; specifically, the ortho-linked chrysophaentin A is more potent than the para-linked chrysophaentin F. The presence of a fifth halogen at C-12’ (20) did not decrease the antibacterial activity further, even when the additional halogen was a bromine, indicating that the atom type at that position is not critical to the observed activity. The absence of a macrocycle, as in 15 and the synthetic fragments 21 and 22, further decreased antibacterial activity. However, an intact asymmetrical macrocycle did not guarantee potency; the positions of halogen diversity also played a role. Chrysophaentin D, which possessed two bromines in place of the chlorines on rings A and C was the least active compound considered in this study, and among the symmetrically linked dimers 18 and 19, the tetrachloro compound 18 was approximately 2.5 times more potent than compound 19, which differs only by replacement of a chlorine atom on ring C by bromine.

To assess specificity as an antimicrobial, we tested chrysophaentin A for cytotoxicity against the human colon tumor cell line HCT-116, the murine leukemia cell line P388, and a control mammalian cell line BSC-1. Interestingly, chrysophaentin A did not inhibit the growth of any of the cancer cell lines at concentrations as high as 74 µM, and did not show cytotoxicity toward the control cells at concentrations as high as 150 µM.
3.7 Discussion

Here we have described a new structural class of antibacterial compounds with potent inhibitory activity against *S. aureus*, drug-resistant *S. aureus*, and community-associated MRSA strains. *S. aureus* has long been a public health concern, and is currently the leading cause of skin, soft tissue, bloodstream, and lower respiratory tract bacterial infections [355]. Most of these strains are methicillin-resistant, and in the early 2000’s, more patients in the US succumbed to lethal MRSA infections than to AIDS, tuberculosis, and hepatitis B combined [31]. The public health threat involving MRSA is not just limited to the spread of antibacterial resistance; the movement of MRSA infections out of hospital settings and into the community is of great concern. These community-associated MRSA (CA-MRSA) strains have become so prevalent in the United States that almost everyone can be considered at risk for these infections [91,92]. CA-MRSA infections present unique challenges to their treatment because they are distinct from hospital-acquired MRSA (HA-MRSA) from an epidemiological, genetic, and clinical perspective [356,357]. CA-MRSA strains generally have a faster growth rate than hospital acquired strains, but are less resistant to antibacterials, contain a unique antibiotic cassette relative to nosocomial *S. aureus*, and are capable of producing an extracellular cytotoxin, Panton-Valentine leukocidin (PVL) [358-360]. CA-MRSA is fully virulent, perhaps even more so than HA-MRSA, and can produce other toxins that vary by geographic location [361,362]. In the United States, there are two distinct community-associated backgrounds that comprise the majority of CA-MRSA infections, namely USA300 and USA400. USA300 has become the predominant infectious CA-MRSA clone, currently accounting for 90-95% of CA-MRSA outpatient isolates in the U.S.
[358,363-367]. One reason for its relative success and apparent fitness advantage may be its increased virulence relative to other CA-MRSA strains [362,368].

The new anti-staphylococcal scaffold comprises two polyhydroxylated, polyhalogenated \(\omega,\omega'\)-diarylbutene units connected through two ether bonds to form symmetrical or asymmetrical macrocycles, or a single ether bond to give rise to acyclic analogs, from the marine chrysophyte alga, \(C. taylori\), from St. John, U.S. Virgin Islands. \(C. taylori\) is a rare organism, originally discovered in the Dry Tortugas off the southern coast of Florida [335], and has since been found in several locations worldwide. Bioactive natural products have been discovered from collections made in two locations. The Puerto Rico location yielded hormothamnione [336] and 6-desmethoxyhormothamnione [337], while the collections from St. John, U.S. Virgin Islands have yielded the ten chrysophaentins, as well as hormothamnione and 6-methoxyhormothamnione. As we have described here, analysis of methanol extracts revealed geographic variability within the \(C. taylori\) collections from St. John. The original collection coming from Round Bay in 2007 yielded chrysophaentins A-H. A second collection at Round Bay two years later contained a similar profile of compounds, indicating temporal stability in the natural compound production at this location. A 2009 collection at Long Bay contained the same ten chrysophaentins in a similar ratio. Both Long Bay and Round Bay are on the southeastern side of the island, and each of these collections was made in shallow protected waters, where the algae were growing on coral sand. The 2009 collection from Hawk’s Nest on the northwestern side of the island yielded a different profile of compounds. It was immediately obvious when comparing the chromatograms that the difference laid in the complete absence of the
asymmetrical chrysophaehtins in the samples collected at Hawk’s Nest. Instead, the most abundant compounds included the acyclic chrysophaehtins E, E2, and E3.

Synthetic efforts to obtain a steady supply of chrysophaehtin A are under way. An early approach that was employed provided two chrysophaehtin fragments that we have shown exhibit similar potencies and spectrum of activity to those of the parent chrysophaehtins. We profiled the anti-staphylococcal activity of several natural chrysophaehtins and the synthetic fragments against five *S. aureus* strains to measure their potency (MIC<sub>50</sub> and MIC<sub>90</sub>) and to determine whether the compounds were bacteriostatic or bactericidal. We found that these compounds have inhibitory activity against three drug-resistant *Staphylococcus* strains and two drug-susceptible strains. Significantly, one of the drug-resistant strains inhibited by the chrysophaehtins is the CA-MRSA strain, USA300-LAC. All chrysophaehtins, natural and synthetic, were bacteriostatic at their MIC<sub>90</sub>; but were bactericidal at concentrations ranging from two to eight fold higher than their respective MIC<sub>90</sub>s. Furthermore, the synthetic fragments showed similar potency to the acyclic chrysophaehtin E (15).

Control antibiotics used in the microbroth dilution assays were oxacillin and vancomycin. Oxacillin is a clinically used antibiotic (trade name Bactocill) that is useful against penicillin-resistant *S. aureus*, but is not used to treat MRSA infections. Vancomycin is widely considered the last line of defense to treat MRSA [369]. In our hands, the MIC<sub>50</sub> of oxacillin against *S. aureus* ATCC 25923 was 0.16 µM, against MRSA BAA-41 was 420 µM, against MDRSA BAA-44 was 540 µM, against *S. aureus* UAMS-1 was 0.37 µM, and against CA-MRSA USA300-LAC was 5.2 µM, while the MIC<sub>50</sub> of vancomycin against *S. aureus* ATCC 25923, *S. aureus* UAMS-1, and CA-MRSA USA300-LAC was 0.26-0.34 µM (data not shown). Our most
potent compound, chrysophaentin A inhibited the growth of all five strains with a concentration between 1.8 and 5.1 µM. While vancomycin was more potent than chrysophaentin A, our compound was able to inhibit the growth of the MRSA strains more potently than oxacillin, and should retain efficacy against strains of vancomycin-resistant *S. aureus* as well, since the chrysophaentins are not affected by the mechanism of vancomycin inactivation, as seen by the ability of chrysophaentin A to inhibit the growth of vancomycin-resistant *E. faecium*.

We have shown that subtle changes to the chrysophaentin structure and composition lead to differences in antibacterial activity. Knowing that the macrocycle is essential for full antibacterial potency, it is not surprising that the synthetic fragments described here have diminished activity compared to the cyclic natural products. However, we were gratified to find that they are indeed antimicrobial and their activities are comparable to the linear chrysophaentins. Their ease of synthesis and antimicrobial activity provide an excellent starting point not only toward the cyclic chrysophaentins, but also for generating more elaborate synthetic analogs.

The biosynthetic pathway leading to production of the chrysophaentins has not been characterized and a genome sequence for any *Chrysophaeum* species has yet to be published. Interestingly, the most closely related natural products include the marchantins, bazzanins, and chlorinated isoplagiochins, all of which were isolated from different species of liverworts [370]. While these macrocyclic compounds are reminiscent of the chrysophaentins, each family possesses defining structural characteristics that differ relative to one another and to the chrysophaentins. The marchantins (for an example of the marchantin structure, see ref. [371]) are bisbibenzyl ethers whose phenol rings are connected by an ethyl group, and thus far none
have contained halogens. The bazzanins [372,373] and chlorinated isoplagiochins [374], on the other hand are chlorinated, but are bisbibenzyls rather than bisbibenzyl ethers. Like the marchantins and all liverwort metabolites, they also contain ethyl linkages. The presence of a butene linkage is unique to the chrysophaentins. While some information is known about the biosynthesis of the liverwort metabolites [375], the pathways are incompletely characterized; moreover, parallels to chrysophaentin biosynthesis cannot be predicted as the producing organisms are not closely related. The lack of biosynthetic information underlines the importance of a synthetic approach that could supply large quantities of the chrysophaentins for further biological studies.

In summary, we have found a new class of halogenated antibacterial compounds with potent activity against drug-susceptible and drug-resistant bacteria. Knowing the structures and spectrum of activity of these compounds provides a starting point for mechanism of action studies that will be explored in the next chapter.

3.8 Materials and methods

3.8.1 Biological material

Samples of the marine algae were collected from Round Bay in July 2007, and Long Bay, Round Bay, and Hawk’s Nest in June 2009, St. John, U.S. Virgin Islands (Collection permit VIIS-2008-SCI-0034 National Park Service). All algae was found growing in shallow waters (-20 ft), and on a coarse coral sand substrate at Round Bay and Long Bay.
3.8.2 Light microscopy

Algae was preserved in 2.5% glutaraldehyde in seawater and viewed by light microscopy on a Zeiss Imager D.1 and images captured with Axiocam.

3.8.3 Chromatographic analysis

Lyophilized material was sequentially extracted with hexanes, CH₂Cl₂, and MeOH. The MeOH extract was analyzed by reverse-phase HPLC with an MS detector (Jupiter Proteo C12, 250 x 4.6 mm, 4 µm, DAD at 220 and 280 nm, flow rate 0.5 mL/min) eluting with a linear gradient of 50-80% methanol in 0.05% TFA-water in 50 minutes to afford compounds 11 (3.5 mg, \( t_R = 28.0 \) min), 12 (0.4 mg, \( t_R = 28.6 \) min), 13 (0.8 mg, \( t_R = 29.4 \) min), 14 (1.8 mg, \( t_R = 32.0 \) min), 15 (2.6 mg, \( t_R = 33.3 \) min), 16 (\( t_R = 34.3 \) min), 17 (\( t_R = 35.3 \) min), 18 (1.5 mg, \( t_R = 41.3 \) min), 19 (1.4 mg, \( t_R = 41.8 \) min), and 20 (0.7 mg, \( t_R = 50.5 \) min).

3.8.4 NMR spectroscopy and physical data

NMR spectra of natural products were recorded in CD₃OD or DMF-\( d_7 \) at 600 MHz with an \( x,y,z \)-shielded gradient triple resonance probe, or at 500 MHz with a cryogenically cooled \( z \)-shielded gradient triple resonance probe. DQF-COSY, 2DHOHAHA, HSQC, HMBC, and ROESY experiments were recorded using standard pulse programs with water suppression (Watergate). HSQC experiments were recorded with dwell times of 1.724 ms (\( ^1 J_{C-H} = 145 \) Hz), and HMBC spectra with dwell times of 31.25 and 50 ms (\( ^2,^3 J_{C-H} = 8 \) and 5 Hz).

**Chrysophaentin A (11).** Colorless amorphous powder; nonoptically active; UV (MeOH) \( \lambda \max (\log \epsilon) \) 210 (4.2), 225 (3.9), 290 (3.4); IR (film) \( \nu \max \) 3384, 1675, 1449, 1203, 1143, 846,
1H NMR (CD$_3$OD) $\delta$H 6.81 (1H, s, H-3), 6.179 (1H, s, H-6), 3.23 (2H, d, $J = 8.7$ Hz, H-7), 5.99 (1H, t, $J = 8.7$ Hz, H-8), 3.39 (2H, br s, H-10), 6.18 (1H, d, $J = 2.8$ Hz, H-12), 6.30 (1H, d, $J = 2.8$ Hz, H-14), 6.84 (1H, s, H-3'), 6.28 (1H, s, H-6'), 3.28 (2H, br d, $J = 8.1$ Hz, H-7'), 6.07 (1H, t, $J = 8.1$ Hz, H-8'), 3.57 (2H, br s, H-10'), 6.16 (2H, br s, H-12' and H-16'). 1H NMR (DMF-$d_7$) $\delta$H 9.90 (1H, s, OH-4), 9.41 (1H, s, OH-13), 9.58 (1H, s, OH-15), 10.1 (1H, s, OH-4'), 9.44 (2H, s, OH-13' and OH-15'). 13C NMR (CD$_3$OD) $\delta$C 148.1 (C-1), 120.0 (C-2), 117.1 (C-3), 150.4 (C-4), 126.7 (C-5), 116.0 (C-6), 30.6 (C-7), 127.7 (C-8), 134.7 (C-9), 33.7 (C-10), 133.0 (C-11), 107.9 (C-12), 155.6 (C-13), 103.8 (C-14), 151.3 (C-15), 135.9 (C-16), 148.9 (C-1'), 121.1 (C-2'), 117.3 (C-3'), 150.7 (C-4'), 127.2 (C-5'), 116.7 (C-6'), 30.4 (C-7'), 127.9 (C-8'), 134.4 (C-9'), 40.6 (C-10'), 136.7 (C-11'), 109.1 (C-12', C-16'), 151.8 (C-13', C-15'), 129.7 (C-14'); HR-ESI-MS 675.0154 [M - H]$^-$ corresponding to a molecular formula of C$_{32}$H$_{24}$Cl$_4$O$_8$ (calcd for C$_{32}$H$_{23}$Cl$_4$O$_8$, 675.0147).

Chrysophaentin B (12). Defining 13C NMR (CD$_3$OD) $\delta$C 109.3 (C-2'); HR-ESI-MS 718.9655 [M - H]$^-$ corresponding to a molecular formula of C$_{32}$H$_{24}$BrCl$_3$O$_8$ (calcd for C$_{32}$H$_{23}$BrCl$_3$O$_8$, 718.9642).

Chrysophaentin C (13). Defining 13C NMR (CD$_3$OD) $\delta$C 108.2 (C-2); HR-ESI-MS 718.9650 [M - H]$^-$ corresponding to a molecular formula of C$_{32}$H$_{24}$BrCl$_3$O$_8$ (calcd for C$_{32}$H$_{23}$BrCl$_3$O$_8$, 718.9642).

Chrysophaentin D (14). Defining 13C NMR (CD$_3$OD) $\delta$C 108.1 (C-2), 109.3 (C-2'); HR-ESI-MS 762.9168 [M - H]$^-$ corresponding to a molecular formula of C$_{32}$H$_{24}$Br$_2$Cl$_2$O$_8$ (calcd for C$_{32}$H$_{23}$Br$_2$Cl$_2$O$_8$, 762.9137).
**Chrysophaentin E (15).** Defining $^1H$ NMR (CD$_3$OD) $\delta_H$ 6.16 (2H, br d, $J = 1.9$ Hz, H-12 and H-16), 6.13 (1H, br d, $J = 1.9$ Hz, H-14), $^1H$ NMR (DMF-$d_7$) $\delta_H$ 9.34 (2H, s, OH-13 and OH-15), 9.52 (1H, s, OH-1'), $^{13}C$ NMR (CD$_3$OD) $\delta_C$ 140.9 (C-11), 108.1 (C-12, C-16), 159.3 (C-13, C-15), 101.6 (C-14); HR-ESI-MS 677.0317 [M - H]⁻ corresponding to a molecular formula of C$_{32}$H$_{26}$Cl$_4$O$_8$ (calcd for C$_{32}$H$_{25}$Cl$_4$O$_8$, 677.00304).

**Chrysophaentin E2 (16).** HR-ESI-MS 720.9786 [M – H]⁻ corresponding to a molecular formula of C$_{32}$H$_{26}$BrCl$_3$O$_8$ (calcd for C$_{32}$H$_{25}$BrCl$_3$O$_8$, 720.9798).

**Chrysophaentin E3 (17).** HR-ESI-MS 764.9282 [M – H]⁻ corresponding to a molecular formula of C$_{32}$H$_{26}$Br$_2$Cl$_2$O$_8$ (calcd for C$_{32}$H$_{25}$Br$_2$Cl$_2$O$_8$, 764.9293)

**Chrysophaentin F (18).** $^1H$ NMR (CD$_3$OD) $\delta_H$ 6.90 (2H, s, H-3 and H-3'), 6.42 (2H, s, H-6 and H-6'), 3.35 (4H, d, $J = 8.3$ Hz, H-7 and H-7'), 5.89 (2H, t, $J = 8.3$ Hz, H-8 and H-8'), 3.54 (4H, br s, H-10 and H-10'), 6.23 (4H, s, H-12, H-16, H-12', and H-16'), $^{13}C$ NMR (CD$_3$OD) $\delta_C$ 148.2 (C-1, C-1'), 120.4 (C-2, C-2'), 117.3 (C-3, C-3'), 150.7 (C-4, C-4'), 126.5 (C-5, C-5'), 115.1 (C-6, C-6'), 31.0 (C-7, C-7'), 127.2 (C-8, C-8'), 134.3 (C-9, C-9'), 39.8 (C-10, C-10'), 137.0 (C-11, C-11'), 110.0 (C-12, C-16, C-12', C-16'), 151.3 (C-13, C-15, C-13', C-15'), 130.0 (C-14); HR-ESI-MS 675.0140 [M - H]⁻ corresponding to a molecular formula of C$_{32}$H$_{24}$Cl$_4$O$_8$ (calcd for C$_{32}$H$_{23}$Cl$_4$O$_8$, 675.0147).

**Chrysophaentin G (19).** Defining $^{13}C$ NMR (CD$_3$OD) $\delta_C$ 108.9 (C-2'); HR-ESI-MS 718.9620 [M - H]⁻ corresponding to a molecular formula of C$_{32}$H$_{24}$BrCl$_3$O$_8$ (calcd for C$_{32}$H$_{23}$BrCl$_3$O$_8$, 718.9642).
**Chrysophaentin H (20).** Defining $^{13}$C NMR (CD$_3$OD) $\delta_{C}$ 106.3 (C-12'); HR-ESI-MS 752.9255 [M - H]$^-$ corresponding to a molecular formula of C$_{32}$H$_{23}$BrCl$_4$O$_8$ (calcd for C$_{32}$H$_{22}$BrCl$_4$O$_8$, 752.9252).

3.8.5 Mass spectrometry and MS/MS fragmentation

The MeOH extract was partitioned between $n$-BuOH-H$_2$O (1:1), and the organic layer was fractionated on Sephadex LH-20. Fractions containing the unknown chrysophaentins were subjected to HR-ESI-MS and MS in-source fragmentation. High resolution accurate mass data was obtained using a Waters LCT Premiere ESI-TOF mass spectrometer equipped with a Z-Spray electrospray ion source. The instrument was operated in the w-mode at a nominal resolution of 10,000 and an internal standard was used as reference. The ionization mode was negative ion and the ESI capillary voltage was -3000V and the cone voltage was 30V. The collisionally activated dissociation (CAD) mass spectra were obtained using a Waters LCT Premiere ESI-TOF mass spectrometer according to the method described by Ren et al. [376]. The ion guide 1 voltage was 65V and the cone voltage was 30V. The instrument was operated in negative ion mode.

3.8.6 Synthesis of chrysophaentin fragments

Synthetic compounds were provided to us by Jared Hammill and Peter Wipf at the University of Pittsburgh. Details on the synthesis are available here [347]. Upon arrival in our lab, compounds 21 and 22 were verified by LCMS and NMR.
3.8.7 Biological assays

Microbroth dilution assays were performed as described in the CLSI guidelines. Minimum inhibitory concentrations (MIC<sub>50</sub> and MIC<sub>90</sub>) were determined using 96-well microbroth dilution assays. Anti-staphylococcal assays were performed as follows. Antimicrobial activity of pure compounds or antibiotic standards was tested by adding serial dilutions to wells containing Mueller Hinton II broth. An overnight growth of <i>S. aureus</i> ATCC 25923, MRSA ATCC BAA-41, MDRSA BAA-44, <i>S. aureus</i> UAMS-1, and CA-MRSA USA300-LAC was diluted in Mueller-Hinton II broth and added to the wells to a final concentration of 5x10<sup>5</sup> colony forming units (CFU) per milliliter. Plates were incubated for 18 hours at 37 °C with shaking at 200 rpm. Assays for <i>E. faecium</i> (ATCC 49032), and vancomycin-resistant <i>E. faecium</i> (ATCC 70022) and <i>Bacillus subtilis</i> (ATCC 49343) were performed as follows. Briefly, single bacterial colonies were used to inoculate 4 mL of BBL Nutrient Broth (<i>Bacillus</i>) or BBL Brain-Heart Infusion (<i>Enterococcus</i>) for overnight growth and inoculum for each were diluted to 5 x 10<sup>5</sup> CFU/mL using the 0.5 McFarland standard. In a 96 well plate (Costar, round bottom), control antibiotics (chloramphenicol for <i>B. subtilis</i> and vancomycin for <i>E. faecium</i> and VREF) or chrysopaentins were added to Muller Hinton II (MHII) broth (<i>Bacillus</i>) or 10% Brain-Heart Infusion in MHII broth (for <i>E. faecium</i> and VRE) in the first column of the well plates, and serially diluted across the plate, reserving wells for a positive growth control (no treatment). 10 µL of diluted bacteria were added to all wells with the exception of those kept for broth blanks. Growth assays were similarly performed for <i>Escherichia coli</i> (ATCC 8739) and envA1 <i>E. coli</i> (Merck).
Plates were incubated at 37 °C overnight and read at absorbance 600 nm on a Molecular Devices plate reader. Growth curves were plotted and MIC$_{50}$ values obtained using Kalidagrapgh software. Curves were fit to a one-site model with Kaleidagraph 4.0 using the equation 
\[ y = \frac{100}{1 + \text{(concentration/MIC$_{50}$)}} \], where MIC$_{50}$ is the concentration at which the growth of bacterial cultures are reduced by 50%. MIC$_{90}$ values were visually calculated by determining the lowest concentration of test compound that allowed no visible growth (no difference of absorption between treated samples and blank controls).

Bacteriostatic or bactericidal properties were determined by plating onto a fresh TSA plate an aliquot from each well that showed no visible growth starting at the MIC$_{90}$. Plates were incubated for 24 hours at 37 °C, and colonies were counted. Bactericidal concentrations were defined as those that resulted in a three logarithmic decrease in the number of viable bacteria relative to the starting inoculum, and the lowest bactericidal concentration was defined as the MBC.

Cytotoxicity assays were performed as described in Section 2.11.4.

3.9 Acknowledgments

I would like to thank Carole Bewley, Michael Bewley, and Marius Clore for collection of the algae, and William Fenical and William Gerwick for helpful discussions on chrysophyte algae. Many thanks to the expertise of Alberto Plaza for his work on the structure elucidation of the chrysoaphentins, John Lloyd for mass spectrometry data and help, Paul Dunman for *S. aureus* strains, Katherine Young for envA1 *E. coli*, and Jared Hammill and Peter Wipf for the synthetic work. This work was supported by the NIH Intramural Research Program (NIDDK),
and the Intramural AIDS Targeted Antiviral Program, Office of the Director, NIH (C.A.B.), and
the Intramural AIDS Research Fellowship, Office of the Director, NIH (J.L.K.).
3.10 Figures and figure legends

Figure 21. Images of *C. taylori* collected from St. John, U.S. Virgin Islands. (A) and (B) The algal mass containing streamers of cells, each with a short independent stalk, which merges and intertwines with others to form the mass. Images acquired with a 20x lens. (C) The structure of the vegetative cell released from the algal mass. Image acquired with a 40x lens. (D) The sulfur yellow color of the alga in life, and the gelatinous streamers that are visible on the edge of the algal mass are shown in this photograph. Dr. Carole Bewley and Michael Bewley are acknowledged for sample collections.
Figure 22. Structures of chrysophentins A-H (11-20).
Figure 23. Structures of fragments I and II comprising chrysophaentin A. Key HMBC correlations are depicted in black arrows, and were used to assign the locations of the hydroxyls. ROESY correlations are in red, and were key in assigning the connectivity between the two fragments, as well as the double bond geometry. Rings have been identified with letters (A-D) to aid in describing the features of the chrysophaentins. I assisted with the NMR and structure elucidation of these compounds, but the majority of the analysis was completed by my collaborator, Dr. Alberto Plaza.
Figure 24. HR-MS fragmentation of chrysophaentins E, E2, and E3. Fragmentation of (A) chrysophaentin E (15) showing individual losses of four chlorine atoms; (B) chrysophaentin E2 (16) showing loss of three chlorine atoms and one bromine atom; (C) the major ion in the spectrum for chrysophaentin E3 (17) corresponds to fragmentation at the ether bond, with weaker ions corresponding to loss of two chlorine and two bromine atoms. The high resolution mass spectrometry and fragmentation pattern analysis was completed by Dr. John Lloyd at the NIH.

Figure on next page.
Figure 24. HR-MS fragmentation of chrysophaentins E, E2, and E3 (legend on previous page).
Figure 25. Locations of *C. taylori* collections on St. John, U.S. Virgin Islands. Collections were made Long Bay in 2009 (green pin), the northern tip of Round Bay in 2007 and 2009 (yellow pin), and on the north side of the island at Hawk’s Nest in 2009 (red pin). Image was created using the Google Earth software package [377].
Figure 26. Geographic distribution of chrysophaeonins analyzed by LCMS. Analytical HPLC chromatograms of MeOH extracts of four collections of *C. taylori* eluting with a linear gradient of 50-80% MeOH in *aq.* 0.05%TFA in 50 min. Panels correspond to *C. taylori* extracts obtained from the following collections sites and dates: (A) Round Bay, 2007; (B) Long Bay, 2009; (C) Round Bay, 2009; (D) Hawk’s Nest, 2009. The expansions shown correspond to the chromatograms at 28-42 mins, absorbance at 280 nm. Peaks are colored as shown in the legend.
Figure 27. Structures of the synthetic fragments mimicking the chrysopaentins. Compound 22 represents half of the structure of all three classes of chrysopaentins (asymmetrical macrocycle, symmetrical macrocycle, and acyclic). These compounds were synthesized by our collaborators Jared Hammill and Dr. Peter Wipf at the University of Pittsburgh. Details on the synthesis can be found here [347].
Figure 28. Microbroth dilution assay curves for chrysopaentin A (11) against (A) drug-susceptible organisms *Bacillus subtilis* and *Staphylococcus aureus* and (B) drug-resistant organisms methicillin-resistant *S. aureus* and multidrug-resistant *S. aureus*. Chrysopaentin A inhibits *B. subtilis* with an MIC$_{50}$ of 11 µM, and the three *S. aureus* strains with MIC$_{50}$s of 1.8-2.7 µM. (C) Time course for the effects of chrysopaentin A to become apparent. *S. aureus* was treated with a bacteriostatic concentration of chrysopaentin A (15 µM). By two hours, the growth inhibitory effects are evident. The definition of bactericidal (a decrease in 10$^3$ CFU/mL) is marked with a red dashed line.
Figure 29. Dose-response curves show the antibacterial activities and reveal a structure-activity relationship for the chrysophaents. Chrysophaents A (11), D (14), and F (18), and fragment 22 were tested for their ability to inhibit the growth of (A) *S. aureus* UAMS-1 and (B) CA-MRSA USA300-LAC in a microbroth dilution assay. Plots represent percentage bacterial growth as a function of compound concentration.
Figure 30. Microbroth dilution assays for (A) chrysophaentin A (11) against Enterococcus faecium (EF) and vancomycin-resistant E. faecium; and (B) chrysophaentin A (11), D (14), and F (18) against permeable E. coli (envA1). Chrysophaentin A inhibits the growth of E. faecium with an MIC$_{50}$ of 5.6 µM, VREF with an MIC$_{50}$ of 4.2 µM, and envA1 E. coli with an MIC$_{50}$ of 27 µM.
Figure 31. The structure-activity relationship (SAR) of the chrysophaentins. SAR analysis of the natural chrysophaentin activity against S. aureus (A) showed chrysophaentin A (11) to be the most potent compound, followed by chrysophaentin F (18). The only difference between these two compounds is the second ether linkage of the macrocyclic structure. Further reinforcing the role of the macrocycle in activity, acyclic chrysophaentin E (15) is even less potent. Finally, chrysophaentin D (14) with bromines in place of some chlorines on the phenolic rings is the least potent compound, indicating the halogens composition also affects the antibacterial activity. SAR analysis of the natural and synthetic chrysophaentins against CA-MRSA (B) showed the same relationship between the natural chrysophaentins, while the synthetic fragments (21 and 22) have similar activity to the acyclic chrysophaentin E (15).
### 3.11 Tables and table legends

**Table 9.** Antibacterial summary of select chrysophyaentins and synthetic fragments against five diverse strains of *Staphylococcus aureus* (all values are in µM; n.d. – not determined)

<table>
<thead>
<tr>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>MBC</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
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Chapter 4
The chrysophaentins inhibit the bacterial cell division protein, FtsZ

4.1 Abstract

A global increase in drug-resistant pathogens demands the development of new antibacterial compounds that meet two criteria. First, the novel compounds should have a distinct structural scaffold; and second, the compound should affect a new pathway, protein, or component that has not been targeted before. These two requirements are often interdependent, and the chrysophaentins, a novel structural class of algal natural products, may fit both of these criteria. The chrysophaentins are structurally distinct from other antibacterial compounds, and features of their structural scaffolds suggest the molecular target of the chrysophaentins may be the bacterial cell division protein, FtsZ. FtsZ is an attractive antimicrobial target because it is conserved in almost all known pathogenic bacteria, and is essential for division, proliferation, and thus, survival. The work here demonstrates the chrysophaentins, particularly chrysophaentin A (11), are specific, competitive inhibitors of FtsZ, and are capable of preventing polymerization and inhibiting GTP hydrolysis in vitro. The synthetic chrysophaentin fragments, compounds 21 and 22, also inhibit the GTPase activities of both E. coli- and S. aureus-FtsZ. Furthermore, these three compounds affect the proper formation of the Z-ring in live bacteria that have been
exposed to these inhibitors. Together, these data suggest the mechanism of antibacterial action of the chrysophaentins and synthetic fragments is to inhibit FtsZ.

4.2 Background

After determining the structure of a novel compound and exploring its biological activity, the next step in a drug discovery program is to investigate the potential mechanism of action and/or molecular target. Our initial work with the macrocyclic chrysophaentins showed these compounds have potent antibacterial activity against a number of Gram-positive organisms, including *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), community-associated MRSA, multidrug-resistant *S. aureus*, *Enterococcus faecium*, vancomycin-resistant *E. faecium*, and *Bacillus subtilis*. The fact that the chrysophaentins inhibit the growth of drug-susceptible and drug-resistant strains at similar concentrations, and are structurally distinct from any clinically used antibiotics suggests the chrysophaentins may have a novel mechanism of action and new molecular target.

The structural features of the chrysophaentins were used as a guide towards identifying potential targets. The chrysophaentins are polyhalogenated, polyoxygenated aromatic compounds. A review of the small molecule antibacterial literature showed somewhat similar structural features in several inhibitors of the bacterial cell division protein, FtsZ (*for a review of FtsZ, and its biochemical and physiological properties, see section 1.5.2-1.5.4*). In particular, berberine, an aromatic natural product derived from the plants barberry and goldenseal [378], and the chlorinated polyphenol zantrin Z1, a synthetic small molecule, share structural features with the chrysophaentins. Both compounds inhibit multiple bacterial strains, and their
antibacterial biological target is FtsZ. These two compounds were reported to inhibit FtsZ GTP hydrolysis and GTP-dependent polymerization [379,380].

There are several mechanisms by which small molecules can inhibit the enzymatic functions of FtsZ. Some compounds, like berberine and zantrin Z1 inhibit both GTP hydrolysis and polymerization of FtsZ. This is similar to the way certain tubulin inhibitors operate, such as nocodazole [381,382]. Others, like the synthetic molecules zantrin Z3 [379], OTBA [383], and PC190723 [142,384] inhibit GTP hydrolysis, but stabilize FtsZ polymers, similar to microtubule-stabilizing agents such as paclitaxel [385]. Inhibition of the biochemical properties of FtsZ by either of these mechanisms can affect the dynamics of FtsZ, and concurrently, its physiological role in division, thereby leading to cell death. In order to explore a possible mechanism of action for the chrysophaentins, we used complimentary biochemical and microscopy techniques to determine the effects of the chrysophaentins on FtsZ.

**4.3 Expression, purification, and functional analysis of recombinant FtsZ**

Initially, we obtained purified, recombinant *E. coli*-derived FtsZ from Cytoskeleton Inc. for experiments. Early promising results warranted further experiments with the protein, and because it was not cost-effective to purchase a large quantity of protein, we developed a route for expressing and purifying FtsZ in our lab. An isopropyl β-D-1 thiogalactopyranoside (IPTG) inducible plasmid that over-expresses *E. coli*-derived FtsZ (abbreviated as EcFtsZ) was given to us by Dr. William Margolin at the University of Texas and has been described in references previously [386,387]. Sequencing using T7 forward (5’-TAATACGACTCACTATAGGG-3’) and T7 terminator reverse (5’-CTAGTTATTGCTCAGCGGT-3’) primers confirmed the FtsZ
protein sequence encoded by this plasmid was identical to *E. coli* FtsZ sequences in the NCBI protein database (one such example is NP_285791, the FtsZ identified from whole genome sequencing of *E. coli* O157:H7 strain EDL933). IPTG-induced expression in competent *E. coli* BL21-DE3(Gold) verified that this pET-11a plasmid allowed for high level protein expression. Because the chrysophaentins showed potent anti-staphylococcal activity, we wanted to express *S. aureus*-derived FtsZ as well to test for potential inhibitory effects, and to determine if there were any species specific differences in FtsZ activity and FtsZ inhibitors. A plasmid expressing *S. aureus* FtsZ (abbreviated SaFtsZ) was not readily available, so a synthetic gene of SaFtsZ was ordered from GeneArt. The protein sequence was found in the NCBI protein database, as AAA16512, the FtsZ from *S. aureus*. The nucleotide sequence was codon-optimized for expression in *E. coli*, and cloned into a pET-11a plasmid. SaFtsZ and EcFtsZ share only 53% amino acid identity with each other (Fig. 32), and not much work has been done on characterizing the biochemical differences between SaFtsZ and EcFtsZ.

Both the EcFtsZ-pET-11a and SaFtsZ-pET-11a plasmids were separately transformed by heat-shock into competent *E. coli* BL21-DE3(Gold) cells for expression. Clones containing the plasmid were selected on solid Luria-Bertani (LB) agar containing 100 µg/mL ampicillin (AMP). Single colonies were chosen, and grown in 10 mL LB broth with 100 µg/mL AMP. Those that expressed FtsZ were frozen as glycerol stocks for future use. A single glycerol stock was thawed and used to inoculate 100 mL of LB broth with AMP. This was grown overnight at 37 °C with shaking at 250 rpm, and diluted into one liter of LB with AMP the following morning. One liter of diluted *E. coli* was split into three 1000 mL baffled Erlenmeyer flasks, and grown with shaking at 37 °C, until the optical density reached 0.4 at an absorbance of 600 nm. Protein
expression was induced by addition of 1 mM IPTG, and the cells were grown for 5 hours with
shaking at 37 °C. These conditions were optimized for BL21-DE3(Gold) E. coli expressing
EcFtsZ. Protein yield was highest when grown in smaller volumes to increase aeration, and
when induced at a low density. However, when the same conditions were used to overexpress
SaFtsZ in BL21-DE3(Gold) E. coli, protein expression was significantly lower relative to
EcFtsZ; and concurrently, the bacteria became filamentous (Fig. 33). The heterologous SaFtsZ
expression in E. coli was detrimental to normal growth and division, which contributed to the
decreased protein yield. The abnormal cell shape was improved by lowering the temperature to
30 °C when inducing with IPTG, which also increased the protein yield.

After induction, cells were harvested by centrifugation (9000xg), and the cell pellet
frozen at -80 °C. Initially, the pelleted cells were lysed with BugBuster® Protein Extraction
Reagent, but after many attempts at purifying the protein, it was determined the detergent in
BugBuster® was detrimental to FtsZ stability and activity. A protocol utilizing repeated cycles
of freezing and thawing was devised [388], where the cell pellet was frozen in a liquid nitrogen
bath, and thawed in an ice bath for three cycles. This method greatly improved stability and
overall yield of FtsZ. Initial functional tests of FtsZ enzymatic activity indicated measurable
GTP hydrolysis, but all attempts at visualizing polymerization failed, indicating perhaps this
method of protein purification was not suitable for FtsZ. Each step of the purification process
was investigated, and after many trials, it was determined the method for breaking the cell pellet
needed to be changed. Therefore, the pelleted cells were resuspended in Lysis buffer (50 mM
Tris pH 8.0, 100 mM KCl, 1 mM EDTA, 1% benzamidine (for protease inhibition), Roche
Protease Cocktail inhibitor, 0.1% β-mercaptoethanol, 1 mM MgSO₄, adapted from Beuria et al.
[209] with modifications), briefly sonicated, and mechanically disrupted with a high pressure Microfluidizer or EmulsiFlex homogenizer. Insoluble cellular debris was removed by centrifugation (29000xg), and the supernatant was supplemented with 5 mM MgCl₂ and additional protease inhibitors to ensure stability and prevent degradation of the protein.

The protein was precipitated at 4 °C with ammonium sulfate to a final concentration of 30% (w/v) ammonium sulfate and the semi-purified protein collected by centrifugation (36000xg). The pellet was resuspended in a low-salt buffer (50 mM Tris pH 7.4, 25 mM KCl, 5 mM MgCl₂, 10% glycerol), and dialyzed overnight against the same buffer to remove excess salt. The dialyzed protein was chromatographed by ion exchange chromatography on a strong anion exchanger column. The isoelectric point (pI) of FtsZ is low (4.63 for EcFtsZ and 4.87 for SaFtsZ), so a buffer with a pH greater than 5 allowed the protein to bind to the positively charged quaternary ammonium groups within the column. The protein was then eluted from the column using a buffer with a high ionic content (50 mM Tris pH 7.4, 1 M KCl, 5 mM MgCl₂, 10% glycerol). Neither EcFtsZ nor SaFtsZ contain tryptophan residues, so the protein has no UV absorbance at 280 nm, which normally can be used to track protein elution. Therefore, fractions that were collected from the anion exchange column were analyzed by gel electrophoresis on a 10-15% SDS-PAGE gradient gel for the presence of FtsZ (Fig. 34).

Fractions containing FtsZ were combined and concentrated in a GE Vivaspin concentrator (5000 mwco), then chromatographed on a gel filtration column using a buffer consisting of 50 mM Tris pH 7.4, 50 mM KCl, 1 mM EDTA, and 10% glycerol from Andreu et al. [384]. Gel filtration is a liquid chromatography method that separates molecules based on size differences, a method also known as size-exclusion chromatography. The column chosen
for FtsZ purification was an Amersham Biosciences High load Superdex 75 16/60, where the superdex packing material is covalently bonded dextran and cross-linked agarose, which forms a gel matrix with a mean particle size of 34 µm, making it suitable for separating molecules that are between 3-70 kDa. Access to the pores in the gel matrix is limited by molecular weight and size, allowing for separation of proteins based on their size.

Fractions eluted from the gel filtration column were analyzed for the presence of FtsZ (Fig. 34), and fractions containing the protein were pooled according to concentration and purity, and frozen at -80 °C. Protein identity was verified by mass spectrometry, since the protein, particularly SaFtsZ, migrated on a gel with an apparent molecular weight larger than its actual molecular weight (Fig. 34). In the initial steps of the purification, glycerol was necessary in order to dialyze and concentrate the protein, and at the gel filtration step, the presence of glycerol was important to ensure a monomeric population of FtsZ. Dialysis prior to the first chromatographic step was necessary to remove excess ammonium sulfate ions, so that the protein was able to interact with the anion exchange column; and the final gel filtration chromatography was necessary to ensure purity of the protein, the presence of a constant amount of potassium in each fraction, and to remove any remaining phosphates from the cell lysis so that the protein could be used in GTPase assays.

The biochemical properties of FtsZ are reviewed in section 1.5.3, and include the ability of FtsZ to bind and hydrolyze GTP, and to polymerize in a GTP-dependent manner. Therefore, protein that had been purified according to the protocol developed here was subjected to GTPase and polymerization assays to ensure its activity before use with potential inhibitors. The purified recombinant protein was subjected to an end-point GTPase assay, where the production of
inorganic phosphate ($P_i$) from GTP hydrolysis was measured based on the change in absorbance of malachite green in the presence of phosphomolybdate ($P_i$ ColorLock Gold from Innova Biosciences). A standard curve utilizing purified inorganic phosphate was constructed so that phosphate production could be quantified from absorbance at 635 nm (Fig. 35A). It was assumed that one mole of inorganic phosphate was produced from the enzymatic hydrolysis of one mole of GTP to one mole of GDP. When 0.25 mM purified GTP from Innova Biosciences was added to 2 µM EcFtsZ in Assay buffer (50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0, 100 mM KCl, 5 mM MgCl$_2$), after ten minutes at 23 °C, between 30 and 40 µmol phosphate was released and detected (Fig. 35B), and was within the linear portion of the standard curve. This corresponded to hydrolysis of between 1.5 and 2 µmol GTP per minute per µmol EcFtsZ, which was similar to activities reported elsewhere [389], as well as to activity observed with the EcFtsZ from Cytoskeleton Inc. EcFtsZ contains a bound nucleotide (GDP) when purified in the manner described here, and the nucleotide cannot be removed without loss of protein stability [390].

The GTPase assay needed to be robust enough to be able to compare the activities of a number of different potential inhibitors, including the chrysopaentins. For this reason, the GTPase assay was optimized for EcFtsZ and SaFtsZ so that the end-point production of inorganic phosphate was similar for both proteins (30-40 µmol $P_i$) when the initial amount of GTP in the reaction mixture was kept constant. A GTPase assay with 2 µM SaFtsZ in the presence of 0.25 mM GTP for ten minutes at 23 °C gave almost undetectable levels of phosphate production. Comparing different concentrations (2 µM, 4 µM, and 6 µM) of SaFtsZ protein in the presence of 0.25 mM GTP for up to two hours showed a significantly lower rate of
hydrolysis by SaFtsZ at 23 °C (averaging 0.03, 0.04, and 0.1 µmol GTP hydrolyzed per minute per µmol SaFtsZ, for each of the three concentrations of SaFtsZ, respectively) (Fig. 35C). From this data, it was obvious that the rate of GTP hydrolysis by SaFtsZ was much lower compared to EcFtsZ. The highest rate of hydrolysis was obtained with 6 µM SaFtsZ, and this concentration was used in all subsequent experiments. In addition, the temperature of the assay was increased to 30 °C to attempt to increase the enzymatic activity of SaFtsZ. Under these conditions, the rate of hydrolysis increased to an average of 0.4 µmol GTP per minute per µmol SaFtsZ. Greater than 40 µmol P

was produced when the assay was terminated at 20 minutes (Fig. 35D). The conditions used in all further inhibition assays were 2 µM EcFtsZ with 0.25 mM GTP for 10 minutes at 23 °C and 6 µM SaFtsZ with 0.25 mM GTP for 20 minutes at 30 °C, both in Assay buffer. These conditions consistently gave rise to an absorbance of 1.5-2.3 at 635 nm. Protein concentrations for all assays were determined by a Bradford Assay (BioRad) utilizing a BSA standard curve, since there are no tryptophans in FtsZ and the extinction coefficient cannot be reliably used for protein quantitation.

FtsZ polymerization was studied using transmission electron microscopy (TEM), ultracentrifugation, and light scattering (Fig. 36). Upon addition of 1 mM GTP, 6 µM EcFtsZ formed a network of single stranded protofilaments within minutes in Assay buffer, and these protofilaments were viewed by TEM after fixation with uranyl acetate (Fig. 36B). SaFtsZ protofilaments were not detected under the same conditions (Fig. 36C). Without GTP, EcFtsZ did not form polymers (Fig. 36A). High-speed analytical ultracentrifugation pelleted FtsZ protofilaments under certain conditions. Protein in the pellet and supernatant was analyzed and quantified by SDS-PAGE. Ultracentrifugation showed that 90% of the EcFtsZ mass was
pelletable at high speeds (95k rpm) in a GTP-dependent manner (Fig. 36D), but only in the presence of a minimum of 10 mM calcium and 1 mM GTP. DMSO did not cause precipitation or aggregation of the protein. Under the same conditions, SaFtsZ did not pellet. Higher levels of calcium induced GTP-dependent-pelleting of SaFtsZ, as did the addition of macromolecular crowders such as diethylaminoethyl-dextran (DEAE-dextran) and trimethylamine N-oxide (TMNO) (Fig. 36E). EcFtsZ protofilaments were not detectable by absorbance, but 90° light scattering was effective for tracking the formation of FtsZ protofilaments. Light scattered by EcFtsZ filaments was shown to increase upon addition of 1 mM GTP (Fig. 36F), but these results could not be reproduced with SaFtsZ (data not shown). Addition of 1 mM GTP to 2 µM EcFtsZ led to the formation of stable polymers that persisted over the course of the one hour light scattering experiment. When the concentration of EcFtsZ was increased to 4 µM or 10 µM, polymers were formed to a larger extent, but the GTP present in solution was used up quicker and the polymers began to dissociate over the course of the experiment.

Taken together, these results showed that EcFtsZ formed detectable and stable protofilaments. However, while SaFtsZ must be polymerizing to some extent (in order to hydrolyze one molecule of GTP, a head-to-tail dimer of FtsZ must form [198]), the protofilaments formed by SaFtsZ are not detectable under many of the standard conditions tried here. In fact, in the absence of crowding or stabilizing agents, such as DEAE-dextran and TMNO, conditions for reproducible and measurable polymerization of SaFtsZ are unknown [384]. Due to this fact, we have evaluated our small molecule inhibitors using an in vitro SaFtsZ GTPase assay, but have not been able to develop assays to investigate SaFtsZ polymerization.
4.4 The chrysophlaentins and synthetic fragments inhibit FtsZ GTPase activity

Chrysophlaentin A (11) and F (18), and their synthetic fragments (21 and 22) were tested in the colorimetric GTPase assay described in Section 4.3 for their ability to inhibit the GTP hydrolysis of EcFtsZ and SaFtsZ. Chrysophlaentin A inhibited the GTP hydrolysis activity of EcFtsZ in a dose-dependent manner with an IC$_{50}$ value of 9.9±2.5 µM (Fig. 37A). Compound 11 was less potent against SaFtsZ, but still inhibited GTP hydrolysis in a dose-dependent manner with an IC$_{50}$ of 67±13 µM. Chrysophlaentin F, the symmetrically-linked macrocyclic compound, inhibited GTP hydrolysis by EcFtsZ and SaFtsZ with IC$_{50}$ values of 160±63 µM and 130±23 µM, respectively. The synthetic chrysophlaentin fragment 21 inhibited EcFtsZ and SaFtsZ with IC$_{50}$s of 37±7 µM and 38±9 µM, respectively, while compound 22 was inhibitory with IC$_{50}$s of 160±26 µM and 120±22 µM, against EcFtsZ and SaFtsZ, respectively.

4.5 Chrysophlaentin A specifically inhibits FtsZ polymerization but does not affect tubulin

Transmission electron microscopy (TEM) was employed to visualize the effects of the most potent compound, chrysophlaentin A, on polymerization of FtsZ. In the presence of 1 mM GTP, 6 µM EcFtsZ polymerized within minutes to form a network of linear single-stranded protofilaments (Fig. 36A and 38A). However, when 50 µM chrysophlaentin A was pre-incubated with EcFtsZ before addition of 1 mM GTP, polymerization was completely inhibited (Fig. 38B). Because FtsZ is a structural homologue of tubulin, and some compounds have similar inhibitory effects on both tubulin and FtsZ [391,392], we conducted a tubulin polymerization assay to determine whether chrysophlaentin A also inhibited tubulin. Chrysophlaentin A had no effect on tubulin polymerization at concentrations between 15-150 µM as shown by a turbidometry assay.
measuring microtubule formation by increase in absorbance at 340 nm, where light that is scattered by microtubules is proportional to the concentration of microtubule polymer \cite{393,394} (Fig. 38C). The velocity \(V_{\text{max}}\) for tubulin polymerization was determined in the presence of several agents. The velocity of normal microtubule formation in standard buffer conditions was 24.4 s\(^{-1}\). The velocity in the presence of paclitaxel, a compound that decreases the time necessary for microtubule nucleation and stabilizes polymers was 79.8 s\(^{-1}\), while the velocity in the presence of nocodazole, a compound that inhibits tubulin polymerization and leads to an overall decrease in microtubule mass, was 11.9 s\(^{-1}\). The velocity of tubulin polymerization in the presence of 150 \(\mu\)M chrysophaentin A was 37.0 s\(^{-1}\), which is similar to the velocity in the presence of a 2.5% DMSO vehicle control (36.8 s\(^{-1}\)), and not significantly different from the velocity of the buffer control. Together these results demonstrate that chrysophaentin A is a FtsZ inhibitor that exhibits at least a 15-fold selectivity for FtsZ over tubulin.

4.6 Chrysophaentin A binds to FtsZ and is competitive with GTP\(\gamma\)S

To identify the regions of chrysophaentin A involved in FtsZ binding, Saturation Transfer Difference (STD) NMR \cite{395} spectra of \textit{11} were recorded in the presence of EcFtsZ. Published experimental conditions were optimized for our instrumentation, and samples typically contained a 100-fold excess of chrysophaentin A relative to EcFtsZ with respective concentrations of 1.5 mM and 15 \(\mu\)M. An STD NMR spectrum shows which protons of the ligand (chrysophaentin A) are in direct contact with the protein (EcFtsZ). Two 1D experiments were recorded, an on-resonance and an off-resonance, and were subtracted from one another. The difference spectrum obtained from the subtraction was then compared to a reference spectrum. Signals in the
difference spectrum with the greatest enhancement are in closest contact to the protein, while signals with low to zero enhancement are further away. An expansion of the aromatic region of a representative difference spectrum (red) and control spectrum (black) is presented (Fig. 39A) and shows strongly enhanced and nonoverlapping aromatic and olefinic protons of 11. Normalization of the signal/s of greatest intensity ($\delta_{11} 6.85$) in the difference spectrum to those of the reference spectrum showed signals for the aromatic protons H-3 (100%), H-14 (100%), H-12 (100%), and H-3' (98%) to display the strongest enhancements, while the overlapped signals of the aromatic and olefinic protons H-6' and H-8 showed a combined enhancement of $\sim 50\%$ (Fig. 39B). Thus, when bound to EcFtsZ, the face of chrysophaentin A displaying protons H-3, H-14, H-12, and H-3' (rings A, B, and C) is in closest proximity to the protein. Under the buffer conditions used to prepare these complexes we were unable to observe signals for the remaining two aromatic protons H-12' and H-16', both of which reside on ring D, in either $^1$H or STD NMR spectra.

To probe the mode of binding of chrysophaentin A to FtsZ, competition STD NMR experiments were performed where increasing amounts of a non-hydrolyzable GTP analog, guanosine 5'-O-(3-thiotriphosphate) (GTP$\gamma$S), known to bind to the GTP binding site of FtsZ with high affinity [396], was added to a 100:1 complex of chrysophaentin A:EcFtsZ. Spectra were recorded as described above on samples containing 1.25 mM 11 in the presence of 12.5 $\mu$M FtsZ. Difference spectra were monitored for a change in intensity of signals belonging to either chrysophaentin A or GTP$\gamma$S during the titration. As seen in the spectral expansions showing the aromatic and olefinic region of the difference spectra (Fig. 39C), addition of 0.5 equivalents of GTP$\gamma$S (625 $\mu$M) resulted in a $\sim 50\%$ uniform decrease in intensity for signals belonging to 11.
Moreover, a new STD NMR signal appeared at $\delta_H 5.84$ (H-1′-GTP$\gamma$S) that was assigned to the anomeric proton of the ribose of GTP$\gamma$S (Fig. 39C). Stepwise addition of two additional equivalents of GTP$\gamma$S to the complex further diminished the signal intensities of 11 concomitant with steady increases in signal intensities for the anomeric and guanosine protons of GTP$\gamma$S, H-1′-GTP$\gamma$S and H-8-GTP$\gamma$S. As seen in Figure 39C, by addition of three equivalents of GTP$\gamma$S relative to 11, signals for chrysophaentin A were imperceptible. Thus, chrysophaentin A and GTP$\gamma$S bind the GTP binding site of FtsZ in a competitive manner.

4.7 Z-ring visualization and effects of the chrysophaentins

Once the effects of the chrysophaentins on FtsZ in vitro were established, we sought to characterize the effects of the compound on FtsZ in bacteria in vivo, using fluorescence microscopy. To do this, it was necessary to develop a model system where FtsZ is fluorescently-tagged, the compounds have efficacy, and the bacteria divide normally in the absence of inhibitor. Several groups have worked with fluorescent constructs of FtsZ, but a construct of FtsZ-YFP (known as Venus) under control of a disrupted arabinose promoter and with chloramphenicol (CAM) selection had the properties necessary for our experiments and was kindly provided to us by Dr. Harold Erickson at Duke University [397-399]. The YFP (yellow fluorescent protein) was attached to the C-terminus of FtsZ via a three amino acid linker. This plasmid (pFtsZ-YFP) was transformed into a permeable strain of E. coli (Escherichia coli K12 C600 leu thr lac (thi) galK lpxC::Tn10) (referred to as envA1) [354] via electroporation. Previous work with fluorescent constructs of FtsZ showed fluorescently-tagged FtsZ localized to mid-cell and co-localized with wild-type untagged FtsZ to form normal Z-rings. However, for
this to occur, expression levels of the fluorescent constructs needed to be maintained at 30% of wild-type levels or less [177].

In order to reconstruct the conditions necessary for normal division in the presence of fluorescently-tagged FtsZ in our system, bacteria were electroporated with pFtsZ-YFP and screened on LB agar with 20 µg/mL CAM for clones that successfully acquired arabinose-inducible pFtsZ-YFP (envA1/pFtsZ-YFP). Replicate plates were made, and those clones that expressed yellow fluorescence at mid-cell after a 15 minute induction with 0.4% L-arabinose in LB broth supplemented with 20 µg/mL CAM were harvested and saved as glycerol stocks and frozen at -80 °C. When needed, a glycerol stock was thawed and grown in 15 mL LB broth with 20 µg/mL CAM for approximately 3 hours at 37 °C with shaking at 200 rpm, or until the OD$_{600}$ was ~0.2. The arabinose promoter is tightly regulated, and there is very little background fluorescence from plasmid expression pre-induction (Fig. 40A). Fluorescent protein expression was induced with 0.4% L-arabinose for 30 minutes (Fig. 40B). After 30 minutes, cells were harvested by centrifugation at 3500 rpm, and resuspended in PBS pH 7.4 with 50 µg/mL tetracycline to wash off the arabinose, and maintain selection of the envA1 mutation for permeability. The centrifugation and wash steps were repeated three times, and after the third centrifugation, cells were washed in LB broth and centrifuged a final time. Bacteria were then resuspended in 25 mL LB broth with 20 µg/mL CAM.

At the time of resuspension, almost all bacteria showed yellow fluorescence localized to mid-cell, indicating the presence of a Z-ring composed of both fluorescently-labeled and untagged wild-type FtsZ (Fig. 40C). Small volumes (1 mL each) were removed and subjected to different treatments, including 1% MeOH (as a vehicle control), chrysophaentin A, and
compounds 21 and 22. This extensive washing system and minimal induction time was necessary to maintain cells with a single Z-ring and normal length. Without the wash step, or with longer expression times, E. coli became largely filamentous, with several Z-rings per filaments cell. Filamentation is indicative of a block at some step in division. The filamentous phenotype can be induced by treatment with cephalexin (data not shown), an antibiotic known to cause filamentation in bacteria without affecting Z-ring structure, because it inhibits a specific penicillin-binding protein (PBP3) necessary for new cell wall synthesis during division [400].

Antibacterial assays with the permeable E. coli K12 strain showed that the concentration of compound 22 necessary to inhibit the growth of the bacteria was not significantly different whether analyzed at 1, 2, 4, or 17 hours after treatment. This indicated that the target of compound 22 was sufficiently affected by one hour after treatment, and the time frame chosen for use in this assay was acceptable. The MICs for chrysophaentin A, compound 21, and compound 22 were determined to be ~25 µM, ~125 µM, and ~75 µM, respectively (data not shown). The concentrations chosen to treat envA1/pFtsZ-YFP were 125 µM for chrysophaentin A, 340 µM for 21, and 370 µM for 22. These concentrations are slightly higher than the respective MIC90 for each compound, and were chosen in order to reproducibly determine whether a phenotype existed in a majority of treated cells. The bacteria treated with 1% MeOH had normal Z-rings at 30 minutes (Fig. 40D). These bacteria looked very similar to those immediately after washing, indicating the presence of the fluorescent construct was not affecting the ability of the bacteria to grow and divide. This E. coli strain has a tendency for the daughter cells to not fully separate after division, as shown by the “chain” of four bacteria in the lower panel of Fig. 40D.
After 30 minutes treatment with chrysophaentin A (11) in methanol, the localization of fluorescent FtsZ differed from that of untreated bacteria (Fig. 40E). FtsZ-YFP was no longer localized to mid-cell, and instead appeared dispersed throughout the cytoplasm, and in patches near the membrane. Thirty minutes of treatment with compound 21 showed a more exaggerated but similar phenotype to the treatment with 11 (Fig. 40F). However, after 30 minutes of treatment with 22, there did not appear to be a noticeable disruption in Z-rings present (Fig. 40G).

Bacteria containing the fluorescent construct and treated with vehicle control maintained a single fluorescently-labeled Z-ring visible at 60 minutes (Fig. 40H). The “chain” morphology of these bacteria was even more pronounced at this time point. This morphology shows that the bacteria are dividing, because earlier time-points did not have these chains. Sixty minutes of treatment with chrysophaentin A (11) showed a further extension of the phenotype described, where FtsZ is no longer localized to mid-cell, and instead shows patches dispersed throughout the cell and particularly around the inner membrane (Fig. 40I). This same phenotype is caused by treatment with compound 21 (Fig. 40J), and dispersed fluorescence is visible with treatment by compound 22 (Fig. 40K). The background fluorescence levels detected in bacteria that have not been induced with arabinose, but treated with compounds 21 (Fig. 40L) and 22 (Fig. 40M) is negligible.

Because filamentation is indicative of a block in cell division, and other groups have reported an increase in cell length concurrent with inhibitor application, we measured the size of E. coli at each time-point and with each treatment. Average cell length was calculated and compared using a student’s t-test. At 30 minutes, vehicle-treated envA1/pFtsZ-YFP averaged 4.1
µm, while those treated with chrysophaeatin A (11) averaged 5.2 µm. envA1/pFtsZ-YFP treated with compound 21 averaged 5.2 µm, and envA1/pFtsZ-YFP treated with compound 22 averaged 4.9 µm. The cell size of envA1/pFtsZ-YFP treated with the two synthetic chrysophaeatin fragment analogs were significantly larger (p<0.01 and p<0.05 for compounds 21 and 22, respectively) than the control cells. Chrysophaeatin A increased the cell size relative to the controls, but there was much more variability in the sizes of cells treated with chrysophaeatin A, and thus, the increase is not significant. At 60 minutes, vehicle-treated control cells average 2.9 µm, while those treated with 11 were 4.1 µm, those treated with compound 21 were 7.6 µm, and envA1/pFtsZ-YFP treated with compound 22 were 5.1 µm. At 60 minutes, the cell size in all three treatment groups was significantly larger than the vehicle-treated control cells (p<0.05, p<0.0001, and p<0.005 for compounds 11, 21, and 22, respectively). The trend in increased cell size continued into longer time-points, namely 90 and 120 minutes. At these longer time points, vehicle-treated bacteria had divided so many times that the population of fluorescently-labeled FtsZ within the bacteria was no longer visible because it was split into smaller and smaller proportions within each cell after each division. However, bacteria treated with inhibitors still remained larger in cell size and possessed mislocalized fluorescent FtsZ since division was not occurring and the fluorescent properties were not diluted.

Taken together, these results show that the chrysophaeatins, including both the natural product chrysophaeatin A, and the synthetic fragment mimics, compounds 21 and 22, affect the proper localization of FtsZ in unfixed bacteria. Prior to treatment with inhibitory compounds, almost all bacteria contained one fluorescently-labeled Z-ring localized at mid-cell. Bacteria that only received a methanol treatment were able to grow and divide, and the location of their single
Z-ring was visible at 30 and 60 minutes. On the other hand, bacteria that were treated with the chrysophaentins no longer contained a single well-resolved Z-ring, and were no longer dividing normally. Instead, these bacteria contained patches of FtsZ-YFP visible throughout the cytoplasm and lining the membrane. This indicates two effects on FtsZ: 1) the chrysophaentins are disrupting previously formed Z-rings, since the majority of bacteria contained Z-rings before but not after treatment; and 2) the mis-localization of FtsZ appears to maintain some association with the membrane.

Fluorescently-tagged FtsZ cannot alone support division; wild-type FtsZ also must be present in order for the bacteria to be able to divide. The fact that treatment with the chrysophaentins impaired the ability of the bacteria to divide in the same manner as untreated bacteria indicates the chrysophaentins were also affecting the wild-type FtsZ in the cell, not solely the fluorescently-tagged FtsZ. Chrysophaentin A and compound 21 show very similar phenotypes, while compound 22 was somewhat different in its effects on envA1/pFtsZ-YFP. After 60 minutes, all compounds affected proper Z-ring formation and localization, but at 30 minutes, there did not appear to be a visual effect on envA1/pFtsZ-YFP treated with compound 22. However, these bacteria were larger than control treated bacteria, indicating there still may be an effect. Other groups have reported finding intact Z-rings that appear to be no longer functional in the presence of inhibitors [383], and that may explain the lack of overt phenotype at 30 minutes. However, by 60 minutes in the presence of compound 22, the Z-rings completely dissociate, revealing a phenotype of diffuse fluorescence.
4.8 Discussion

Here we have described a possible mechanism of action for the chrysopaentins and their synthetic fragment analogs. Based on the structural features of the chrysopaentins, we hypothesized that their molecular target could be the bacterial cell division protein, FtsZ. Approximately three dozen different small molecules have been described recently as FtsZ inhibitors. These small molecules fall into six broad structural classes, including guanine derivatives; carboxylic acids; phenol and polyphenolic compounds; benzamides; N-heterocycles; and others, which contain a number of hydrophobic dyes and other unrelated compounds [401]. The loose relatedness between many FtsZ inhibitor structures shows it is entirely plausible to hypothesize a molecular target based on structural features. At the same time, many of these compounds exhibit different modes of inhibition in their interaction with FtsZ, demonstrating that not all FtsZ inhibitors operate in the same manner. In order to explore whether the chrysopaentins inhibit FtsZ, and determine their mode of inhibition, we expressed recombinant EcFtsZ and SaFtsZ. Attempts at expression of heterologous SaFtsZ in E. coli did not yield consistent high yields. Heterologous expression of FtsZ has been shown to be detrimental to the ability of the expressing bacteria to divide when expressing FtsZ from Rhizobium meliloti [402], Brevibacterium lactofermentum [403], Neisseria gonorrhoeae [404], Thermoplasma acidophilum [405], and Corynebacterium glutamicum [406]. The expression of SaFtsZ was not toxic, however, since lowering the expression temperature alleviated some of the problems encountered with over-expression in this system.

Recombinant EcFtsZ was purified and its functions were analyzed by GTPase and polymerization assays. EcFtsZ hydrolyzed approximately 2 µmol GTP/min/µmol protein, and
formed protofilaments that were monitored by TEM, ultracentrifugation, and light scattering. On the other hand, when purified in a similar manner, recombinant SaFtsZ only hydrolyzed approximately 0.4 μm GTP/min/μmol protein, and did not form stably detectable protofilaments. The enzymatic interface of FtsZ is formed by the association of two monomers. The nucleotide binds to the N-terminal domain of one monomer, in the nucleotide binding pocket. The T7-loop on the bottom of a second monomer inserts into the nucleotide binding pocket to activate hydrolysis of the nucleotide. The nucleotide binding pockets of EcFtsZ and SaFtsZ are very similar and well conserved, while the bottom face of the monomer where the T7-loop is located is less conserved. Furthermore, the C-terminal region shares even less identity between EcFtsZ and SaFtsZ. In particular, SaFtsZ contains a series of arginine residues that are not present in EcFtsZ, or B. subtilis FtsZ. These divergences indicate that there may be significant differences in the way EcFtsZ and SaFtsZ are regulated. EcFtsZ which can stably polymerize on its own in vitro and during division may only require other proteins in the cell to localize the Z-ring and prevent association at improper times. On the other hand, SaFtsZ may require associated proteins to facilitate polymerization and/or stabilize polymers as it seems unable to do so on its own in vitro. Furthermore, the divisome complex in S. aureus has not been studied as well as that of E. coli and all the components are not known. The slower rate of GTP hydrolysis and turnover of SaFtsZ may not be surprising, considering S. aureus divides at a much slower rate than E. coli. The filamentation of competent E. coli expressing SaFtsZ further demonstrates that there are significant differences in the way SaFtsZ and EcFtsZ operate, since E. coli cannot properly divide when SaFtsZ is present.
Chrysophyaentin A inhibited the GTP hydrolysis activity of EcFtsZ and SaFtsZ in a dose-dependent manner, and was four-fold more potent against EcFtsZ than SaFtsZ. A similar trend in activities was observed for compound 21, one of the synthetic chrysophyaentin analogs. Chrysophyaentin F (18) and compound 22 showed similar, less potent activities against both EcFtsZ and SaFtsZ. It is interesting to note that chrysophyaentin A inhibited the growth of multiple bacterial strains with MIC<sub>50</sub> values that were considerably lower than the <i>in vitro</i> IC<sub>50</sub> values observed in GTPase assays. This phenomenon has been observed for several other FtsZ inhibitors, including unrelated compounds totarol [407] and OTBA [383]. Because formation of the Z-ring is an initial step in bacterial cell division, this differential activity has been attributed to amplification of the effect of inhibiting FtsZ polymerization. Similar effects are observed with microtubule inhibitors where micromolar concentrations are required for <i>in vitro</i> inhibition of tubulin polymerization, while nanomolar concentrations disrupt microtubule assembly <i>in vivo</i> [408].

Several inhibitors of FtsZ have been shown to be specific for this protein over tubulin. Notably, the competitive nucleotide analog inhibitors are specific for FtsZ [147,409], while known tubulin inhibitors, such as albendazole, colchicines, nocodazole, paclitaxel, 3-methoxybenzamide, and thiabendazole have no effect on FtsZ [125]. Chrysophyaentin A was shown to be specific for FtsZ over tubulin, as treatment of tubulin with chrysophyaentin A had no effect on tubulin polymerization into microtubules. While a similar assay based on turbidometry cannot be done with FtsZ since it forms single-stranded protofilaments instead of the larger aggregate of microtubules, visualization of the FtsZ polymers by TEM is a useful method to determine whether a compound prevents protofilament formation. In the case of chrysophyaentin
A, polymerization of FtsZ was efficiently and completely inhibited. FtsZ immediately polymerizes into protofilaments following GTP binding; hydrolysis of the nucleotide occurs immediately after; and inorganic phosphate release and subunit turnover occurs later [200,201]. Polymerization is dependent on GTP binding, so if polymerization is completely prevented, GTP binding may have also been prevented. These results are supportive of the idea that the chrysophaentins prevent GTP binding by competitively blocking the nucleotide binding pocket.

STD NMR experiments with chrysophaentin A verified this compound to be a competitive inhibitor of FtsZ. Prior to this work, the only known competitive inhibitors of FtsZ were nucleotide analogs. Two small synthetic molecules, unrelated structurally to GTP or the chrysophaentins, may also be competitive, as one is reported to inhibit FtsZ with competitive kinetics, but data to support this claim and the experimental conditions for obtaining it were not provided; and a second has resistant mutants whose mutations map to the nucleotide binding pocket [410]. Several attempts were made to experimentally determine whether compound 21 and 22 were similarly competitive. However, compounds 21 and 22 have limited solubility in the aqueous conditions necessary for STD NMR. We were unable to determine their mode of binding in this manner. There is one report of using a fluorescent GTP analog (mant-GTP) to look at competitive binding, but with this method it is necessary to have highly pure mant-GTP and an empty nucleotide binding pocket [411]. The commercially available mant-GTP (AnaSpec) is highly fluorescent even in aqueous conditions, without binding in a hydrophobic protein pocket, and is not suitable for use. Furthermore, while the FtsZ of certain organisms such as Methanocaldococcus jannaschii can be purified in its apo-form, it cannot be done with E. coli FtsZ [390]. We also attempted to quantify binding with isothermal titration calorimetry
(ITC), but the bound nucleotide ligand of *E. coli* created substantial problems in this method as well.

To further evaluate the mode of binding of chrysopaentin A to FtsZ, a model of chrysopaentin A docked to an *E. coli* FtsZ homology model (based on the 2.1 Å crystal structure of *Pseudomonas aeruginosa* FtsZ [146] since the crystal structure of *E. coli* FtsZ has not been solved) was created. Docking simulations placed chrysopaentin A in the GTP binding site. Moreover, the docked conformation and protein-ligand interactions observed were entirely consistent with the STD NMR results. In this docked model, chrysopaentin A occupies the triphosphate region of the GTP binding site and partially occludes the guanine-binding site as well. The docked model indicates specific amino acids that are within hydrogen bonding distances of the side chain or backbone nitrogen and oxygen atoms of FtsZ. This model can provide a starting point for identifying amino acids that should be mutated in order to further verify the binding region of chrysopaentin A, and the model supports the notion that chrysopaentin A inhibits the GTPase activity and polymerization of FtsZ through binding to the nucleotide binding site in a competitive manner with GTP.

The competition between chrysopaentin A and GTP for the binding site of FtsZ is the potential mechanism of action both *in vitro* and *in vivo*. *In vitro*, the effects are seen as a decrease in the ability of FtsZ to hydrolyze GTP and inhibition of polymerization. *In vivo*, the effects are manifested as a disruption in Z-ring localization and integrity. Bacteria that were treated with the chrysopaentins no longer contained a single well-resolved and properly located Z-ring, as the control bacteria did. Many of the control bacteria possessed a single fluorescently labeled Z-ring at mid-cell, both before and after treatment with vehicle-control solvents. On the
other hand, after treatment with the chrysophaentins, many of the bacteria had patches of fluorescence visible throughout the cytoplasm and lining the membrane. This phenotype, along with the persistence of a larger cell length relative to the control bacteria, show that the chrysophaentins are preventing division in envA1/pFtsZ-YFP. Furthermore, the chrysophaentins disrupt previously formed Z-rings, prevent the formation of new Z-rings, and cause FtsZ to mis-localize and remain near the membrane, rather than self-associate at mid-cell in a ring structure. The chrysophaentins do not cause overt filamentation, as seen by other groups in the presence of inhibitors [412], but due to the fact that filamentation can be caused by a number of different disruptions, such as DNA damage [178] or inhibition of penicillin-binding protein 3 [400], the lack of extreme filamentation is not troubling. The chrysophaentins are inhibiting division, as seen by the fact that treated bacteria remain as larger singles with visibly mis-localized fluorescent FtsZ, while control-treated bacteria form a “chain” with the recently divided daughter cells attached to one another, and their division dilutes the fluorescent FtsZ to negligible levels by two hours.

4.9 Materials and methods

4.9.1 Protein purification

Escherichia coli FtsZ was purified as described in Section 4.3. Briefly, E. coli BL21-DE3 (Gold) was grown in LB with AMP and FtsZ overexpression was induced with 1 mM IPTG for 5 hours at 37 °C. Cells were harvested by centrifugation, lysed in Lysis buffer (50 mM Tris pH 8.0, 100 mM KCl, 1 mM EDTA, 1% benzamidine (for protease inhibition), Roche Protease Cocktail inhibitor, 0.1% β-mercaptoethanol, 1 mM MgSO4, adapted from Beuria et al. [209]),
sonicated, and mechanically broken. Insoluble debris was removed by centrifugation, and soluble protein was precipitated with 30% ammonium sulfate. Protein was centrifuged and resuspended in a low salt buffer (50 mM Tris pH 7.4, 25 mM KCl, 5 mM MgCl₂, 10% glycerol) and purified by two chromatography steps. The first employed a ResQ anion exchange column, and the second a Superdex gel filtration column. Protein was eluted from the gel filtration column in 50 mM Tris pH 7.4, 50 mM KCl, 1 mM EDTA, and 10% glycerol. Protein was quantified by Bradford assay, and frozen at -80 °C until use.

SaFtsZ was purified in a similar manner, except protein expression was induced with addition of 1 mM IPTG at 30 °C for five hours.

4.9.2 Protein quantification

EcFtsZ and SaFtsZ concentrations were quantified by Bradford Assay (Bio-Rad), using a standard protocol performed in the 250 µL microplate assay format according to the manufacturer’s instructions. The linear range of the assay with a BSA standard is 125-1000 µg/mL. A standard curve with BSA was constructed by diluting a 2 mg/mL BSA standard reagent. Five microliters of each standard and unknown sample were pipetted into separate microplate wells. 250 µL of the 1X dye reagent (Quick Start Bradford Reagent) was added to each well, and mixed on a microplate mixer. The plate was incubated at room temperature for five minutes, then the absorbance was measured on a Molecular Devices plate reader at 595 nm. The standard curve constructed was fit with a linear line of best fit, which gave the equation $y=0.0006x+0.0197$, where $y$ is the absorbance at 595 nm, and $x$ is the concentration in µg/mL.
4.9.3 GTPase activity assay

GTPase activity of recombinant EcFtsZ and SaFtsZ was performed as described in Section 4.3. Briefly, the assay was measured in 96-well plate format using a colorimetric assay that measures inorganic phosphate production. Solutions containing EcFtsZ (2 μM) and SaFtsZ (6 μM) in Assay buffer (50 mM MES, pH 6.0, 100 mM KCl, and 5 mM MgCl₂) were treated with serial dilutions of 11, 18, 21, and 22 (final concentrations 0-600 µM in 5% DMSO or 5% MeOH), followed by addition of GTP (Roche) added to a final concentration of 0.25 mM at 23 °C for EcFtsZ and 30 °C for SaFtsZ. Reactions were quenched 10 min (EcFtsZ) or 20 min (SaFtsZ) later by addition of malachite green/ammonium molybdate (P, ColorLock Gold, Innova Biosciences) and the color developed for 30 min. Inorganic phosphate was quantified by absorbance at 635 nm, and compared to a standard curve with the equation y=0.057x+0.1186. Controls included DMSO and MeOH (no significant difference in GTPase activity up to 8% DMSO and 7% MeOH); FtsZ alone in buffer plus compound; FtsZ alone in buffer; and GTP alone in buffer.

4.9.4 Polymerization assays

Protofilament formation of FtsZ was assessed by polymerization assays.

Transmission electron microscopy (TEM)

Aliquots of stock solutions were added to Assay buffer (final volume 50 µL) to give a final concentration of recombinant EcFtsZ (6 μM) or SaFtsZ (10 μM) with 5% DMSO, or 50 μM chrysopaentin A in 5% DMSO (with EcFtsZ only), for 2 min at room temperature, followed by addition of 1 mM GTP (Cytoskeleton Inc.). Following an additional 5 min incubation period at
room temperature, aliquots (5 μL) were adsorbed onto carbon films on lacey carbon supports on
400 mesh copper grids, rinsed with H2O, and exposed to 3% uranyl acetate for 5 min for
negative staining. Images were acquired with an FEI Morgani transmission electron microscope,
operating at 80 kV, and equipped with an AMT Advantage camera, at 44 000× magnification.

**Analytical ultracentrifugation**

EcFtsZ (6 μM) or SaFtsZ (10 μM) was diluted in Polymerization buffer (50 mM MES
pH 6.0, 100 mM KCl, 10 mM MgCl2) and incubated at room temperature for 3 min. 1 mM GTP
was added, and CaCl2, if required (see Figure 36) to a total volume of 50 μL. The solution was
incubated at 37 °C for two minutes, and centrifuged at 95k rpm for 20 minutes in a Beckman
Coulter TLA 100 rotor. The supernatant was removed, and the pellet was resuspended in 50 μL.
10 μL each of the supernatant and resuspended pellet were removed and analyzed for the
presence of FtsZ by SDS-PAGE, and compared to 10 μL of a 6 μM EcFtsZ or 10 μM SaFtsZ
solution. The protocol described here is based on [209] and [165].

**Light scattering**

EcFtsZ (2, 4, or 10 μM) in Polymerization buffer was incubated in the light scattering
cuvette for 10 minutes. Readings were taken until the baseline stabilized. Then 1 mM GTP was
added, and readings immediately resumed. The polymerization reaction was monitored by 90°
light scattering in a spectrofluorometer at room temperature with both the excitation and
emission wavelengths set at 350 nm and a slit width of 1.5 nm. The net change in light
scattering following nucleotide addition was plotted as a function of time.
4.9.5 Tubulin polymerization assay

A tubulin polymerization assay was conducted based on the manufacturer’s protocol (Cytoskeleton Inc.), and was capable of assessing inhibitors to the nucleation, growth, and steady state equilibrium phases of microtubule polymerization. The assay volume was 100 μL and utilized a spectrophotometer path length of 0.5 cm in a half area microtiter plate. The polymerization reaction was started by the increasing the temperature from 4 °C to 37 °C upon transfer of the reaction mixture to pre-warmed microtiter wells. The microtiter plate was prewarmed to 37 °C for 30 minutes prior to the beginning of the assay. Cold (4 °C) tubulin polymerization buffer was made with a final concentration of 80 mM PIPES pH 6.9, 2 mM MgCl₂, 0.5 mM EGTA, 15% glycerol, and 1 mM GTP. General tubulin buffer (80 mM PIPES pH 6.9, 2 mM MgCl₂, 0.5 mM EGTA) was warmed to room temperature. Controls included buffer only wells, the known stabilizing agent paclitaxel (10 μM final concentration), and inhibitor nocodazole (20 μM final concentration). Tubulin was thawed in a room temperature water bath, then kept on ice. The tubulin was diluted with ice cold tubulin polymerization buffer to a final concentration of 3 mg/mL. 100 μL of diluted tubulin in tubulin polymerization buffer was added to the wells of the microtiter plate. The microtiter plate was immediately place in the Molecular Devices plate reader, at 37 °C, and measurements were recorded at 340 nm as a function of time (60 minutes).

4.9.6 STD NMR

Saturation transfer difference (STD) NMR experiments were recorded with the carrier set at -1 ppm for on-resonance irradiation and 40 ppm for off-resonance irradiation. Control spectra
were recorded under identical conditions on samples containing free compound 11 to test for artifacts. Selective protein saturation (2 s) was accomplished using a train of 50 ms Gauss-shaped pulses, each separated by a 1 ms delay, at an experimentally determined optimal power (49 dB on our probe); a T1E filter (30 ms) was incorporated to suppress protein resonances. Experiments were recorded using a minimum of 1024 scans and 32K points. On- and off-resonance spectra were processed independently, and subtracted to provide a difference spectrum. GTPγS was obtained from Sigma.

4.9.7 Fluorescence microscopy

The fluorescent plasmid provided to us by Dr. Erickson at Duke University was derived from the pBAD plasmid, which has an arabinose-inducible promoter. *FtsZ* genes were blunt cloned into the SmaI site. The yellow fluorescent protein (YFP) was the variant Venus [413], which is superior to other green fluorescent proteins for fusion to *FtsZ* [414].

For light microscopy, *envA1/pFtsZ-YFP* were grown in Luria broth (LB)-based medium containing 20 µg/mL chloramphenicol (Sigma) until the optical density at 600 nm reached 0.2, then were induced with 0.4% arabinose (Sigma) for 30 minutes (see Section 4.7). Cells were visualized on a polylysine coated slide with differential interference contrast and fluorescence microscopy using a Zeiss LSM700 confocal scanning laser unit with an inverted microscope (AXIO Observer.Z1) with a 100X objective, 1.3 numerical aperture lens. Fluorescent wavelengths utilized the laser set to excite at 488 nm, and emission filters at 558 nm. Images were acquired through the microscope software package.
4.10 Acknowledgments.

I would like to thank Dr. Alberto Plaza for his work on the STD NMR experiments and the docked homology model. I also thank Kent Thurber and Rob Tycko for assistance with and use of the Morgani TEM, Dan Sackett for help with and use of light scattering instrumentation, Nancy Dwyer and John Hannover for training on and use of the Zeiss microscope, Paul Dunman for \textit{S. aureus} strains, Katherine Young for \textit{envA1 E. coli}, William Margolin for the EcFtsZ overexpression plasmid, and Yaodong Chen and Harold Erickson for the fluorescent EcFtsZ-YFP plasmid. This work was supported by the NIH Intramural Research Program (NIDDK), and the Intramural AIDS Targeted Antiviral Program, Office of the Director, NIH (C.A.B.), and the Intramural AIDS Research Fellowship, Office of the Director, NIH (J.L.K.).
### 4.11 Figures and figure legends

**Figure 32.** Sequence alignment of *E. coli* FtsZ (NCBI Reference Sequence NP_285791) with *S. aureus* FtsZ (NCBI Protein Database Accession No. AAA16512). FtsZ is a conserved protein across bacterial species, but there are amino acid changes between the two proteins. Sequence alignment was performed by the SIM tool available on the ExPASy Proteomics Server (https://www.expasy.ch/cgi-bin/sim.pl?prot). The comparison matrix is BLOSUM62 with no gap penalties. Red asterisks indicate identical amino acids between the two sequences. Residues reported to be involved in binding the nucleotide are highlighted in yellow.

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<td>ECFZ</td>
<td>TVVI-GT-SLDPDM-NDELRV-TVVATG-IGMD-KRP------E----IT-----LV-T--N</td>
<td>SAFZ</td>
<td>MI-FGTGV-INEPEQ-DEI-VVTVIATGF-------DK-PTSHGRKGS-TGPGTS-VNTSSN</td>
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<td>ECFZ</td>
<td>--KQVQQPMTRYQQHMA-PLTQEQKPVKVKN---DNAPQ-T-A-------KEPDYL</td>
<td>SAFZ</td>
<td>ATSK----------D-E----SF-T---------S--NSS-NA-QATDSVSRHTTKE-D-</td>
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<td>ECFZ</td>
<td>DIPAPLR-KQAD----------</td>
<td>SAFZ</td>
<td>DIPFSIRNRE-ERRSRRTT</td>
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Figure 33. Competent *E. coli* BL21-DE3(Gold) expressing SaFtsZ. While some bacteria remain normal, many become filamentous due to the heterologous protein expression. The presence of SaFtsZ is detrimental to normal *E. coli* division. These light microscope images were acquired at 20x magnification. White arrowheads indicate an example of filamentous *E. coli* in each panel. The black arrowhead in the top left image shows an example of normal *E. coli* cell size. Scale bars are shown in each image (50 µm).
Figure 34. Purity and molecular weight of EcFtsZ and SaFtsZ. (A) Fractions of EcFtsZ analyzed on a 10-15% gradient SDS-PAGE gel (left) and mass spectrum showing the molecular weight of EcFtsZ (experimental 40326 kDa, theoretical 40323.9 kDa) (right). (B) Fractions of SaFtsZ analyzed on a 10-15% gradient SDS-PAGE gel (left) and mass spectrum showing the molecular weight of SaFtsZ (experimental 41037 kDa, theoretical 41036.9 kDa) (right). (C) Gels showing the expression levels of SaFtsZ (left), and purification of SaFtsZ using anion exchange (center) followed by gel filtration (right).
Figure 35. GTPase activity assays of EcFtsZ and SaFtsZ. (A) Phosphate standard curve showing correlation between µmol phosphate in each well and absorbance at 635 nm. (B) GTPase activity assay of 2 µM EcFtsZ with 0.25 mM GTP at 23 °C for 0-30 min. (C) GTPase activity assay of 2 µM (black), 4 µM (blue), and 6 µM (red) SaFtsZ with 0.25 mM GTP at 23 °C for 0-120 min. (D) GTPase activity assay of 6 µM SaFtsZ with 0.25 mM GTP at 30 °C for 0-45 min.
Figure 36. Polymerization experiments with EcFtsZ and SaFtsZ.

(A) 6 µM EcFtsZ in Assay buffer (50 mM MES pH 6.0, 100 mM KCl, 5 mM MgCl₂), visualized by TEM at 44000x magnification, with negative staining, black scale bar is 100 nm. (B) 6 µM EcFtsZ with 1 mM GTP in Assay buffer, visualized by TEM at 44000x magnification, with negative staining, black scale bar is 100 nm. (C) 10 µM SaFtsZ with 1 mM GTP in Assay buffer, visualized by TEM at 44000x magnification, with negative staining, black scale bar is 100 nm.

(D) 10 µM EcFtsZ in Polymerization buffer (50 mM MES pH 6.0, 100 mM KCl, 10 mM MgCl₂) analyzed by ultracentrifugation. Each gel panel contains one lane with a protein standard marker (15-150 kDa Novagen Perfect Protein). Lanes labeled with an S are supernatant, P are pellet, and L are 10 µM EcFtsZ loading controls. Additives to solution prior to centrifugation are labeled by number in the right panel. EcFtsZ only pellets in the presence of 10 mM CaCl₂ and 1 mM GTP, and is not precipitated by DMSO. (E) 10 µM SaFtsZ in Polymerization buffer analyzed by ultracentrifugation. Each gel panel contains one lane with a 15-150 kDa protein standard marker. Lanes labeled with an S are supernatant, P are pellet. Additives to solution prior to centrifugation are labeled by number in the right panel. SaFtsZ only pellets partially in the presence of high calcium and crowding agents.

(F) Polymerization of EcFtsZ monitored by light scattering. 2 µM (red), 4 µM (blue), and 10 µM (black) EcFtsZ were incubated at room temperature in polymerization buffer in the light scattering cuvette, and monitored until the baseline stabilized. At time 0, 1 mM GTP was added to the cuvette, and the polymerization reaction monitored for one hour. 2 µM EcFtsZ formed stable polymers that persisted for one hour, while 4 µM and 10 µM EcFtsZ both polymerized to a greater extent, but quickly used up the available GTP, and the polymers began to dissociate over the hour (4 µM) and by 15 minutes (10 µM).

Figure on next page.
Figure 36. Polymerization experiments with EcFtsZ and SaFtsZ (legend on previous page).
Figure 37. GTPase inhibition assay curves for EcFtsZ and SaFtsZ. (A) Chrysophaentin A (11) inhibits the GTP hydrolysis activity of EcFtsZ and SaFtsZ in a dose-dependent manner with IC$_{50}$ values of 9.9±2.5 µM and 67±13 µM, respectively. (B) Chrysophaentin F (18) inhibits the GTPase activity of EcFtsZ and SaFtsZ with IC$_{50}$ values of 160±63 µM and 130±23 µM, respectively. (C) Compound 21 inhibits the GTP hydrolysis activity of EcFtsZ and SaFtsZ in a dose-dependent manner with IC$_{50}$ values of 37±7 µM and 38±9 µM, respectively. (D) Compound 22 inhibits the GTPase activity of EcFtsZ and SaFtsZ with IC$_{50}$ values of 160±26 µM and 120±22 µM, respectively.
Figure 38. The effect of chrysophaentin A on FtsZ and tubulin polymerization. (A) 6 µM EcFtsZ polymerizes in Assay buffer upon addition of 1 mM GTP to form linear, single-stranded protofilaments. Protofilaments are visualized by TEM at 44000x magnification with negative staining. (B) In the presence of 50 µM chrysophaentin A, EcFtsZ polymerization is completely inhibited. (C) Tubulin polymerization was monitored by increase in absorbance at 340 nm. 10 µM paclitaxel increases the velocity (79.8 s\(^{-1}\)) and extent of tubulin polymerized, while 20 µM nocodazole decreases both velocity (11.9 s\(^{-1}\)) and polymer mass. 150 µM chrysophaentin A has no effect on tubulin polymerization, and the velocity (37 s\(^{-1}\)) is not different from the 2.5% DMSO-buffer control (36.8 s\(^{-1}\)).
Figure 39. STD NMR spectra of chrysophaentin A (11) and EcFtsZ. (A) Reference (black) and STD NMR difference (red) spectra of chrysophaentin A in complex with EcFtsZ. Spectral expansion shows nonoverlapped aromatic and olefinic signals of 11 displaying the strongest enhancements upon binding to EcFtsZ. Overlaid spectra were normalized to the signal for H-3 ($\delta_H$ 6.85), which gave the strongest enhancement. (B) Structure of chrysophaentin A (11) with STD enhancements overlaid (H-3 100%; H-14 100%; H-12 98%; H-3' 98%; H-8 50%; H-6' 50%; H-6 40%). (C) Competition of chrysophaentin A and GTP$\gamma$S binding to EcFtsZ by STD NMR. Expanded $^1$H STD NMR spectrum of chrysophaentin A (1.25 mM) in the presence of EcFtsZ (12.5 µM). Addition of 0.5, 1.0, 2.0, and 3.0 equivalents of GTP$\gamma$S (final concentrations of GTP$\gamma$S were 625 µM, 1.25 mM, 2.5 mM, and 3.75 mM, respectively) are shown below. Signals for chrysophaentin A protons are clearly decreasing with each addition, while signals for GTP$\gamma$S H-8 and H-1' are increasing. I thank Dr. Alberto Plaza for performing the STD NMR experiments.
Figure 40. Fluorescence microscopy of envA1 E. coli containing an arabinose inducible plasmid encoding FtsZ-YFP (pFtsZ-YFP). All panels show two images, one above the other; the left image is fluorescence only (ex. 488, em. 558); right image is fluorescence overlaid on DIC. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner. White arrowheads indicate examples of intact Z-rings.

Figure 40 A-C: Optimized conditions for fluorescent Z-ring visualization in envA1/pFtsZ-YFP (page 178)

Figure 40 D-G: Treatment of envA1/pFtsZ-YFP with inhibitors or solvent control for 30 minutes (page 179).

Figure 40 H-K: Treatment of envA1/pFtsZ-YFP with inhibitors or solvent control for 60 minutes (page 180).

Figure 40 L-M: Treatment of envA1 E. coli with inhibitors (page 181).

Figures on next pages.
Figure 40 A-C: Optimized conditions for fluorescent Z-ring visualization in envA1 E. coli transformed with the FtsZ-YFP plasmid. All panels show two images, one above the other; the left image is fluorescence only (ex. 488, em. 558); right image is fluorescence overlaid on DIC. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner. White arrowheads indicate examples of intact Z-rings. (A) envA1/pFtsZ-YFP prior to induction. (B) envA1/pFtsZ-YFP induced with 0.4% L-arabinose for 30 minutes. (C) envA1/pFtsZ-YFP induced with 0.4% arabinose for 30 minutes, then washed in PBS with 50 µg/mL tetracycline. Additional caption information on page 177.
Figure 40 D-G: Treatment of envA1/pFtsZ-YFP with inhibitors or solvent control for 30 minutes. Bacteria were transformed with pFtsZ-YFP, induced with 0.4% arabinose for 30 minutes, and then washed in PBS with 50 µg/mL tetracycline. envA1/pFtsZ-YFP were then grown for an additional 30 minutes in LB supplemented with 20 µg/mL chloramphenicol in the following conditions: (D) 1% MeOH as a vehicle-control; (E) 125 µM chrysophaentin A in 1% MeOH; (F) 340 µM compound 21 in 1% MeOH; and (G) 370 µM compound 22 in 1% MeOH. Additional caption information on page 177.
Figure 40 H-K: Treatment of *envA1/pFtsZ-YFP* with inhibitors or solvent control for 60 minutes. Bacteria were transformed with pFtsZ-YFP, induced with 0.4% arabinose for 30 minutes, and then washed in PBS with 50 µg/mL tetracycline. *envA1/pFtsZ-YFP* were then grown for an additional 60 minutes in LB supplemented with 20 µg/mL chloramphenicol in the following conditions: (H) 1% MeOH as a vehicle-control; (I) 125 µM chrysophaentin A in 1% MeOH; (J) 340 µM compound 21 in 1% MeOH; and (K) 370 µM compound 22 in 1% MeOH. Additional caption information on page 177.
**Figure 40 L-M:** Treatment of *envA1 E. coli* with inhibitors. As controls, bacteria were treated with compounds 21 and 22 for 30 minutes to visualize any background fluorescence. All panels show two images, one above the other; the left image is fluorescence only (ex. 488, em. 558); right image is fluorescence overlaid on DIC. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner. (L) *envA1 E. coli* treated with compound 21. (M) *envA1 E. coli* treated with compound 22. Additional caption information on page 177.
Chapter 5
Biological activity of commercial FtsZ inhibitors

5.1 Abstract

There have been several dozen small molecules described as FtsZ inhibitors, but to date, no crystal structure of FtsZ with a non-nucleotide inhibitor has been solved and released. Thus, many gaps in knowledge remain about where small molecule inhibitors bind to FtsZ, whether the binding site correlates to effect on polymerization, and how inhibitor structure plays a role in mechanism of action and/or mode of inhibition. The previous chapter described the chrysophaentins and two synthetic analogs as FtsZ inhibitors, and in particular, chrysophaentin A inhibited FtsZ polymerization and was competitive with GTP for the nucleotide binding pocket. It is difficult to elucidate how the chrysophaentins fit into the larger picture of FtsZ inhibitors without comprehensive studies on multiple compounds. The work here describes in-depth studies on three commercially available FtsZ inhibitors using the experimental set-up and conditions developed in our lab for use with the chrysophaentins. This work provides a broader understanding on the effects of perturbations of FtsZ by small molecules; while at the same time underlining the complexities innate to targeting FtsZ for antibacterial development.

5.2 Background

While there is some structural similarity among certain classes of FtsZ inhibitors, it is largely unknown whether there is any correlation between mode of inhibition and structure. This gap in knowledge is due to the fact that extensive studies on multiple FtsZ inhibitors have not been completed. Several recent reviews have highlighted the fact that for the field of FtsZ
inhibition to move toward development of clinical inhibitors, more comprehensive studies need to be completed [401,415]. Similar to tubulin inhibitors, FtsZ inhibitors can be classified into two broad mechanistic categories: those that inhibit FtsZ polymerization, and those that inhibit FtsZ depolymerization, or stabilize the polymers. Affecting FtsZ polymerization in either manner changes the polymerization dynamics and leads to a change in the rate of GTP hydrolysis, usually manifested as a decrease.

Within these two main categories, there are many ways of further classifying FtsZ inhibitors. The specific mode of inhibition can be described by effects on GTP hydrolysis rates, Z-ring formation and integrity, antibacterial activities, and binding site. Comprehensive data in the literature on all these effects is sparse, and information on only some of the effects of a small molecule FtsZ inhibitor does not allow one to predict the remaining effects with any certainty. This disparity arises because of the many unanswered questions in the field. For instance, is there any correlation between mode of FtsZ inhibition and whether a compound is bacteriostatic or bactericidal to the microorganism? Do the \textit{in vivo} effects of a compound on Z-ring localization and integrity reveal anything about \textit{in vitro} effects on FtsZ polymerization, and \textit{vice versa}? How many drug binding pockets does FtsZ have, and does that correlate with mode of inhibition? Are all polymerization inhibitors competitive? What features of a compound allow it to be specific for FtsZ, and not affect tubulin? Will potent FtsZ inhibitors be specific for certain related organisms, or can broad-spectrum inhibitors be found? Currently, most FtsZ inhibitors have come from screening efforts of natural products and chemical libraries, and targeted hypothesis-driven \textit{in vitro} assays. Discovering the answers to some of the questions in this
A paragraph could allow for rational design of specific, potent FtsZ inhibitors with predictable modes of inhibition.

In order to investigate how the chrysopaentins fit into the larger picture of FtsZ inhibitors, we chose a selection of commercially available FtsZ inhibitors to study using the same conditions and experimental design used for the chrysopaentins and synthetic chrysopaentin fragments. Commercially available inhibitors that were purchased included zantrin Z1 (23) and zantrin Z3 (24) [379], berberine (25) [380,416], OTBA (26) [383], and curcumin (27) [417,418] (Fig. 41). Zantrin Z1 and berberine were chosen because of their structural similarities to the chrysopaentins, and all three compounds are FtsZ polymerization inhibitors. We reasoned comparing the activities of these three compounds would provide information on whether structure plays a role in mode of FtsZ inhibition, and whether all polymerization inhibitors generally operate in the same manner and are competitive with the nucleotide. On the other hand, compounds 24 (zantrin Z3) and 26 (OTBA) were chosen because they stabilize FtsZ polymers. Curcumin (27) was chosen since it was reported to increase the rate of GTP hydrolysis while inhibiting polymerization, the only E. coli FtsZ inhibitor reported to have this effect. There is another class of compounds reported to increase the GTP hydrolysis rate of FtsZ, but those particular compounds are specific for Mycobacterium tuberculosis and M. tuberculosis FtsZ [419]. Initial experiments with these five inhibitors revealed that OTBA was not soluble enough for use in the GTPase assays, and curcumin reacted in a detrimental way with the malachite green reagent used in the assay. Thus, in-depth studies were only completed on zantrins Z1 and Z3, and berberine.
5.3 Zantrin Z1

The original publication describing the small molecule zantrin Z1 (23) as a FtsZ inhibitor found that 23 was a broad-spectrum antibacterial agent that inhibited the growth of *E. coli* with an MIC of 20 µM and was more potent against Gram-positive organisms, inhibiting both *S. aureus* and MRSA with an MIC of 2.5 µM. Compound 23 decreased the GTPase activity of EcFtsZ in a dose-dependent manner with an IC₅₀ of 5 µM, and similarly affected *M. tuberculosis* FtsZ with an IC₅₀ of 55 µM. Compound 23 destabilized EcFtsZ polymers, and *in vivo*, perturbed Z-ring formation and integrity. In experiments utilizing an anti-FtsZ antibody and a fluorescent secondary antibody, some bacteria treated with 23 were characterized by a mislocalization of FtsZ to the poles of the cell rather than mid-cell [379]. While this paper described an early effort in the investigation of FtsZ inhibitors, some areas were not addressed, such as where zantrin Z1 binds, whether it has an effect on tubulin, and whether zantrin Z1 was bactericidal or bacteriostatic.

To address some of the remaining questions, we tested zantrin Z1 in antibacterial assays, evaluating for MIC₅₀, MIC₉₀, and the minimum bactericidal concentration (MBC). Zantrin Z1 inhibited the growth of *S. aureus* with an MIC₅₀ of 0.58±0.2 µM (Fig. 42A). Its MIC₉₀ was 1 µM in a microbroth dilution assay performed with two-fold serial dilutions of inhibitor. When aliquots of the treated *S. aureus* were plated out on fresh agar, it was found that zantrin Z1 was bacteriostatic at its MIC₉₀, since many colonies were observed on plating. At concentrations of 10 µM and above, zantrin Z1 was bactericidal; thus, the MBC was 10 µM. Zantrin Z1 also inhibited the growth of MRSA with an MIC₅₀ of 0.48±0.1 µM, and MDRSA with an MIC₅₀ of 0.18±0.03 µM (Fig. 42A). Zantrin Z1 was indeed broad-spectrum, as it inhibited the growth of
E. coli with an MIC$_{50}$ of 7.8±3.2 µM (Fig. 42B). In a growth inhibition assay with permeable E. coli, zantrin Z1 was even more potent, and inhibited envA1 E. coli with an MIC$_{50}$ of 1.7±0.7 µM (Fig. 42B).

In vitro enzymatic assays with recombinant FtsZ (purified as described in Section 4.3) showed that zantrin Z1 inhibited the GTPase activity of EcFtsZ with an IC$_{50}$ of 4.8±1.7 µM (Fig. 43A), which is very similar to the reported value. We also tested zantrin Z1 for the ability to inhibit the GTPase activity of SaFtsZ, and found 23 inhibited the hydrolysis activity of SaFtsZ with an IC$_{50}$ of 2.4±0.7 µM (Fig. 43A). Interestingly, under certain conditions, zantrin Z1 increased the rate of GTP hydrolysis of SaFtsZ relative to a DMSO control in a dose-dependent manner. This result was unexpected, and further experimentation was required to provide an explanation as to what conditions caused this increase. We found that the increased GTP hydrolysis correlated to the presence of a truncated SaFtsZ that “naturally” formed because of cellular proteases. To verify the correlation between increased GTPase activity in the presence of zantrin Z1 and the truncated form of SaFtsZ, we created a mutated SaFtsZ by inserting a stop codon via site-directed mutagenesis at position 370. This mutated SaFtsZ was missing the last twenty amino acids, corresponding to the same truncation spot as the proteolytic cleavage site. The mutated SaFtsZ (abbreviated SaFtsZ-20) hydrolyzed GTP at a similar rate as wild-type SaFtsZ (Fig. 43B – black line). When SaFtsZ-20 was incubated with 23 prior to addition of GTP, we found that higher concentrations of zantrin Z1 increased the amount of GTP hydrolyzed in 30 minutes at 30 °C relative to the control (Fig. 43B – blue line). This is indirect evidence that the last twenty amino acids play a role in either the zantrin Z1 binding pocket or modulating its activity. Several attempts were made to further characterize the binding of 23, but this
compound had limited solubility in the stricter aqueous conditions necessary for STD NMR and ITC, and thus, we were unable to use these methods.

To evaluate the effects of **23** on FtsZ polymerization, we used transmission electron microscopy (TEM). Upon addition of 1 mM GTP, 6 µM EcFtsZ forms a network of protofilaments visible by TEM upon fixation with uranyl acetate (Fig. 44A). Addition of 240 µM zantrin Z1 completely inhibited EcFtsZ polymerization (Fig. 44B). We then tested whether this compound had a similar effect on tubulin in a polymerization assay, and found that this same concentration of zantrin Z1 (240 µM) decreased both the rate of tubulin polymerization ($V_{\text{max}}$ 5.13 s$^{-1}$ in the presence of **23**, and 35.9 s$^{-1}$ for 2% DMSO buffer control) and the extent of tubulin polymerized (Fig. 44C). To confirm whether tubulin inhibition resulted in effects on eukaryotic cells, zantrin Z1 was tested in a cytotoxicity assay with HCT-116, a human colorectal cancer cell line, and 4T1, a mouse metastatic breast cancer cell line. Compound **23** was cytotoxic to both eukaryotic cancer cell lines with an IC$_{50}$ of 5.1±0.7 µM against HCT-116, and an IC$_{50}$ of 24±5 µM against 4T1.

The cellular structure of Z-ring formation in the presence of zantrin Z1 was investigated in vivo with envA1/pFtsZ-YFP (as described in Section 4.7). Even though **23** inhibited the growth of wild-type *E. coli*, we chose to use permeable envA1 *E. coli* so that the experimental setup was the same as that used to investigate the effects of the chrysophaentins. Treatment with 4.9 µM (~3x envA1 *E. coli* MIC$_{50}$) zantrin Z1 for 30 and 60 minutes showed a mislocalization of the FtsZ fluorescence (Fig. 45). At 30 minutes, FtsZ-YFP was no longer localized to mid-cell in a ring, as observed for the DMSO-treated controls (Fig. 45A), and instead appeared as spiral-shaped structures radiating to the cell poles (Fig. 45B). By 60
minutes, most of the fluorescence was localized to the polar region of cells treated with 23 (Fig. 45C). Compound 23 had no inrinsic fluorescence at the wavelengths utilized for fluorescence microscopy (Fig. 45D). Average cell length was measured at each time point and compared using a student’s t-test. At 30 minutes, vehicle-treated control cells averaged 4.1 µm, while those treated with zantrin Z1 averaged 4.5 µm. At 60 minutes, vehicle-treated control cells average 2.9 µm, while those treated with zantrin Z1 were 4.6 µm. The increased cell size of envA1/pFtsZ-YFP treated with zantrin Z1 for 60 minutes was significantly larger (p<0.0001) than the control cells.

5.4 Zantrin Z3

From the same chemical library screening effort that identified zantrin Z1 (23) as a FtsZ polymerization inhibitor, another compound, zantrin Z3 (25) was found to stabilize polymers formed by FtsZ. Zantrin Z3 only affected Gram-positive organisms, inhibiting S. aureus and MRSA with MICs of 5 µM and 10 µM, respectively, but had no effect on E. coli up to 80 µM. Compound 25 inhibited the GTPase activity of EcFtsZ with an IC50 of 15 µM, and was less potent against M. tuberculosis FtsZ with an IC50 of 50 µM. In vivo, Z-ring formation and integrity was perturbed [379]. Similar to 23, many aspects of FtsZ inhibition were not addressed, including binding site, tubulin cross-reactivity, and bacteriostatic or bactericidal effects.

To further contribute to knowledge of the activity of zantrin Z3, we tested 25 in antibacterial assays, evaluating for MIC50, MIC90, and the MBC. Zantrin Z3 inhibited the growth of S. aureus with an MIC50 of 2.4±1.2 µM (Fig. 46A). Its MIC90 was 7.3 µM in a microbroth dilution assay using two-fold serial dilutions of the inhibitor. When aliquots of the treated
bacteria were plated out on fresh agar, it was found that zantrin Z3 was bactericidal at this concentration, therefore, the MBC was 7.3 µM. Compound 25 also inhibited the growth of MRSA with an MIC50 of 1.8±1 µM, and MDRSA with an MIC50 of 2.0±1 µM (Fig. 46A). Zantrin Z3 was ineffective against *E. coli* up to 230 µM, but was able to inhibit the growth of envA1 *E. coli* with an MIC50 of 26±8 µM.

*In vitro* enzymatic assays with recombinant FtsZ showed that zantrin Z3 inhibited the GTPase activity of EcFtsZ with an IC50 of 58±12 µM, and SaFtsZ with an IC50 of 100±21 µM (Fig. 46B). To confirm the stabilizing effects of 25 on FtsZ polymerization, we used TEM. Incubation of 230 µM zantrin Z3 with EcFtsZ did not affect the ability of EcFtsZ to form stable polymers after addition of 1 mM GTP. In fact, some of the polymers appeared thicker than the control (Fig. 44A), indicating perhaps the protofilaments were bundled or paired (Fig. 47A), similar to the previous report. Because TEM only gives a snapshot of polymer status and is not suitable for time course experiments, we verified the stabilizing effect of zantrin Z3 through analytical ultracentrifugation. EcFtsZ only forms a pelletable mass in the presence of GTP and CaCl2, a known stabilizing agent [208], as shown previously in Fig. 36. However, with zantrin Z3, EcFtsZ formed a pelletable mass without calcium, indicating 25 was playing a stabilizing role in forming pelletable EcFtsZ (Fig. 47B). We then tested whether this compound had a similar effect on tubulin in a polymerization assay, and found that zantrin Z3 increased both the velocity of tubulin polymerization (Vmax 97.9 s⁻¹) and the extent of tubulin polymerized (Fig. 47C). Again, this cross-reactivity with tubulin made 25 cytotoxic to eukaryotic cancer cells, with an IC50 of 4.4±1.6 µM against HCT-116, and an IC50 of 13±8 µM against 4T1.
The process of proper Z-ring formation in the presence of zantrin Z3 was investigated in vivo. Treatment with 120 µM (~5x envA1 E. coli MIC50) zantrin Z3 for 30 and 60 minutes showed a mislocalization of the FtsZ fluorescence in envA1/pFtsZ-YFP (Fig. 48). At 30 minutes, FtsZ-YFP was no longer localized to mid-cell, and instead showed foci of fluorescence throughout the cell (Fig. 48A). At 60 minutes, the patchy fluorescent phenotype remained, but it was also evident that the morphology of the cell membrane had changed (Fig. 48B). The cells looked shriveled and bumpy in comparison to vehicle treated controls (Fig. 48C - top panel). Compound 25 had no intrinsic fluorescence at the wavelengths utilized for fluorescence microscopy (Fig. 48C - bottom panel).

To investigate the morphological change to the cell membrane that was evident, the lipophilic dye FM5-95 was employed to stain the membrane, using a previously published procedure [412]. Normal cells that have been stained with FM5-95 are shown in Fig. 48D (top panel) and appear as red hollow ovals. The membranes of permeable E. coli treated with zantrin Z1 stain normally with FM5-95, and the mislocalization of FtsZ to the poles does not affect FM5-95 staining (data not shown). However, envA1/pFtsZ-YFP treated with zantrin Z3 and stained with FM5-95 show a different phenotype, where the membrane integrity has been disrupted (Fig. 48D – bottom panel). Average cell length was measured at each time point and compared using a student’s t-test. At 30 minutes, vehicle-treated control cells averaged 4.1 µm, while those treated with zantrin Z3 averaged 6.1 µm. At 60 minutes, vehicle-treated control cells average 2.9 µm, while those treated with zantrin Z3 were 6.1 µm. The increased cell size of envA1/pFtsZ-YFP treated with zantrin Z3 for 30 and 60 minutes was significantly larger (p=0.003 at 30 minutes, and p<0.0001 at 60 minutes) than the control cells.
5.5 Berberine

The natural product berberine has historically been used in traditional medicines for its antimicrobial properties [378,420,421], but its mechanism of action was unknown. One group speculated it could target FtsZ, and using light scattering, TEM, GTPase assays, STD NMR experiments, ITC, FtsZ-GFP fluorescence, and competition assays provided several lines of evidence for its putative mechanism of action [380]. Our work with berberine has shown that its mechanism may not be as clear cut as initial studies seemed to suggest. We first evaluated berberine (24) in antibacterial assays to determine the MIC$_{50}$ and MIC$_{90}$. Berberine inhibited the growth of *S. aureus* with an MIC$_{50}$ of 180±27 µM (Fig. 49A). Its MIC$_{90}$ was 340 µM in a microbroth dilution assay with two-fold serial dilutions. When aliquots of the treated *S. aureus* were plated out on fresh agar, it was found that berberine was bacteriostatic at this concentration. Compound 24 also inhibited the growth of MRSA with an MIC$_{50}$ of 170±70 µM (Fig. 49A), and was inactive against MDRSA up to 1.4 mM. Berberine is a known substrate for multidrug-resistant pumps [422], which may explain the resistance of our MDRSA strain to berberine. Berberine was also ineffective against *E. coli* up to 1.4 mM, but was able to inhibit the growth of *envA1 E. coli* with an MIC$_{50}$ of 280±90 µM (Fig. 49A).

*In vitro* enzymatic assays with recombinant FtsZ showed that berberine inhibited the GTPase activity of EcFtsZ with an IC$_{50}$ of 230±23 µM and SaFtsZ with an IC$_{50}$ of 280±58 µM (Fig. 49B). It is worth noting that the potency of berberine in our hands is significantly lower than reported [380]. To investigate the effects of 24 on FtsZ polymerization, we used TEM. Incubation of 1.4 mM berberine with EcFtsZ decreased the polymer mass after addition of 1 mM GTP (Fig. 49C), but did not completely abolish FtsZ polymerization. Other groups have
suggested that the remaining protofilaments are present because the inhibitor may act by destabilizing bonds between FtsZ subunits and decreasing self-association, rather than by preventing de novo FtsZ assembly [379,380]. We attempted to analyze effects on tubulin, but the spectral properties of berberine interfered with the tubulin polymerization assay. Furthermore, these same properties made it impossible to analyze polymer formation by light scattering, even under the conditions reported in the original paper. There was a decrease in the amount of light scattered by EcFtsZ in the presence of GTP and 24, perhaps indicating a decrease in GTP-dependent polymerization. However, when the background signal of berberine alone was subtracted, there was no difference in the amount of light scattered in the presence or absence of berberine (data not shown). While there is a literature report that berberine does not affect tubulin polymerization at concentrations up to 100 µM [423], we found berberine to have cytotoxic effects on eukaryotic cells, with an IC₅₀ of 73±10 µM against HCT-116, and an IC₅₀ of 125±53 µM against 4T1.

We were able to use STD NMR to evaluate berberine binding to EcFtsZ. We used experimental conditions similar to those reported for the chrysopaentins (Section 4.6), and where samples typically contained a 50-fold excess of berberine relative to EcFtsZ with respective concentrations of 500 µM and 10 µM. An expansion of a representative difference spectrum (top panel - blue) and control spectrum (red) is shown (Fig. 50A) to include the enhanced protons of 24. Normalization of the signal of greatest intensity (δH 6.8) in the difference spectrum to that of the reference spectrum showed signals for the aromatic protons H-3 (100%), H-11 (94%), H-1 (87%), H-10 (84%), and H-12 (77%) to display the strongest
enhancements, while the isoquinolinium proton H-7 (51%) and methylene 2-CH₂ (63%) showed slightly lower enhancement (Fig. 50A) (proton numbering system adapted from [380]).

To probe whether berberine was competitive with the nucleotide, competition STD NMR experiments were performed where increasing amounts of non-hydrolyzable GTPγS was added to a 50:1 complex of berberine:EcFtsZ. Spectra were recorded as described previously on samples containing 500 µM 24 in the presence of 10 µM EcFtsZ. Difference spectra were monitored for a change in intensity of signals belonging to either berberine or GTPγS during the titration. As seen in the spectral expansions showing the aromatic region of the difference spectra (Fig. 50B), addition of 1 equivalent of GTPγS (500 µM) resulted in no significant change in signals belonging to 24. Addition of increasing amounts of GTPγS up to 3 mM (six-fold excess) resulted in no change in the signals of 24. Moreover, STD NMR signals for the anomeric and guanosine protons of GTPγS appeared, and increased in intensity in a concentration-dependent manner. Similar results were seen when 1.5 mM GTPγS was bound to EcFtsZ first, then 1 equivalent of berberine added. Thus, this data suggests that berberine and GTPγS are not competing for the same binding site.

To investigate in more detail the lack of competition between berberine and the nucleotide, we analyzed GTP hydrolysis rates of EcFtsZ in the presence of 8-128 µM of the substrate, GTP, and increasing amounts of berberine (0, 40.8, 204, 408, 815 µM). The velocity of GTP hydrolysis was calculated as the amount of inorganic phosphate produced per minute and the inverse of the velocity was graphed as a function of the inverse of the substrate concentration. The series of lines obtained for each concentration of berberine were roughly parallel, indicating the mechanism of inhibition of berberine was uncompetitive (Fig. 51).
Proper Z-ring formation in the presence of berberine was investigated in vivo. In previous studies, berberine caused diffuse fluorescence visible throughout treated cells containing a FtsZ-GFP construct. This result, concurrent with an increase in cell size (filamentation), led the authors to conclude that berberine caused a disruption of Z-ring formation, and a decrease in the number of Z-rings per cell [380,416]. Treatment of envA1/pFtsZ-YFP with berberine led to the diffuse fluorescence phenotype (Fig. 52A). However, treatment with berberine on permeable E. coli that does not express fluorescent FtsZ revealed that the diffuse fluorescence was due to the intrinsic autofluorescence of berberine (Fig. 52B). To more fully investigate this discrepancy between literature reports and our results, we replaced the yellow fluorescent protein in pFtsZ-YFP with a red fluorescent protein (RFP), known as tdTomato. Expression was induced with arabinose, cells were pelleted and washed, and resuspended in LB as described previously (Section 4.7). Unfortunately, FtsZ-RFP impaired normal division in envA1 E. coli, as shown by the largely increased cell size (Fig. 52C). While this strain is not suitable for extended experimentation, we could nevertheless use it to test whether berberine affected Z-ring localization in the manner reported. Treatment of envA1/pFtsZ-RFP with 680 µM berberine for 60 minutes revealed intact, visible red Z-rings, separate from the yellow berberine intrinsic fluorescence (Fig. 52D). On a separate occasion, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) to show nucleic acid content within the bacteria. The blue fluorescence of DAPI stained in a separate pattern from berberine localization as well (data not shown). Berberine does affect the cells to some extent, because by 60 minutes, envA1/pFtsZ-RFP that were not treated with berberine recovered to their normal size and the amount of fluorescent protein present decreased with each division until it was no longer
visible, while those treated with berberine still retained a fluorescent Z-ring and were larger than normal (data not shown).

5.6 Other FtsZ inhibitors

While OTBA and curcumin were unsuitable for testing in *in vitro* GTPase assays, they could still be used in antibacterial assays. Thus, we analyzed growth inhibition of *S. aureus* with these two compounds, as well as two others, cinnamaldehyde and 3-methoxybenzamide. Cinnamaldehyde is reported to be a FtsZ polymerization inhibitor [424], while 3-methoxybenzamide has been shown to bundle *Bacillus subtilis* FtsZ polymers in a stabilizing manner [384]. In our hands, OTBA and 3-methoxybenzamide were both bactericidal to *S. aureus* at their respective MIC₉₀s (MBC=MIC₉₀), while curcumin and cinnamaldehyde were bacteriostatic to *S. aureus* at their respective MIC₉₀s (MBC>MIC₉₀). This data, taken together with our findings on zantrin Z1, Z3, berberine, and chrysophaentin A show there is a correlation between effect on FtsZ polymerization, and whether the MBC is equal to or greater than the MIC₉₀. Of the compounds tested, those that inhibit FtsZ polymerization are bacteriostatic at their MIC₉₀, while those that stabilize FtsZ polymers are bactericidal.

While an interesting and novel finding, it is unknown whether this correlation will be predictive of mechanism of action, which further highlights the gaps in knowledge of FtsZ inhibitors. For instance, in many of the published studies, researchers use one set of organisms for qualitative antibacterial studies, and often another species’ FtsZ for *in vitro* work, and still a third species for any *in vivo* work, if completed. In fact, when the available literature values of antibacterial activity of FtsZ inhibitors and inhibition of FtsZ GTPase activity are plotted against
one another, there is a very poor correlation between the two values (Fig. 53A – blue line, 
$R^2=0.003$) because the data come from studies on divergent organisms and FtsZ proteins. The 
data obtained in my studies on seven compounds (Fig. 53A – black line, $R^2=0.53$) shows much 
improved correlation between MIC$_{50}$ and IC$_{50}$ because all data used in this graph comes from S. 
aureus antibacterial and SaFtsZ, and envA1 E. coli antibacterial and EcFtsZ. Individual data sets 
(S. aureus and SaFtsZ in green, $R^2=0.76$; envA1 E. coli and EcFtsZ in red, $R^2=0.69$) are shown in 
Fig. 53B, and show an even greater degree of correlation between antibacterial activity and \textit{in vitro} GTPase inhibition when the same organism is used, highlighting the necessity for 
complementary experiments to take place within a constant model system.

\textbf{5.7 Discussion}

The work described here continues to illustrate the difficulty in designing experiments 
suitable to test multiple classes of FtsZ inhibitors. However, we are able to contribute some 
further knowledge to the larger picture of FtsZ inhibition by small molecules. For instance, we 
found zantrin Z1 to be a potent antibacterial agent and to inhibit the GTPase activity of SaFtsZ 
and EcFtsZ in a similar manner. As reported, zantrin Z1 inhibited EcFtsZ polymerization, but 
we have found this compound was also an inhibitor of tubulin polymerization. Similarly, 
zantrin Z3, a published FtsZ stabilizing agent, also stabilized and promoted tubulin 
polymerization. Both zantrin Z1 and Z3 disrupted proper Z-ring formation in bacteria \textit{in vivo}, 
but zantrin Z3 also disrupted membrane integrity. Berberine, another reported FtsZ 
polymerization inhibitor, was in our hands significantly less active than reported, and its intrinsic 
spectral properties make it very difficult, if not impossible, to use in certain experiments.
However, we were able to determine using STD NMR and enzyme kinetics that berberine inhibited FtsZ in a manner that is uncompetitive with the nucleotide.

Overall, while it may seem that bactericidal agents that are capable of killing an organism would make better antibacterials than bacteriostatic compounds, this is not necessarily the case. Many antibiotics used in the clinic are bacteriostatic, such as chloramphenicol and tetracycline. Some groups working on FtsZ inhibitors have implied that because FtsZ inhibition causes filamentation, and filaments bacteria will eventually lyse, FtsZ inhibitors must cause lysis, and this is a bactericidal occurrence [415,425]. However, in a study using antisense RNA to inhibit antibacterial targets, one group found that inhibition of \textit{ftsZ} in Mycobacteria led to a moderate growth inhibition, and behaved in a bacteriostatic manner [426]. These two results imply that there may be more than one outcome to inhibiting FtsZ and that both bacteriostatic and bactericidal agents could potentially be useful.

In their work on the binding site of curcumin, one group hypothesized that inhibitors of FtsZ polymerization must bind to the same site as the nucleotide [418]. This seems logical, since polymerization is dependent on nucleotide binding [200,201]. However, we have shown that this simplistic mechanism may not hold true for all polymerization inhibitors. We have shown by two methods, STD NMR and enzyme kinetic analysis, that berberine, a FtsZ polymerization inhibitor, is uncompetitive with the nucleotide. Another group has shown that totarol [407], sanguinarine [427], and cinnamaldehyde [424], all of which are FtsZ polymerization inhibitors, are not competitive with mant-GTP in a competition assay [411]. Furthermore, compounds that increase the GTPase activity of FtsZ cannot be competitive for the nucleotide because hydrolysis is necessarily occurring over the course of the assay.
The increase in GTPase activity of SaFtsZ-20 in the presence of zantrin Z1 is an interesting phenomenon. In a study on *M. tuberculosis* FtsZ, an arginine residue at the C-terminal end is required for polymerization but not GTPase activity, since a truncated form can hydrolyze GTP normally, but does not form polymers [428]. Figure 5 shows the sequence alignment of *M. tuberculosis* FtsZ and SaFtsZ. The arginine required for *M. tuberculosis* FtsZ polymerization is amino acid 378, and this arginine residue is conserved in SaFtsZ at amino acid position 378, and in fact, all bacterial FtsZ sequences shown in Fig. 5. This arginine may play a similar role in the function of SaFtsZ, and when it is missing, could explain the differential activity of SaFtsZ in the presence of zantrin Z1.

At this time, FtsZ has two known drug binding pockets: one which overlaps with the nucleotide binding pocket [346], and the second which is analogous to the taxol binding pocket of tubulin [142,384,429]. Our work suggests there may be several more, since berberine and zantrin Z1 both exhibit significant differences in their mode of binding relative to chrysophaentin A. The original report of berberine suggested that its binding pocket overlapped with the nucleotide binding pocket since berberine was competitive with 5,5’-bis-(8-anilino-1-naphthalenesulfonate) (bis-ANS), a dye shown to bind to hydrophobic regions of FtsZ, including the nucleotide binding pocket. However, bis-ANS has been suggested to have as many as five binding sites on FtsZ (one high affinity and 3.59 low affinity, similar to tubulin) [392], and berberine could be competitive with any one of them.

Competitive agents, which would have the greatest chance of inhibiting both FtsZ and tubulin since the nucleotide binding pocket is the most highly conserved region between the two proteins, have been identified that are specific for FtsZ. Nucleotide analogs [147,409] and
chrysophaentin A are the only verified competitive inhibitors identified thus far, and both are specific for FtsZ over tubulin. Stabilizing agents OTBA [383] and PC190723 [142] are also both specific for FtsZ over tubulin. High resolution crystal structures of these small molecules in complex with FtsZ are needed to determine the structural components necessary for specific inhibitors. Our work with zantrin Z1 and zantrin Z3 have found that both of these agents affect tubulin in the same way they affect FtsZ. Zantrin Z1 inhibited both FtsZ and tubulin polymerization, while zantrin Z3 promoted and stabilized both FtsZ and tubulin polymerization. Both compounds had cytotoxic effects on eukaryotic cells as well, and zantrin Z1, also known as tricycline B, has been shown to also exert antifungal activities [430].

The benzamide derivatives are specific for certain species of FtsZ, namely, B. subtilis FtsZ and SaFtsZ. This specificity arises because susceptible organisms have a hydrophobic valine at position 307 within the putative binding cleft of the molecules, while non-susceptible organisms have a basic arginine or histidine residue at position 307 [142]. The group working on these molecules has hypothesized that because a change as small as a single amino acid substitution can result in species specificity within inhibitors, it may be difficult to find broad-spectrum FtsZ inhibitors. Our work with SaFtsZ and EcFtsZ shows that for many of the inhibitors, the IC\textsubscript{50}s of SaFtsZ and EcFtsZ are very similar under the conditions used in our assays. Therefore, the only limit to identifying broad-spectrum FtsZ inhibitors is the ability to find compounds with broad-spectrum antibacterial activity, i.e. the ability to penetrate the membrane of Gram-negative organisms.

Finally, with our in vivo work, we have been unable to propose a correlation between effects on Z-ring localization and in vitro effects on FtsZ. Each of the inhibitors investigated,
albeit a small number, showed a slightly different phenotype and effect on Z-ring formation, localization, and integrity. The phenotypes observed included diffuse fluorescence and membrane localization of FtsZ (caused by compound 11, 21, and 22), helical disruption and polar localization (23), and patches of fluorescence concurrent with membrane disruption (25). This work has contributed to filling in some of the gaps of knowledge within FtsZ inhibitors, and has led to a broader understanding of how the chrysophaentins are similar to and different from other FtsZ inhibitors.

5.8 Materials and methods

5.8.1 Chemicals

3-methoxybenzamide (M10050), berberine chloride (B3251), cinnamaldehyde (W228613), curcumin (08511), L-arabinose (A3256), and DAPI (32670) were obtained from Sigma-Aldrich. GTPγS (NU-412) was purchased from Jena Bioscience. FM5-95 (T-23360) was obtained from Invitrogen. OTBA (5670490) was purchased from the small molecule library ChemBridge, and zantrin Z1(0075-0073) and zantrin Z3 (000A-0025) were obtained from the compound library ChemDiv.

5.8.2 Site-directed mutagenesis of SaFtsZ

Site-directed mutagenesis on a pet-11a plasmid containing a synthetic gene encoding SaFtsZ was performed using the Agilent Technologies QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer’s instructions. Forward and reverse primers with respective sequences of 5’ – CGAACGTACCATACCAACAAAGAATAAGATATTCCGAGC – 3’ and 5’
– GCTCGGAATATCTTATTCTTTGGTGGTATGGGTACGTTCG – 3’ were used for the PCR reactions to insert a stop codon after the 370th amino acid, and were PAGE purified before use (Lofstrand Labs, Gaithersburg). High resolution mass spectrometry was used to confirm the truncation.

5.8.3 STD NMR

Solutions containing free berberine (500 µM), or complexes of berberine (500 µM) in the presence of EcFtsZ (10 µM) were prepared in 20 mM sodium phosphate pH 6.8, 50 mM KCl, 5 mM MgCl₂, with 10% D₂O. NMR data were acquired at 298 K on a Bruker Avance 500 MHz spectrometer equipped with a z-shielded gradient triple resonance cryoprobe. Water suppression was carried out using a 3-9-19 WATERGATE sequence. ¹H STD NMR experiments were recorded as described previously [395]. Spectra were acquired with 3,072 scans, 32,768 points, and a relaxation delay of 2.5 s, with on- and off-resonance carrier frequencies set a 0 and 40 ppm, respectively. Protein saturation (2 s) was accomplished using a train of 50-ms Gaussian pulses followed by 1-ms delays. Difference spectra were obtained from subtracting on- from off-resonance spectra. Relative intensities were obtained by normalizing peak integrals to the strongest signal/s in each spectrum. For titration experiments with GTPγS, because berberine has limited stability in aqueous solutions beyond the 24 hours necessary for each experiment, fresh complexes of berberine:EcfTsZ were made prior to addition of additional equivalents of GTPγS.
5.8.4 Enzyme kinetics

To test for competitive or not competitive inhibition, berberine was prepared in Assay buffer (50 mM MES pH 6.0, 100 mM KCl, 5 mM MgCl2) at concentrations below, equal to, and above its IC50. EcFtsZ was prepared in the same Assay buffer to a final concentration of 3 µM in a 96 well flat bottom plate, and GTP prepared to several concentrations between 8 and 128 µM. Time and GTP concentrations were chosen so that initial velocity and phosphate production were linear. All other conditions were the same as previously described for GTPase assays (Section 4.3 and 4.9.3). Double reciprocal plots of 1/v and 1/S, where v is the initial velocity for each reaction condition, and S is the substrate concentration were created.

5.8.5 Construction of FtsZ-RFP

Site-directed mutagenesis on the arabinose-inducible pFtsZ-YFP was performed using the Agilent Technologies QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer’s instructions. Forward and reverse primers with respective sequences of 5’ – GCTGATGGATCCACTAGTATGGTGAGCAAGGGC – 3’ and 5’ – GCCCTTGCTCACCATACTAGTGGATCCATCAGC – 3’ were used for the PCR reactions to insert a SpeI restriction site between FtsZ and YFP, and were PAGE purified before use (Integrated DNA Technologies). Sequencing by Eurofins using pBad forward (5’ – ATGCCATAGCATTTTTATCC – 3’) and pBad reverse (5’ – GATTTAATCTGTATCAGG – 3’) primers confirmed the presence of the SpeI site and an additional amino acid in the linker region, resulting in GSTS between FtsZ and YFP. The gene sequence for YFP was removed using SpeI and SphI restriction enzymes (New England Biolabs), and the vector backbone was purified by
gel extraction. TdTomato (Clontech) was amplified from the purchased plasmid by PCR using forward and reverse primers with respective sequences of 5' –

GACTAGTATGGTAGCAAGGGCCGAG – 3' and 5' –

ACATGCATGCATCCTACTTGTACAGCT – 3' with Pfu Ultra High Fidelity polymerase (Agilent Technologies) according to the manufacturer’s instructions. The amplified tdTomato was cleaned using Promega Wizard PCR preps DNA purification system, cut with SpeI and Sphl restriction enzymes, and ligated into the vector backbone with T4 ligase. The ligation mixture was transformed by heat stock into DH5α E. coli (New England Biolabs). The plasmid was extracted by mini-prep protocol, and sequenced by Eurofins using the same primers as above. pFtsZ-RFP was electroporated into permeable E. coli using 25 µFD, 200 Ω, and 2.5 kV (time constant 4 ms).

5.8.6 Fluorescence microscopy (RFP)

For fluorescence microscopy, envA1/pFtsZ-RFP were grown in Luria broth (LB)-based medium containing 20 µg/mL chloramphenicol until the optical density at 600 nm reached 0.2, then were induced with 0.4% arabinose for 30 minutes (see Section 4.7). Cells were visualized on a polylysine coated slide with differential interference contrast and fluorescence microscopy using a Zeiss LSM700 confocal scanning laser unit with an inverted microscope (AXIO Observer.Z1) with a 100X objective, 1.3 numerical aperture lens. Fluorescent wavelengths utilized the laser set to excite at 555 nm, and emission filters at 630 nm. Images were acquired through the microscope software package.
5.8.7 *All other experimental conditions and techniques are described in Section 3.8 and 4.9.*

5.9 **Acknowledgements**

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5.10 Figures and figure legends

**Figure 41.** Structures of commercially available FtsZ inhibitors used in this study.

![Chemical structures](image-url)

- **23 Zantrin Z1**
- **24 Zantrin Z3**
- **25 Berberine**
- **26 OTBA**
- **27 Curcumin**
Figure 42. Microbroth dilution assay curves for (A) three *S. aureus* strains, and (B) two *E. coli* strains with zantrin Z1 (23). The lines indicate the inhibition curves used to calculate the minimum inhibitory concentration (MIC). Zantrin Z1 inhibited the growth of *S. aureus* (black) with an MIC$_{50}$ of 0.58±0.2 µM, MRSA (blue) with an MIC$_{50}$ of 0.48±0.1 µM, and MDRSA (red) with an MIC$_{50}$ of 0.18±0.03 µM. It also inhibited the growth of *E. coli* with an MIC$_{50}$ of 7.8±3.2 µM, and permeable *envA1 E. coli* with an MIC$_{50}$ of 1.7±0.7 µM.
Figure 43. GTPase inhibition assay curves for EcFtsZ and SaFtsZ with zantrin Z1 (23). (A) Zantrin Z1 inhibited the GTP hydrolysis activity of EcFtsZ and SaFtsZ in a dose-dependent manner with IC$_{50}$ values of 4.8±1.7 µM and 2.4±0.7 µM, respectively. (B) A truncated form of SaFtsZ (SaFtsZ-20) hydrolyzed GTP at the same rate as wild-type SaFtsZ (black line, lower x-axis). Addition of zantrin Z1 (23) increased GTP hydrolysis activity of SaFtsZ-20 in 30 minutes at 30 °C relative to a DMSO control (blue line, upper x-axis).
Figure 44. The effect of zantrin Z1 (23) on EcFtsZ and tubulin polymerization. (A) 6 μM EcFtsZ polymerized in Assay buffer upon addition of 1 mM GTP to form single-stranded protofilaments. Images acquired by TEM with 44000x magnification and negative staining. (B) In the presence of 240 μM zantrin Z1, EcFtsZ polymerization was completely inhibited. Images acquired by TEM with 44000x magnification and negative staining. (C) Tubulin polymerization was monitored by increase in absorbance at 340 nm. The 2% DMSO-buffer control is graphed in blue and shows the normal amount of tubulin polymerized in 30 minutes (velocity of polymerization is 35.9 s⁻¹). 240 μM zantrin Z1 (23) decreased both the velocity (5.13 s⁻¹) and extent of tubulin polymerization. These results show zantrin Z1 inhibits both EcFtsZ and tubulin polymerization.
Figure 45. Fluorescence microscopy of *envA1 E. coli* containing an arabinose inducible plasmid encoding FtsZ-YFP treated with zantrin Z1. All panels show two images, one above the other; left image is fluorescence only (ex. 488, em. 558); right image is fluorescence overlaid on DIC. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner. White arrowheads show examples of polar localization of fluorescence. *EnvA1 E. coli* was transformed with pFtsZ-YFP, induced with 0.4% arabinose for 30 minutes, washed in PBS with 50 µg/mL tetracycline, then resuspended in LB with the following treatments:

(A) *envA1/pFtsZ-YFP* grown for 30 minutes in LB supplemented with 20 µg/mL chloramphenicol, and in the presence of 1% DMSO as a vehicle-control.

(B) *envA1/pFtsZ-YFP* grown for 30 minutes in LB supplemented with 20 µg/mL chloramphenicol, and in the presence of zantrin Z1 in 1% DMSO.
**Figure 45 (continued).** Fluorescence microscopy of *envA1 E. coli* containing an arabinose inducible plasmid encoding FtsZ-YFP treated with zantrin Z1. All panels show two images, one above the other; left image is fluorescence only (ex. 488, em. 558); right image is fluorescence overlaid on DIC. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner. White arrowheads show examples of polar localization of fluorescence.

(C) *envA1/pFtsZ-YFP* induced with 0.4% arabinose for 30 minutes, washed in PBS with 50 µg/mL tetracycline, then grown for 60 minutes in LB supplemented with 20 µg/mL chloramphenicol, and in the presence of zantrin Z1 in 1% DMSO.

(D) *envA1 E. coli* treated with zantrin Z1 as a background fluorescence level control.
Figure 46. Antibacterial and *in vitro* GTPase activity of zantrin Z3 (25). (A) Microbroth dilution assay curves for three *S. aureus* strains. The lines indicate the inhibition curves used to calculate the minimum inhibitory concentration (MIC). Zantrin Z3 (25) inhibited the growth of *S. aureus* (black) with an MIC$_{50}$ of 2.4±1.2 µM, MRSA (blue) with an MIC$_{50}$ of 1.8±1 µM, and MDRSA (red) with an MIC$_{50}$ of 2.0±1 µM. (B) GTPase inhibition assay curves for EcFtsZ and SaFtsZ. Zantrin Z3 (25) inhibited the GTP hydrolysis activity of EcFtsZ and SaFtsZ in a dose-dependent manner with IC$_{50}$ values of 58±12 µM and 100±21µM, respectively.
Figure 47. The effect of zantrin Z3 (25) on EcFtsZ and tubulin polymerization. (A) In the presence of 230 µM zantrin Z3, EcFtsZ polymerization was stabilized. Some protofilaments appeared bundled and are marked with black arrowheads. Images acquired by TEM with 44000x magnification and negative staining. (B) 10 µM EcFtsZ in Polymerization buffer (50 mM MES pH 6.0, 100 mM KCl, 10 mM MgCl₂) analyzed by ultracentrifugation. Lanes labeled with an S are supernatant, and P are pellet. Additives to solution prior to centrifugation are labeled by number in the right panel. EcFtsZ only pelleted in the presence of 10 mM CaCl₂ and 1 mM GTP. However, addition of 230 µM zantrin Z3 (25) caused pelleting with the presence of 1 mM GTP and no calcium, indicating zantrin Z3 was playing a stabilizing role and could substitute for calcium. (C) Tubulin polymerization was monitored by increase in absorbance at 340 nm. The 2% DMSO-buffer control is graphed in blue and shows the normal amount of tubulin polymerized in 30 minutes (velocity of polymerization is 35.9 s⁻¹). 230 µM zantrin Z3 (25) increased both the velocity (97.9 s⁻¹) and extent of tubulin polymerization. These results show zantrin Z3 acts as a stabilizing agent for both EcFtsZ and tubulin polymerization.
**Figure 47.** The effect of zantrin Z3 (25) on EcFtsZ and tubulin polymerization (legend on the previous page).

A

B

1 & 2 10 μM FtsZ + 1 mM GTP + 10 mM CaCl₂
3 15-150 kDa protein standard
4 & 5 10 μM FtsZ + 10 mM CaCl₂
6 & 7 10 μM FtsZ + 1 mM GTP
8 10 μM FtsZ
9 & 10 10 μM FtsZ + 1 mM GTP + 10 mM CaCl₂ + 230 μM 25
11 & 12 10 μM FtsZ + 1 mM GTP + 230 μM 25

C

Tubulin Polymerization Assay

Absorbance at 340 nm

Time (minutes)
Figure 48. Fluorescence microscopy of *envA1 E. coli* containing the arabinose inducible pFtsZ-YFP treated with zantrin Z3. All panels show two images, one above the other; left image is fluorescence only (ex. 488, em. 558); right image is fluorescence overlaid on DIC. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner. *envA1 E. coli* was transformed with pFtsZ-YFP, induced with 0.4% arabinose for 30 minutes, then washed in PBS with 50 µg/mL tetracycline, and resuspended in LB. Additional treatments are as follows:

(A) *envA1/pFtsZ-YFP* grown for 30 minutes in LB supplemented with 20 µg/mL chloramphenicol, and in the presence of zantrin Z3 in 1% DMSO.

(B) *envA1/pFtsZ-YFP* grown for 60 minutes in LB supplemented with 20 µg/mL chloramphenicol, and in the presence of zantrin Z3 in 1% DMSO.
Figure 48 (continued). Fluorescence microscopy of envA1 E. coli containing an arabinose inducible plasmid encoding FtsZ-YFP treated with zantrin Z3. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner.

(C) Top panel: DIC image showing morphological changes evident to E. coli treated with zantrin Z3. Bottom panel: envA1 E. coli treated with zantrin Z3.

(D) Top panel: envA1 E. coli stained with membrane dye FM5-95. Left image is fluorescence alone (ex. 555, em. 630); right image is DIC alone. Bottom panel: envA1/pFtsZ-YFP treated with zantrin Z3 for 60 minutes, then stained with membrane dye FM5-95. Left image is fluorescence alone (ex. 555, em. 630); right image is DIC alone.
Figure 49. Effects of berberine (24) in antibacterial and *in vitro* FtsZ GTPase and polymerization assays. (A) Microbroth dilution assay curves for three bacterial strains. The lines indicate the inhibition curves used to calculate the minimum inhibitory concentration (MIC). Berberine (24) inhibited the growth of *S. aureus* (black) with an MIC$_{50}$ of 180±27 µM, MRSA (blue) with an MIC$_{50}$ of 170±70 µM, and *envA1 E. coli* (red) with an MIC$_{50}$ of 280±90 µM. (B) GTPase inhibition assay curves for EcFtsZ and SaFtsZ. Berberine (24) inhibited the GTP hydrolysis activity of EcFtsZ and SaFtsZ in a dose-dependent manner with IC$_{50}$ values of 230±23 µM and 280±58 µM, respectively. (C) The effect of berberine (24) on EcFtsZ polymerization. In the presence of 1.4 mM berberine, EcFtsZ polymerization was decreased, but not completely inhibited. Images acquired by TEM with 44000x magnification and negative staining. Black scale bar is 100 nm.
Figure 50. STD NMR spectra of berberine (24) and EcFtsZ. (A) Reference (blue) and STD NMR difference (red) spectra of berberine in complex with EcFtsZ. Spectral expansion shows nonoverlapped aromatic signals of 24 displaying the strongest enhancements upon binding to EcFtsZ. Overlaid spectra were normalized to the signal for H-3 ($\delta_H$ 6.85), which gave the strongest enhancement. Proton signals are labeled as in Figure 41. (B) Competition experiments of berberine and GTPγS (GSP) binding to EcFtsZ by STD NMR. Expanded $^1$H STD NMR spectrum of berberine (500 µM) in the presence of EcFtsZ (10 µM). Addition of 1.0, 2.0, 4.0, and 6.0 equivalents of GTPγS (final concentrations of GTPγS were 500 µM, 1.0 mM, 2.0 mM, and 3.0 mM, respectively) are shown below. Signals for berberine (labeled in black) protons are not changing with each addition, while signals for GTPγS H-8 and H-1' (labeled in blue) are increasing.
Figure 51. Enzyme kinetic analysis of EcFtsZ in the presence of berberine (24). Double reciprocal plot of the inverse of substrate concentration (µM GTP) versus the inverse of the velocity (µmol inorganic phosphate hydrolyzed by 3 µM EcFtsZ per minute). The series of parallel lines obtained in the absence and presence of increasing concentrations of berberine, 40.8-815 µM indicate a particular type of not competitive inhibition by berberine of EcFtsZ, uncompetitive inhibition.
Figure 52. Fluorescence microscopy of *envA1 E. coli* containing a fluorescently-labeled FtsZ treated with berberine. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner. Each panel shows two images, one above the another; the left hand panel is fluorescence alone (ex. 488, em. 558); the right image is fluorescence overlaid on DIC.

(A) *envA1/pFtsZ-YFP* induced with 0.4% arabinose for 30 minutes, washed in PBS with 50 µg/mL tetracycline, then grown for an additional 30 minutes in LB supplemented with 20 µg/mL chloramphenicol, and in the presence of berberine in 1% DMSO.

(B) *envA1 E. coli* treated with berberine.
Figure 52 (continued). Fluorescence microscopy of envA1 E. coli containing a fluorescently-labeled FtsZ treated with berberine. All images acquired with a 100x lens, and scale bar is shown in lower right hand corner. Examples of Z-rings are labeled with white arrowheads. envA1 E. coli was transformed with pFtsZ-RFP, then induced with 0.4% arabinose for 30 mintues, washed in PBS with 50 µg/mL tetracycline, then resuspended in LB. Additional treatments are described below.

(C) envA1/pFtsZ-RFP was grown for 30 minutes in LB supplemented with 20 µg/mL chloramphenicol, and in the presence of 1% DMSO as a vehicle control. There are two images, one above the another; the left hand panel is fluorescence alone (ex. 555, em. 630), and the right panel is fluorescence overlaid on DIC.

(D) envA1/pFtsZ-RFP was grown for 30 minutes in in LB supplemented with 20 µg/mL chloramphenicol, and in the presence of berberine in 1% DMSO. All images are two channel fluorescence (channel 1: ex. 488, channel 2: ex. 555, em. 605). Examples of Z-rings (red) are visible through the berberine fluorescence (yellow).
**Figure 53.** Correlation between antibacterial activity and *in vitro* activity. (A) Antibacterial activity as minimum inhibitory concentration (MIC$_{50}$) graphed against *in vitro* FtsZ GTPase activity (IC$_{50}$). Data obtained from my work graphed in black ($R^2$=0.53); data obtained from the literature graphed in blue ($R^2$=0.003). Because of the many divergent organisms used in the literature, there is very little correlation between antibacterial and *in vitro* data. (B) Data obtained from all my work graphed in black; data from *S. aureus* growth inhibition and SaFtsZ GTPase inhibition in green; and data from *envA1 E. coli* growth inhibition and EcFtsZ GTPase inhibition in red. When antibacterial and *in vitro* assays are completed on the same organisms, there is much better correlation between the two values.
Chapter 6
Conclusions & Future Directions

6.1 *Summary of results and future directions*

The first clinically available antibacterial compounds provided man with a way to manage, treat, and even cure previously life-threatening and life-ending infections. Penicillin cured staphylococci infections, and streptomycin cured tuberculosis. Unfortunately, these diseases were not eradicated, and due to the global rise in drug resistance among pathogens, many of these same infections are today almost untreatable, due to the emergence of strains such as totally-drug-resistant tuberculosis (TDR-TB) and vancomycin-resistant *Staphylococcus aureus* (VRSA). Infections caused by drug-resistant bacteria are similar in their clinical manifestations to those caused by drug-susceptible bacteria; the difference lies in the available options for treatment. One way to circumvent the current resistance pathways are to use antibiotics from new structural classes that are not susceptible to these resistance mechanisms. Incremental changes to lead compound scaffolds can result in increased potency and spectrum but generally do not provide a permanent solution to combating resistance. Thus, new antibiotic compounds should differ structurally from earlier generation antibacterials. A compound with a novel scaffold will be more likely to inhibit a bacterial target beyond the classically targeted pathways of cell wall biosynthesis, protein biosynthesis, DNA replication and repair, and folate coenzyme synthesis. Finding new antibacterial agents belonging to a novel structural class with activity against potentially novel targets can provide insight into the role that these new targets will play in the development of antibiotics for future use.
In the twenty-five year span prior to 2006, 60% of the small molecule new drugs were either natural products or synthetic molecules inspired by natural products. Marine organisms in particular have been the focus of drug discovery efforts by many research groups, because many of the compounds isolated from these organisms do not have structural similarities to any compounds found in the terrestrial environment, and bioassay analyses have shown that up to 10 percent of marine organisms contain bioactive compounds. Nature provides us with many compounds that are privileged structures, in that they are molecularly compatible with binding to druggable targets, and can provide medicinal chemists with logical starting points for optimization and inspiration. Our lab, among others, has used bioassay-guided fractionation to find a number of biologically active compounds from marine organisms, and these compounds can be detected directly in the producing organism, or the host of the producing organism. The compounds we have found comprise many different structures and corresponding biological activities, and the same techniques used to find these previous compounds will allow us to find other compounds through screening for activity against a panel of human pathogenic bacteria. The logical extension of drug discovery efforts of any new compound encompasses an examination of its mechanism of action, and the structures of the isolated compounds can give us an idea of possible mechanisms based on similarities to other known agents.

The hypothesis of this project was that the chemical structures of antibacterial compounds can give insight into their antibacterial targets. Compounds with unique structures that do not fall into known classes of antibiotics may have new targets. In this work, we have made several contributions to the field of marine natural products and the discovery of novel antibacterial compounds.
Even though few antibacterial compounds had previously been identified from sponges of the Theonellidae family, it was fully expected that these sponges could contain antibacterial agents; and perhaps, should contain antibacterial agents since sponges are known to be resistant to bacterial decomposition and contain many species of symbiotic microorganisms. Our work with a Fijian specimen of *Siliquariaspongia* allowed us to isolate a suite of long chain brominated acids with antibacterial activity against drug-susceptible and drug-resistant *Staphylococcus aureus* and *Enterococcus faecium*, that we have named motualevic acids A-F. The suite of natural motualevic acids allowed us to determine an initial structure-activity relationship. We deduced that the free carboxylic acid and (4E) geometry were essential for the antibacterial activity. Within the natural population of motualevic acids, there exists the potential for a biosynthetic relationship between motualevic acid A and motualevic acid F. Motualevic acid A, with its glycine moiety, could serve as a precursor for and nitrogen donor in the biosynthesis of azirine rings, such as that present in motualevic acid F. This warrants further exploration in future studies.

To further test the structure-activity relationship, we synthesized analogs of motualevic acid A in order to test the effects of chain length, the presence of bromines, charge, hydrophobicity, and flexibility. We found that the ω-brominated lipid (E)-14,14-dibromotetradeca-2,13-dienoic acid present in all of the natural products is required for low micromolar antibacterial activity, even with attempts at optimization through synthesis. This study allowed us to discover several additional antibacterial agents, with improved or comparable potencies to the natural motualevic acid A. In general, more hydrophobic amino acid conjugates were found to be more potent antibiotics, as opposed to conjugates that contain polar amino acids or more
than one negative charge, both of which decreased antibacterial activity. These results indicate that motualevic acid analogs with improved antibacterial potency can be constructed, and that their mode of action can be altered with composition. Furthermore, analogs with functional probes were designed that retained some activity and could be used to site-specifically label potential binding partners through bioorthogonal “click” chemistry. A future project could entail determining the mechanism of action of these compounds, and/or a finding the biological target using the probes and tools synthesized here. Given the structural similarity of the motualevic acids to some kinase inhibitors, such studies might start by investigating the effects of the motualevic acids on the autophosphorylation of protein histidine kinases.

In the second project, we have identified a suite of structurally unrelated antibacterial agents from a marine chrysophyte alga, with potent inhibitory activity against *S. aureus*, drug-resistant *S. aureus*, and community-associated MRSA strains. The new anti-staphylococcal scaffold comprises two polyhydroxylated, polyhalogenated $\omega,\omega'$-diarylbutene units connected through two ether bonds to form symmetrical or asymmetrical macrocycles, or a single ether bond to give rise to acyclic analogs. The producing organism is rare, and found in St. John, U.S. Virgin Islands, as well as several other locations worldwide. Analysis of methanol extracts from four locations on St. John revealed geographic variability within the collections. In particular, while there was temporal stability in chrysopaentin production in alga collected from certain locations, there was geographic variability evident in other locations, as seen by the fact that a particular collection of *C. taylori* that lacked the asymmetrically linked macrocyclic chrysopaentins.
Synthetic efforts to obtain a steady supply of chrysopaentin A are under way. An early approach that was employed provided two chrysopaentin fragments that we have shown exhibit similar potencies and spectrum of activity to those of the parent chrysopaentins. All chrysopaentins, natural and synthetic, were bacteriostatic at their MIC$_{90}$; but were bactericidal at concentrations ranging from two to eight fold higher than their respective MIC$_{90}$s. Furthermore, the synthetic fragments showed similar potency to the acyclic chrysopaentin E. We have shown that subtle changes to the chrysopaentin structure and composition lead to differences in antibacterial activity. Knowing that the macrocycle is essential for full antibacterial potency, it is not surprising that the synthetic fragments described here have diminished activity compared to the cyclic natural products. However, we were gratified to find that they are indeed antimicrobial and their activities are comparable to the linear chrysopaentins. Their ease of synthesis and antimicrobial activity provide an excellent starting point not only toward the cyclic chrysopaentins, but also for generating more elaborate synthetic analogs.

The biosynthetic pathway leading to production of the chrysopaentins has not been characterized and a genome sequence for any Chrysophaeum species has not been reported. Future work for this project could involve the discovery of the biosynthetic genes necessary and responsible for chrysopaentin production, and engineering those enzymes and pathways into laboratory-adapted expression strains. Furthermore, in lieu of a total synthesis, it may be possible to use building blocks similar to the synthetic chrysopaentin fragments as precursors in a semi-synthetic, semi-enzymatic assembly. More compound, and a steady supply of compound
is necessary before any *in vivo* animal model studies can be completed for these novel antibacterial agents.

Knowing the structures and spectrum of activity of the chrysophaentin compounds provided a starting point for mechanism of action studies that were explored. Based on the structural features of the chrysophaentins, we hypothesized that their molecular target could be the bacterial cell division protein, FtsZ. This work, coupled with the broad similarity between certain structural classes of other FtsZ inhibitors shows it is entirely plausible to hypothesize a molecular target based on structural features. On the other hand, many of these same compounds exhibit differences in their particular mode of interaction with FtsZ, demonstrating that not all FtsZ inhibitors operate in the same manner. In order to explore whether the chrysophaentins inhibit FtsZ, and to investigate their mode of inhibition, we expressed recombinant EcFtsZ and SaFtsZ. Recombinant EcFtsZ hydrolyzed approximately 2 µmol GTP/min/µmol protein, and formed protofilaments that were monitored by TEM, ultracentrifugation, and light scattering. On the other hand, when purified in a similar manner, recombinant SaFtsZ only hydrolyzed approximately 0.4 µmol GTP/min/µmol protein, and did not form stably detectable protofilaments. These differences could be attributed to significant differences in the way EcFtsZ and SaFtsZ are regulated, particularly in the potential necessity of other proteins and factors to contribute to stable polymerization of SaFtsZ. Future work could entail delving further into the differences between how EcFtsZ and SaFtsZ function *in vitro* and *in vivo*. The structure and components of the divisome complex of *S. aureus* have not been worked on to any extent, and it is unknown what causes the differences in rate of GTP hydrolysis of EcFtsZ and SaFtsZ, and the apparent lack of stability of SaFtsZ protofilaments.
We found chrysophaentin A inhibited the GTP hydrolysis activity of EcFtsZ and SaFtsZ in a dose-dependent manner, and was four-fold more potent against EcFtsZ than SaFtsZ. Chrysophaentin A was shown to be specific for FtsZ over tubulin, as treatment of tubulin with chrysophaentin A had no effect on its ability to polymerize into microtubules. On the other hand, chrysophaentin A efficiently and completely inhibited polymerization of FtsZ. FtsZ immediately polymerizes into protofilaments following GTP binding; hydrolysis of the nucleotide occurs immediately after, followed by inorganic phosphate release and subunit turnover. Polymerization is dependent on GTP binding, and one mechanism of completely preventing polymerization would be to prevent GTP binding. This mechanism is supportive of the hypothesis that chrysophaentin A prevents GTP binding by competitively blocking the nucleotide binding pocket, a scenario that was confirmed by STD NMR experiments. In vivo, chrysophaentin A affects the localization and integrity of Z-rings. Chrysophaentin A inhibits division of envA1/pFtsZ-YFP and disrupts previously formed Z-rings, prevents the formation of new Z-rings, and causes FtsZ to mis-localize and maintain association with the membrane, rather than self-associate at mid-cell in a ring structure.

My work with commercially available FtsZ inhibitors has expanded our understanding of the larger picture of FtsZ inhibition by small molecules. For instance, we found zantrin Z1 to be a potent antibacterial agent and to inhibit the GTPase activity of SaFtsZ and EcFtsZ in a similar manner. As reported, zantrin Z1 inhibits EcFtsZ polymerization, but we have found this compound is also an inhibitor of tubulin polymerization. Similarly, zantrin Z3, a published FtsZ stabilizing agent, also stabilizes and promotes tubulin polymerization. Both zantrin Z1 and Z3 disrupt proper Z-ring formation in bacteria in vivo, but zantrin Z3 also disrupts membrane
integrity. Berberine, another reported FtsZ polymerization inhibitor, is in our hands significantly less active than reported, and its intrinsic spectral properties make it impossible to be used in certain experiments. However, using STD NMR and enzyme kinetics, we were able to determine that berberine inhibits FtsZ in a manner that is uncompetitive with the nucleotide. Our work with SaFtsZ and EcFtsZ shows that for many of the inhibitors, the IC₅₀s of SaFtsZ and EcFtsZ are very similar under the conditions used in our assays. Therefore, the only limit to identifying broad-spectrum FtsZ inhibitors is the ability to find compounds with broad-spectrum antibacterial activity.

This work has contributed to filling in some of the gaps of knowledge within FtsZ inhibitors, and has led to a broader understanding of how the chrysophhaentins are similar to and different from other FtsZ inhibitors. This work also illustrates the difficulty in designing experiments suitable to test multiple FtsZ inhibitors. A major remaining gap in knowledge about FtsZ inhibitors is the analysis of binding affinities and mode of binding. While STD NMR is a powerful tool for analyzing binding and completing competition experiments, the ligands must be amenable to NMR conditions, and relatively soluble, which procludes the use of many small molecule inhibitors. Furthermore, while there are benefits to having continuity within a model system (i.e. testing on SaFtsZ and S. aureus, EcFtsZ and E. coli), apo-FtsZ, which can only be obtained from certain species of FtsZ, is powerfully useful in binding experiments, such as ITC or surface-plasmon resonance (SPR). Future work with EcFtsZ and SaFtsZ should find a way to quantitatively calculate binding affinities; and work with the chrysophhaentins, in particular, should incorporate the homology model generated by our collaborators, which suggest starting
points for identifying amino acids that should be mutated in order to further verify the binding region of chrysophaentin A.

6.2 Concluding statement

In conclusion, we have found two structurally distinct classes of antibacterial compounds from marine organisms, using techniques that combine antimicrobial assay-guided fractionation, selection of organisms that are known producers of bioactive compounds, and dereplication techniques to find novel halogenated compounds. The compound structures presented here represent completely novel structural scaffolds. Intraclass differences have allowed us to propose a structure activity relationship for each to explain their potencies against drug-resistant bacterial strains. Furthermore, the chemical structure of the chrysophenitins further gave us insight into their potential mechanism of action and biological target. Based on structural similarities between the chrysophenitins and a few other compounds, we proposed that the chrysophenitins inhibit the bacterial cell division protein FtsZ. Through several biochemical and microscopy techniques, we verified the molecular target of the chrysophenitins is FtsZ. Our in vitro techniques allow us to propose the chrysophenitins competitively compete with the natural substrate, GTP, for the nucleotide binding pocket. Prevention of nucleotide binding leads to a decrease in FtsZ polymerization and protofilament formation. Without proper polymerization and without substrate binding, hydrolysis of GTP by FtsZ cannot occur. Our in vivo model, using envA1/pFtsZ-YFP, is the first time this plasmid and fluorescent protein expression system has been used in an inhibitor model system, and we have shown it can be used with several diverse inhibitors. In particular, we have shown that our most potent compound, chrysophenitin
A, prevents division of \textit{envA1/pFtsZ-YFP}, disrupts preformed Z-rings, and causes FtsZ to mislocalize within treated cells, so that it no longer forms an intact ring at mid-cell for the division event. Taken together, chrysophaentin A is bacteriostatic to Gram-positive organisms, because it can access the intracellular molecular target and affect the biochemical properties of FtsZ. These effects lead to an inhibition of the physiological role of FtsZ in division and a decrease in the ability of treated bacteria to divide.
Chapter 7
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