A Dive into the Pacific Fish Microbiome: Exploration of Antibiotics from a Unique Ecosystem

by
Molly Clair Austin

A THESIS

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Oregon State University

Honors College

in partial fulfillment of
the requirements for the
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Honors Baccalaureate of Science in Chemistry
(Honors Scholar)

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Molly Clair Austin for the degree of Honors Baccalaureate of Science in Chemistry presented on November 26, 2019 . Title: A Dive into the Pacific Fish Microbiome: Exploration of Antibiotics from a Unique Ecosystem

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Abstract approved:_____

Sandra Loesgen

With the rise of resistant bacterial pathogens, currently prescribed antibiotics are rendered useless. In recent years, marine natural products (MNPs) have emerged as novel sources for drug leads. The ocean is a complex environment where marine animals cohabitate with many, and sometimes harmful microorganisms and viruses, but resist infections. Developed defenses, specifically mucous on fish scales, has been found to contain a variety of immunological chemical defenses and host a diverse microbial community. Recently, cultures from the exterior fish microbiome has been found to inhibit growth of human and fish pathogenic bacteria and fungi.

In exploration for new antibiotics, bacteria were isolated from the mucous of Pacific fishes and screened for their antibiotic-producing potential. Mucous swabs from juvenile fish yielded 47 different microbial strains. Strains were grown and extracted with organic solvent to obtain chemical extracts. Extracts were tested in antimicrobial microbroth assays against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* at 125 µg/mL. Nineteen extracts yielded potent inhibitory activity in at least one assay. In a case study of a particularly potent extract (PF-1-D), three inhibitors against methicillin resistant *S. aureus* and one metabolite with moderate inhibition of colon carcinoma were identified. Metabolites were isolated using multiple chromatographic techniques and elucidated by nuclear magnetic resonance and mass spectrometry. Overall, the potent anti-infective activity of these strains showcase bioactive metabolites that can be found within the Pacific fish microbiome, a potentially untapped source of novel antibiotics.

Key Words: marine natural products, fish mucous, antibiotics, *Pseudomonas* sp., antimicrobial resistance

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: IMPORTANCE OF NATURAL PRODUCTS

1.1 Antimicrobial resistance

Due to the rise of antimicrobial resistance (AMR) in both hospital and community settings, current prescribed antibiotics are losing their ability to be useful to fight infections.[1] In the 1900's, antibiotics nearly doubled the human lifespan[2,3], however the increased reliance on antibiotics among other factors has enabled bacterial pathogens to quickly adapt to modern medicines. Inappropriate and frequent use of antibiotics has contributed to pathogen resistance.[4] One notable case is methicillin-resistant *Staphylococcus aureus* (MRSA), which is attributed to nearly 20,000 patient deaths per year in the US.[5] New chemical entities with novel mechanisms of action are needed for the next generation of antibiotics.[4,6] Without efficient drug interventions, deaths are estimated to reach 10 million worldwide by 2050, surpassing the current mortality count of cancer victims[7] **(Figure 1.1)**.

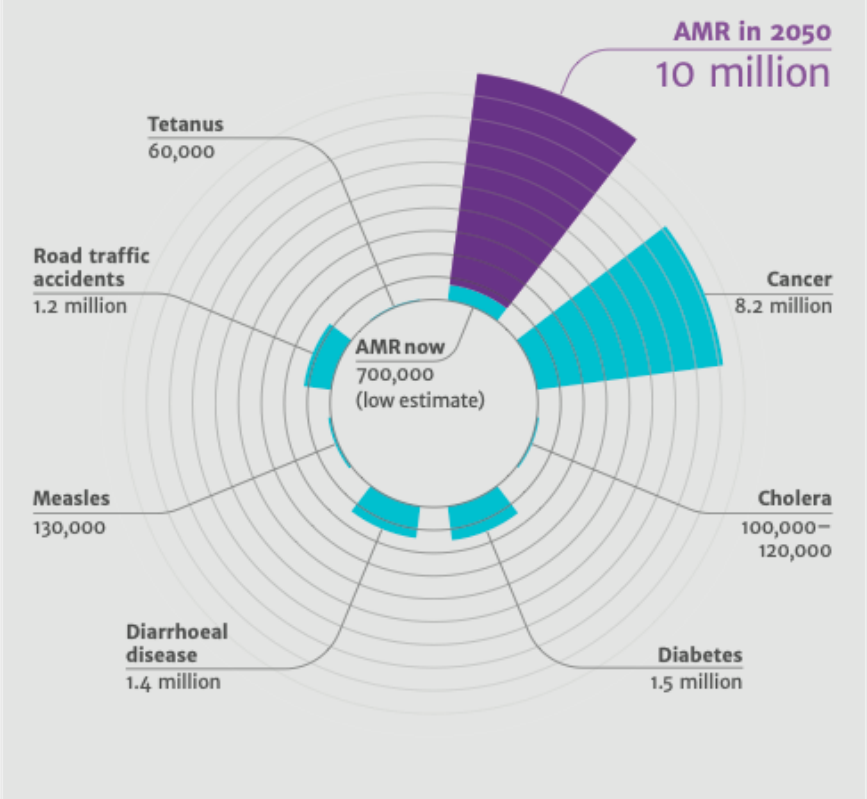


Figure 1.1 Worldwide deaths attributed to antimicrobial resistance (AMR) compared to other major causes. Deaths due to AMR without new drugs are projected to increase to 10 million by 2050.[7]

On November 7th, 2019 the Center for Disease Control declared antibiotic resistant '*one of the biggest public health challenges of our time'*.[8] ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are bacterial species of specific concern.[7,9,10] The mechanisms of resistance include reducing penetration of antibiotics (*e.g.* efflux pumps), target modification (*e.g.* mutations on binding sites), or molecule modification (*e.g.* degradation).[6] Potential therapeutic routes to overcome these resistance mechanisms could include either developing new chemical scaffolds e.g. inspired by new natural products.

1.2 Role of natural products in drug discovery

Natural products, interchangeably called secondary metabolites, are organic compounds produced by organisms not directly involved in normal growth & development.[11] Although the absence of these compounds does not result in immediate death, impairment of an organism's survivability, mating, and aesthetics could occur.[11,12] Currently, modern medicine utilizes natural products and natural product analogs from microbial and eukaryotic sources across medical specialties. Isolated from willow bark, the natural small molecule salicylic acid **(1)** served as the inspiration for many synthetic anti-inflammatory derivatives such as aspirin **(2)**[13] Cyclosporine **(4)**, a cyclic peptide isolated from *Tolypocladium inflatum*, is utilized as an immunosuppressant.[14] Antibiotics such as erythromycin **(3)** and neomycin **(5)** are aminoglycoside and macrolide antibiotics produced by *Streptomyces fradiae* and *Saccharopolyspora erythraea*[15,16] **(Figure 1.2)**. The breadth of chemical structures, therapeutic applications, and biological origins of natural products make them a powerful pharmacological tool. As of 2016, it is estimated that 50-70% of FDA-approved therapeutics were derived from natural product sources or natural product inspired scaffolds, which stresses the importance of secondary metabolites in medicine past and future[17,18] **(Figure 1.3)**.

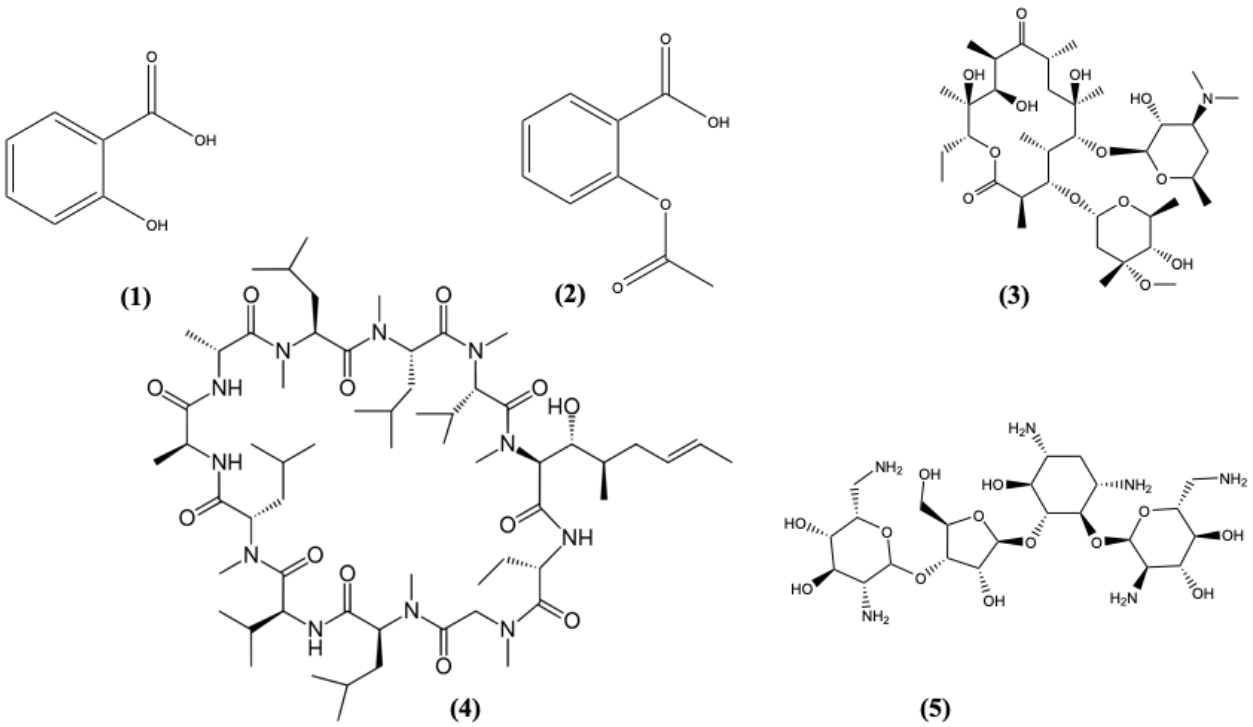


Figure 1.2 Structures of natural products **1-5** used in modern medicine.[13-16]

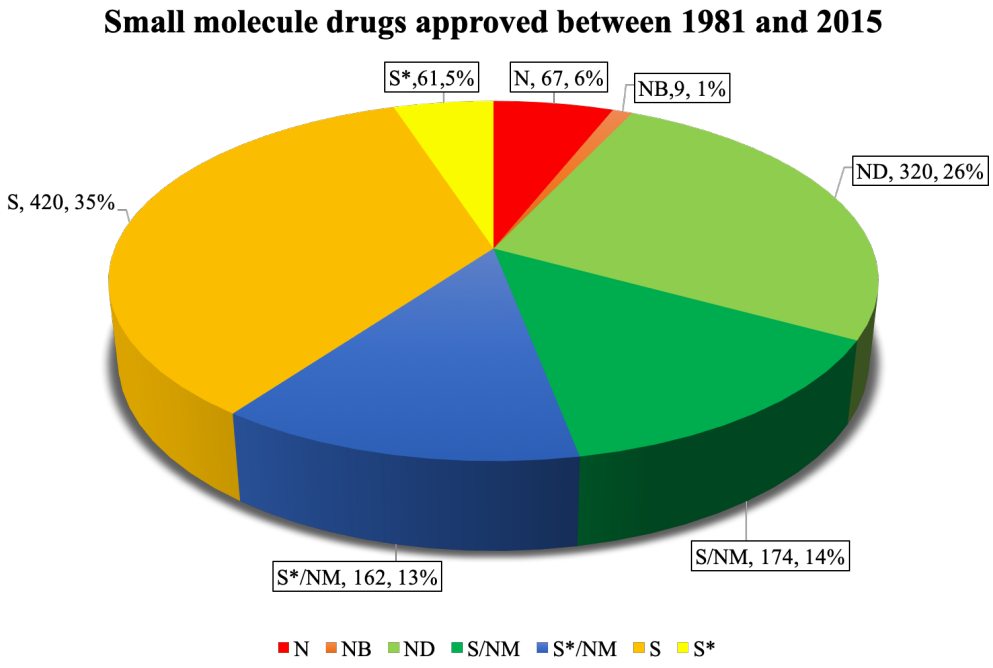


Figure 1.3 Source of small molecule drugs approved between 1981 and 2014 by type: N = Natural product, ND = Natural product derivative, NB = Botanical drug, /NM = Natural product mimic, S = Synthetic drug, S* = Synthetic drug (natural product pharmacophore).[17] Boxed categories: natural products and inspired chemistry making up 65% of drugs.

1.3 Marine chemistry: an untapped source

Terrestrial natural products (TNPs) have been a traditional source for medicine since records of Mesopotamian civilization using plant-based oils.[19] Several well-known medicines were derived from terrestrial sources including the botanical quinine **(6)**[20] which is used to treat malaria and common antibiotics such as penicillin (**7**)[21], and vancomycin (**8**)[22] from terrestrial microorganisms[19] **(Figure 1.4)**. With oceans and seas occupying 70% of the earth's surface, targeting marine sources for marine natural products (MNPs) may provide much needed chemical diversity for drug development. TNPs and MNPs occupy different chemical spaces, meaning the overlap in scaffolds and bioactive properties differs.[23] MNPs tend to have lower relative solubility, be larger in mass, and contain more heteroatoms such as nitrogen and the halogens.[11] This makes them more physicochemically favorable for drug development.[23] Today, several FDA-approved medicines originated from marine sources, including ziconotide (**9**)[24] a powerful analgesic from a cone snail and vidarabine (**10**)[25], one of the first antiviral clinical drug found in sponges[23] **(Figure 1.5)**.

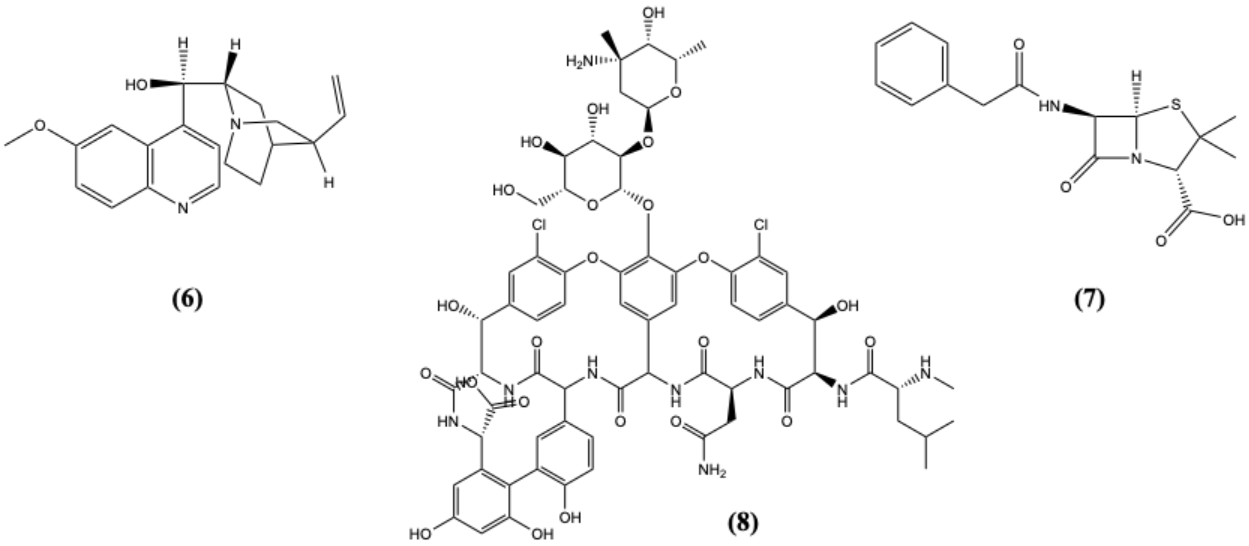


Figure 1.4 Structure of **6-8,** known terrestrial natural products (TNPs)[20-22]

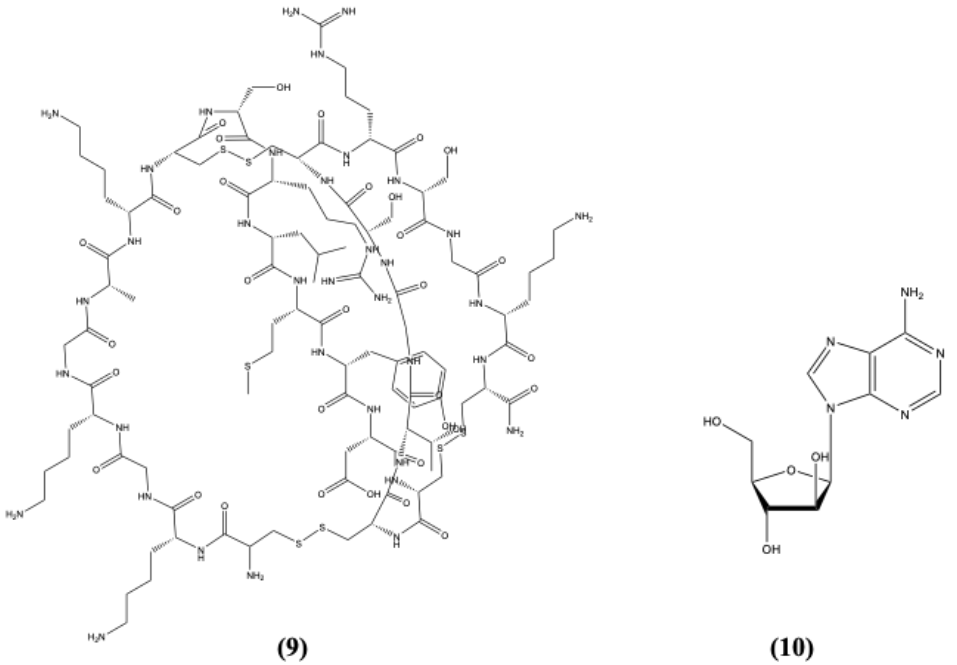


Figure 1.5 Structures of **9** and **10**, FDA approved marine natural products. [24,25]

Metabolites isolated from marine bacteria makeup a majority of antibiotic compounds and are a promising source for discovery of new natural products. In a survey of marine bacteria, approximately 50% exhibited antibacterial activity[26] Additionally, the discovery of bacterial natural products active against resistant pathogens has steadily increased since 2001[4] **(Figure 1.6)**. From 1997 to 2008, 660 new marine bacterial compounds were identified.[27] Many reported compounds include potent anti-MRSA active metabolites from marine bacteria such as marinopyrrole A **(11)** and B **(11a)** from *Streptomyces sp.*[28,29] with reported MIC values <1 μg/mL **(Figure1.7)**.

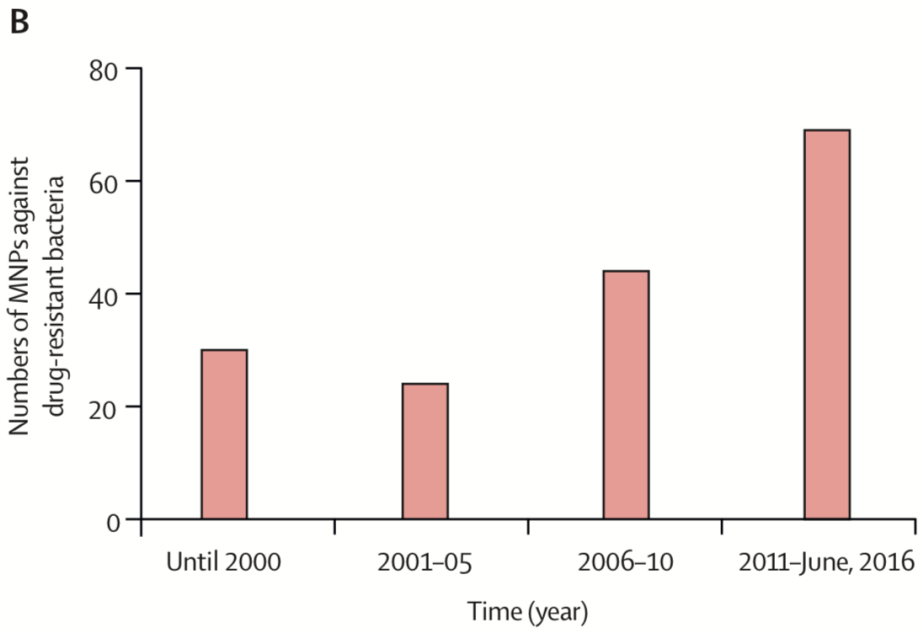
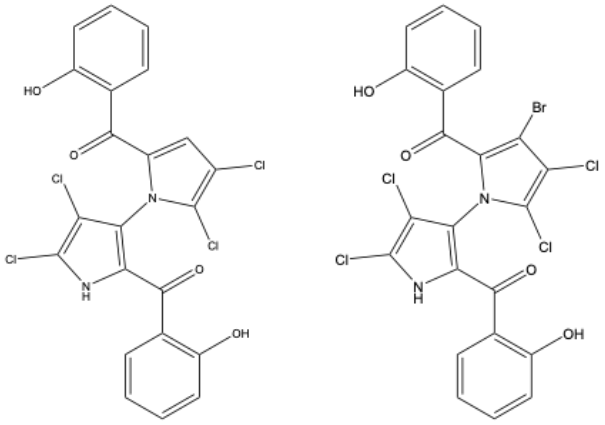


Figure 1.6 Number of marine natural products (MNPs) activate against drug-resistant bacteria, showing an increasing trend in isolation.[4]



(11) **(11a)**

Figure 1.7 Marine natural products **11** and **11a.**[28,29]

1.4 Innate fish defenses

The ocean is a complex environment rich with microbial diversity.[26,30] Consequently, for fish and marine species this implies cohabitating with harmful bacteria and viruses that present immunological risks and deadly infections.[30.31] Despite the threat of pathogens in their immediate environment, fish resist infection due to uniquely evolved immune mechanism.[30.31] Their skin provides a mechanical barrier that is covered in a multifunctional mucous layer. The mucous traps and prevents pathogenic adherence to the epithelial tissue.[30,32,33] Mucous membranes lining the gut, skin, gills, and olfactory organs are also known to host microbial communities, termed the fish microbiome.

Studies have shown that microbes can protect the host body from infection and even act as a source for new antibiotics.[34,35] In the human microbiome, the new antibiotic lugdunin **(12)** isolated from *Staphylococcus lugdunesis* was found in the human nose.[35] New natural products have also been found in the gut microbiome of marine mammals. Phocoenamicin **(13)**, a potent compound against MRSA and *Clostridium difficile*, was isolated from *Micromonospora auratinigra* found in the gut of a harbor porpoise.[36] **13** exemplifies the potential for beneficial bacteria residing in the microbiome to produced anti-infectives to protect the host against pathogens common to their mucosal site. Studies of the fish gut microbiome found twenty different *Streptomyces* strains isolated from freshwater fish in Iran, which exhibited a range of antibiotic activity against fish and human pathogens.[37,38] In addition, the discovery of sebastenoic acid **(14)**, an antibacterial lipid that was isolated from the stomach microbiota of a Canadian Rock Cod from Santa Cruz provided further evidence for antibiotics originating from the fish microbiome[34] **(Figure 1.8)**.

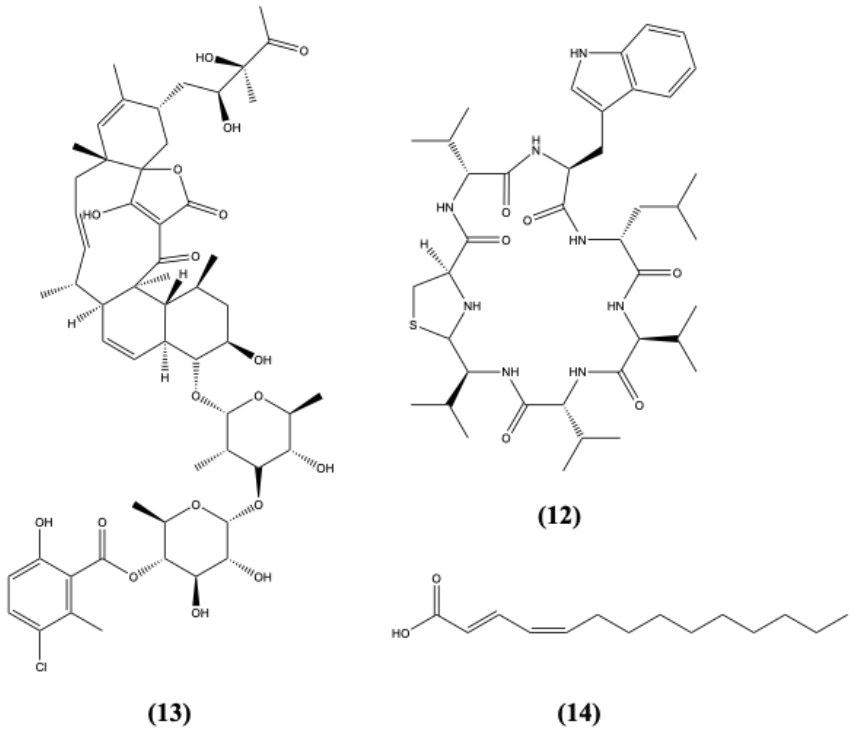


Figure 1.8 Marine natural products isolated from human **(12)** or marine **(13** and **14)** microbiomes.[34-36]

With successful discoveries of antibiotic activity from the internal fish microbiome, research has expanded into determining patterns and potential of the external mucous membranes. Mucous removed from the skin of a turbot fish resulted in increased mortality of the fish pathogen *Vibrio anguillarum.*[30] Microbiota of marine skin mucous was found to have prolific microbial diversity, potentially driven by phylosymbiosis, diet, and competition of cohabitating microbes.[39] Compared to the gut, gills, and olfactory microbiomes, skin had the greatest phylogenetic diversity with nearly three times the number of operational taxa units as the gut.[40] Skin mucous specifically is also known to contain a variety of immunological chemical defenses such as antibacterial peptides, proteases, phosphatases and lysozyme have been isolated from skin secretions.[30,33,41] Production of antibacterial substances has been linked to the host,

but astonishingly few research has been conducted on secondary metabolites from microorganisms living in fish mucous that care selected for and act as fish protection.

Potential for the dynamic and manifold skin mucous microbiome to exhibit inhibition of pathogens has been tested in various co-culture experiments. Mucous extracts from the epidermal surface of *Channa striatus* (snakehead fish) inhibited the growth of human pathogens *Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* in addition to the known fish pathogen *Aeromonas hydrophila.*[42] Bacteria isolated from *Oncorhynchus mykiss* (rainbow trout) mucous exhibited antifungal activity against aquatic pathogens *Saprolegnia australis* and *Mucor hiemalis*.[40] Bacterial fish pathogens, *Flavobacterium psychrophilum* and *Flavobacterium columnare,* which causes columnaris disease*,* were competitively antagonized by bacteria originating in *Salvelinus fontinalis* (brook trout) skin mucous.[43] Mucous strains showing strong antagonism induced decreased mortality in fish with columnaris disease.[43] The antimicrobial potential of the external microbiome of Pacific Ocean fish was investigated to determine the multifunctionality of fish surface-mucous and its role in housing the external fish microbiome. 47 bacteria from mucous samples were isolated and their chemotype analyzed and tested for antibiotic activity. As a case study, bioactive metabolites from a single strain were isolated, their structures elucidated, and tested for antibiotic potential.

CHAPTER 2: BIOACTIVITY SCREENING OF BACTERIAL EXTRACTS DERIVED FROM PACIFIC FISHES

2.1 Introduction

The surface level mucous of many fish in the ocean is made up of a microbially-rich environment that has remained understudied in terms of its microbiology and chemical ecology.[30] As part of the innate immune system, the mucous is employed as a multifunctional layer that traps and prevents pathogenic adherence to the epithelial tissue.[30] While Fish mucous primarily acts as a mechanical barrier, it is also known to contain a variety of chemical defenses and host microbiome.[30] Microbial species in the skin mucous of fish have been shown to inhibit human pathogens such as *Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa.*[42] Studies suggest microbes from the external fish microbiome are capable of inhibiting various species of pathogens. With resistant human pathogens emerging, new natural products are needed to combat MRSA and fluconazole resistant *C. albicans*. Here, I surveyed the chemistry and bioactivity of bacterial strains originated from the mucous of Pacific fish in search of new antibiotics.

2.2. Experimental materials and methods

2.2.1 Microbial isolation from mucous swabs

Swab samples from the external surface of 70 different juvenile fish from 27 different genera were used to isolate bacteria. Juvenile fish usually produce more mucous as protection as their immune system is not fully developed yet and relies on innate mechanisms.[30] A broad spectrum of various fishes from both coastal and deep-sea environments in southern California were sampled **(Appendix 1)**. Identification of each fish by species or genus were noted for each swab sample to trace potential patterns of bacterial composition or bioactivity back to the fish.

Fish mucous and scale swab samples were received from Dr. Misty Paig-Tran, marine biologist at California State University, Fullerton. Fish swabbed included species of eel, toadfish, lanternfish, and octopi. Juvenile fish were handled using gloves and mucous samples were collected using a sterile swab which was capped for shipment. For bacteria isolation, each swab was streaked in a zig-zag motion onto SYP-SW agar plates (soluble starch (10 g/L), yeast extract (4 g/L), peptone (2 g/L), and InstantOcean® (33.3g/L), agar (15 g/L)).[44] Agar plates were incubated at 28˚C for 3-5 days and checking every 1-2 days for microbial growth.

2.2.2 Species isolation on agar

Bacterial colonies of unique morphology and/or pigment production were isolated from agar plates using a sterile loop or toothpick and streaked onto new antifungal SYP-SW agar plates containing nystatin and cycloheximide, each at 0.01 mg/mL, to avoid yeast or fungal growth. Antifungal SYP-SW plates were incubated at 28˚C for 3-5 days and restreaked until pure colonies were obtained. Glycerol stocks were made for each bacterium with 300 µL of sterile glycerol solution (in 50% water) and 700 µL liquid bacterial culture grown in SYP-SW liquid medium. Strains were given an internal designation following a PF-1-A pattern: the number is referring to the swab number and the letter refers to the respective colony.

2.2.3 Small scale culture and extract preparation

Forty-seven different microbial strains were isolated and grown in small scale to produce an extract for the in-house extract library. Each strain was grown in duplicates in 50 mL SYP-SW liquid broth under constant shaking at 23.0°C for 5-7 days. Duplicates with identical phenotype in liquid and on agar, were combined for extraction. If growth in duplicates resulted in two morphologically different strains, cultures were extracted separately and differentiated by adding the number 1 or 2 to the ID. Cultures were extracted with equal volumes of ethyl acetate

(EtOAc) and dried under vacuum to obtain an organic extract.

2.2.4 Antimicrobial and cytotoxicity testing

All organic extracts were screened for antimicrobial and cytotoxic activity in cell-based assays against a human colon carcinoma cell line (HCT-116), fungal pathogen *Candida albicans* (ATCC 90027), gram negative bacterium *Escherichia coli* (ATCC 8739), and gram positive bacterium *Staphylococcus aureus* (ATCC 25923). Extracts active against *Staphylococcus aureus* were also tested against methicillin-resistant *S. aureus* (MRSA, ATCC BAA-41). Extracts active against *Candida albicans* were tested against amphotericin B-resistant *C. albicans* (ATCC 200955) and fluconazole-resistant *C. albicans* (ATCC 10231). Assays were conducted in 96 well plate format that hold a maximum of 27 samples when tested in duplicates.

Microbroth single dose assays against *Staphylococcus aureus* (ATCC 25923), methicillin- resistant *S. aureus* (ATCC BAA-41), *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 90027), amphotericin B-resistant *Candida albicans* (ATCC 200955), and fluconazole-resistant *Candida albicans* (ATCC 10231) were used to test bioactivity of extracts, fractions, or pure compounds. Bacterial pathogens were T-streaked on trypticase soy agar II (TSA II) while fungal pathogens were grown on Sabouraud dextrose agar. Pathogens on agar were incubated at 37˚C for 16-24 hours. Single colonies were selected and inoculated in 4 mL of appropriate broth, shaking at 37˚C for 16-24 hours **(Table 2.1)**. 1.25 µL of each sample, DMSO, and the appropriate positive control is added in duplicates to the plate **(Table 2.1, Figure 2.1)**. To prepare inoculum, culture was diluted to an OD_{600} value of 0.10 for bacteria ($1.5x10^8$ CFU/mL) and 0.15 for fungi ($1x10^6$ CFU/mL). Using the concentration determined by OD_{600} value, inoculum was diluted to $5x10^5$ CFU/mL using the appropriate test media. Inoculum at $5x10^5$ CFU/mL concentration was used to preparing an inoculum solution with a 5:95 ratio of

inoculum to media **(Table 2.1)**. Finally, 98.75 µL inoculum was added to every well with a multichannel pipette, except for two wells filled with 100 µL of media only. The plate(s) were incubated at 37˚C for exactly 18 hours and absorbance of 620 nm was read on a BioTek Synergy plate reader.

Pathogen	Liquid Media	Test Broth	Positive Control
Staphylococcus aureus (ATCC 25923)	Trypticase Soy broth	Muller Hinton II (MH2)	Kanamycin
Methicillin- resistant *S. aureus* (ATCC BAA-41)	Trypticase Soy broth	MH2	Vancomycin
Escherichia coli (ATCC 8739)	LB Luria broth	MH2	Ampicillin
Candida albicans (ATCC 90027)	Sabouraud liquid broth	RPMI 1640 broth	Amphotericin B
Amphotericin B-resistant *Candida albicans* (ATCC 200955)	Sabouraud liquid broth	RPMI 1640 broth	Fluconazole
Fluconazole-resistant *Candida albicans* (ATCC 10231)	Sabouraud liquid broth	RPMI 1640 broth	Amphotericin B

Table 2.1 Pathogen media and antimicrobial positive controls for antimicrobial testing.

96 wells	1	2	3	4	5	6	7	8	9	10	11	12
A	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum
B	Media + Inoculum	Sample 1	Sample 1	Sample 7	Sample 7	Sample 13	Sample 13	Sample 19	Sample 19	Sample 25	Sample 25	Media + Inoculum
C	Media + Inoculum	Sample 2	Sample 2	Sample 8	Sample 8	Sample 14	Sample 14	Sample 20	Sample 20	Sample 26	Sample 26	Media + Inoculum
D	Media + Inoculum	Sample 3	Sample 3	Sample 9	Sample 9	Sample 15	Sample 15	Sample 21	Sample 21	Sample 27	Sample 27	Media + Inoculum
E	Media + Inoculum	Sample 4	Sample 4	Sample 10	Sample 10	Sample 16	Sample 16	Sample 22	Sample 22	Pos. Control	Pos. Control	Media + Inoculum
F	Media + Inoculum	Sample 5	Sample 5	Sample 11	Sample 11	Sample 17	Sample 17	Sample 23	Sample 23	1.25uL DMSO	1.25 uL DMSO	Media + Inoculum
G	Media + Inoculum	Sample 6	Sample 6	Sample 12	Sample 12	Sample 18	Sample 18	Sample 24	Sample 24	Media Only	Media Only	Media + Inoculum
H	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum	Media + Inoculum

Figure 2.1 96 well plate layout for bioassay fitting 27 samples per plate.

Cytotoxic activity of extracts, fractions, and pure compounds was assessed using a human colorectal carcinoma model (HCT-116) by measuring the reduction of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) by metabolically active cells. HCT-116 cells were plated into 96-well plates at a density of 7,000 cells/well and maintained overnight for treatment. Samples were prepared at 10 mg/mL in DMSO and diluted in media to 100 µg/mL for addition to the wells. For single dose treatment, samples were tested at a final concentration of 10 µg/mL in each well. After 48 hours, 10 µL of a 5 mg/mL of MTT reagent in PBS is added to the wells and incubated in the same conditions for 2 more hours. The purple formazan crystals are solubilized by the addition of 50 µL DMSO.[45] Cell viability was assessed with a Bioteck Synergy 96-well absorbance plate reader at 550 nm. Metabolic activity of vehicle-treated cells (0.1% DMSO) was defined as 100% cell growth. Etoposide (250 µM) was used as a positive control.

2.3. Results and discussion

47 microbial extracts were tested in seven different antimicrobial assays to survey the bioactivity of bacterial extracts isolated from various Pacific fish microbiomes. Potency of the extracts in biological assays were measured as percent survival and compared to their respective controls **(Table 2.2)**. Extracts with 40% cell survival or less are considered active. Bioactivity testing revealed unique patterns of activity among the strain collection, with 40% of strains showing activity in at least one of the assays. DNA from four strains with notable activity were sequenced by 16S rRNA amplification for species identification (**Table 2.3**).

Pathogen	Positive Control
Staphylococcus aureus (ATCC 25923)	Kanamycin
methicillin- resistant *S. aureus* (ATCC BAA-41)	Vancomycin
Escherichia coli (ATCC 8739)	Ampicillin
Candida albicans (ATCC 90027)	Amphotericin B
Amphotericin B-resistant *Candida albicans* (ATCC 200955)	Fluconazole
Fluconazole-resistant *Candida albicans* (ATCC 10231)	Amphotericin B
HCT-116 colon carcinoma (ATCC CCL-247)	Etoposide

Table 2.2 Antibiotic, antifungal, or cytotoxic agent used as positive control for each bioassay.

Pacific Fish ID	16S Identification	Assay Inhibition
1-D	*Pseudomonas aeruginosa*	*S. aureus, MRSA,* colon carcinoma cells
62-A	*Psychrobacter celer*	*S. aureus, C. Albicans,* amphotericin B-resistant *C. albicans*, fluconazole-resistant *C. albicans,* colon carcinoma cells
74-A	*Pseudoalteromonas carrageenovora*	*S. aureus, MRSA,* colon carcinoma cells
85-A	*Streptomyces bacillaris*	*S. aureus, MRSA,* colon carcinoma cells

Table 2.3 Identification of bioactive strains by 16S rRNA sequencing.

A complete overview of the assay data for all 47 strains is detailed in **Appendix 2**. Bioactivity varied significantly among the extracts tested. 40% of strains showed activity in at least one assay **(Figure 2.2)**. Five strains were active against *C. albicans,* five were inhibiting colon carcinoma, and fourteen strains were active against *S. aureus*. Some strains showed selective inhibition against a single pathogen including PF 6-C, 6-D, 18-A, 24-B, 33-B, 37A, 43-A, 70-A, and 83-B, which were all selective against *S. aureus*. Anti-eukaryotic activity was observed in PF 71-A, 72-A, 75-A-2 and 87-A with selectivity against *C. albicans*. *Pseudomonas aeruginosa* (1-D and 37-A), *Pseudoalteromonas carrageenovora* (74-A) and *Streptomyces bacillaris* (85-A) exhibited both cytotoxic activity against HCT-116 cells and antibiotic activity against MRSA. Finally, *Psychrobacter celer* (PF-62-A) was the only strain to exhibit antifungal, anticancer, and antibacterial activity.

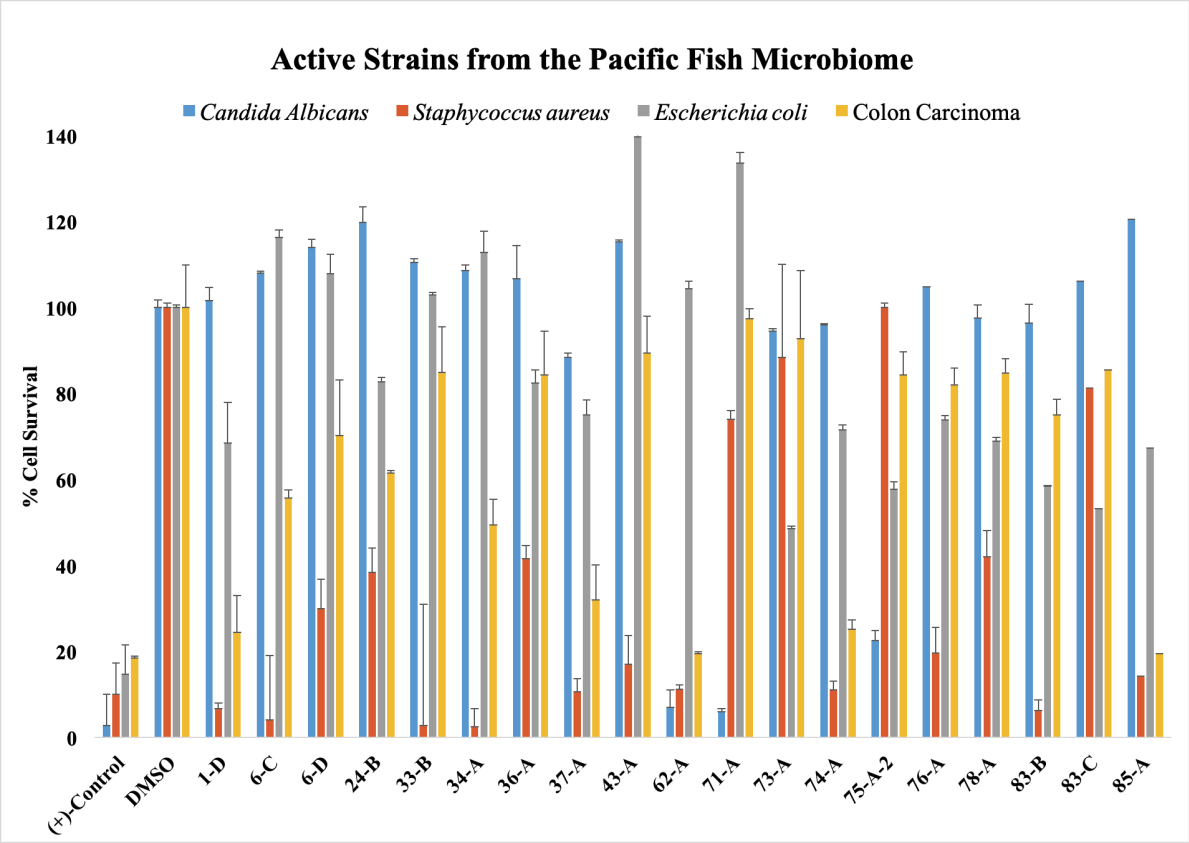


Figure 2.2 Bacterial extracts with activity against *Candida albicans* (ATCC 90027), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 8739), and human colon carcinoma (HCT-116).

Extracts that showed strong activity against *S. aureus* and *C. albicans* were further tested against corresponding resistant strains. Fourteen extracts derived from our Pacific fish microbial library that showed activity against *S. aureus* were tested against a methicillin-resistant *Staphylococcus aureus* strain (MRSA). Four out of the fourteen strains showed strong inhibition against MRSA indicating potential metabolites capable of combating resistance (**Figure 2.3**). Five strains active against *C. albicans* were tested against two drug resistant strains (**Figure 2.4**). All five strains were active against the amphotericin B-resistant strain of *C. albicans*, only *Psychrobacter celer* (PF-62-A) was active against the fluconazole B-resistant *C. albicans*.

Results from the *Escherichia coli* bioassay were obstructed by cell clumping. After an initial reading on the plate reader, well plates were gently mixed with a pipette to break up cell clusters and then read again to compare the data. Percent cell survival varied greatly after mixing, but no strains showed activity with less than 40% cell survival. Differences between pathogen growth were visibly distinguishable but quantifying the results of inhibition remain unreliable. However, PF-73-A extract induced 49% cell survival for in *E. coli*, and could be a potentially active strain in a repeated and improved assay.

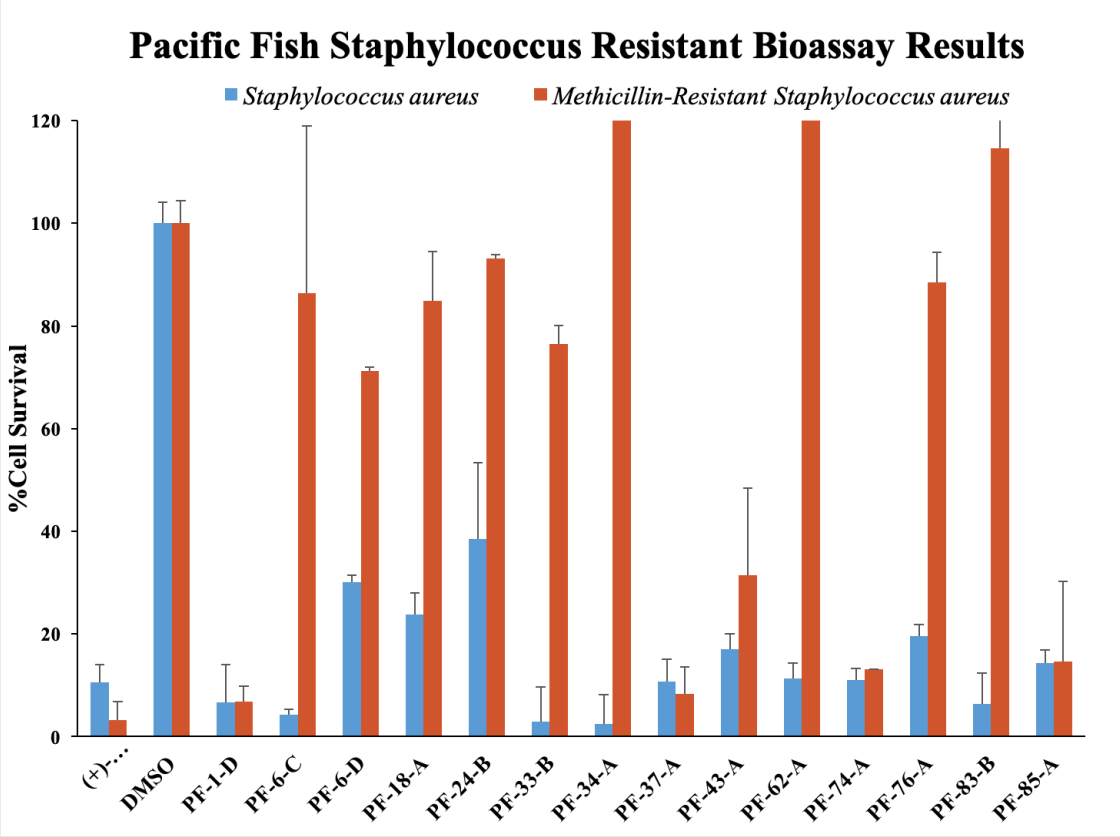


Figure 2.3 Activity of the Pacific fish microbiome against *Staphylococcus aureus* (ATCC 25923) and methicillin-resistant *Staphylococcus aureus* (ATCC BAA-41) (MRSA).

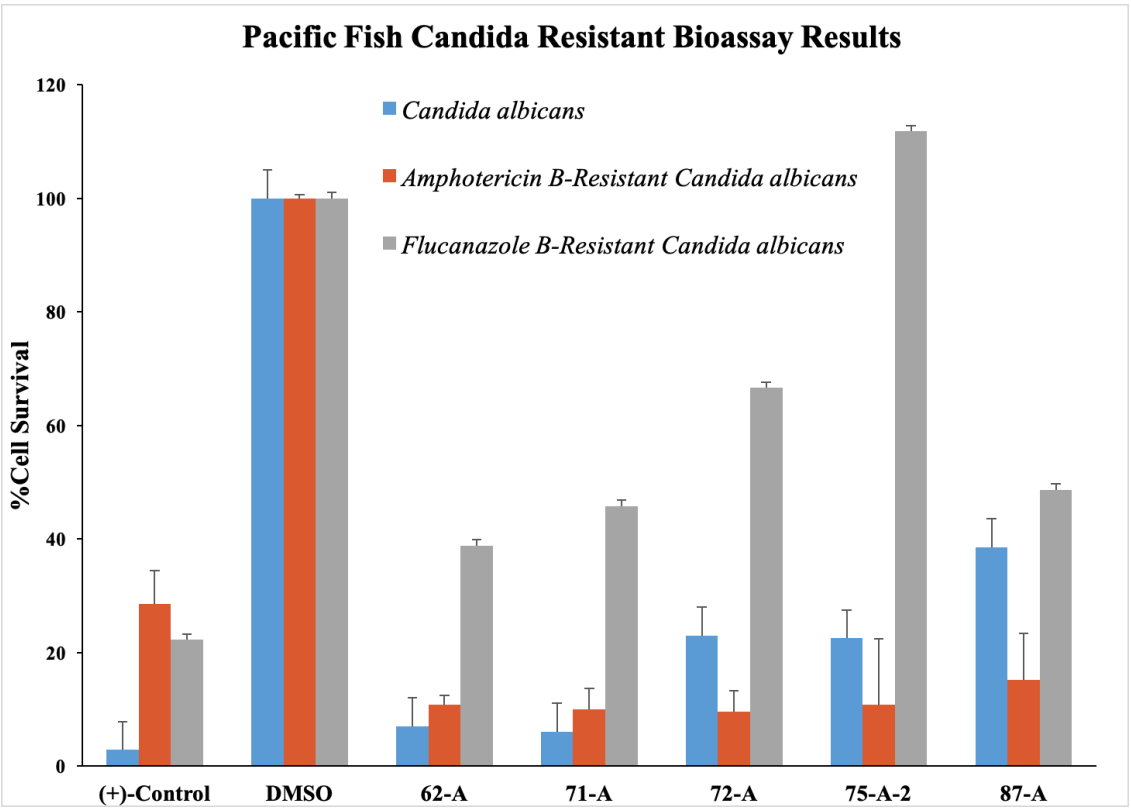


Figure 2.4 Activity of the Pacific fish microbiome against amphotericin B-resistant (ATCC 200955) and fluconazole B-resistant *Candida albicans* (ATCC 10231) compared to the non-resistant strain (ATCC 90027).

2.4 Conclusion

In our study, we explored the antimicrobial and cell inhibitory activities of bacterial extracts, isolated from the Pacific fish microbiome. Microbial samples from 70 different juvenile fish and 27 different genera were analyzed. Bacterial cultures were grown, metabolites were extracted, and their activity tested against gram positive and gram negative bacteria, yeast, and a colon carcinoma cell line. We found activity against highly relevant human pathogens concluding that extracts derived from fish mucous bacteria can serve as a source for bioactive natural products. In total, 47 strains were extracted to prepare extracts for bioactivity assessment. 14 strains were active against *Staphylococcus aureus*, five were active against *Candida albicans,* five were active against colon carcinoma cells, and one had moderate activity against *Escherichia coli.* Some strains exhibited selective activity in only one assay, e.g. metabolites

from 73-A have potential unique metabolites targeting Gram negative bacteria. *Pseudomonas aeruginosa* (1-D and 37-A), *Pseudoalteromonas carrageenovora* (74-A) and *Streptomyces bacillaris* (85-A) were capable of producing natural products with selective antibacterial and cytotoxic activity. One strain, *Psychrobacter* sp. (PF-24-A) showed lethal effects in all assays, and initial chemical analysis hints to the production of endotoxins, known toxic lipopolysaccharides from *Psychrobacter*.

The collected Pacific fish derived bacteria will be added to the Loesgen Lab strain collection and extracts will serve future screening campaigns. Currently, *Pseudoalteromonas carrageenovora* (PF-74-A), a marine genus of Gammaproteobacteria, and *Streptomyces bacillaris* (PF-85-A) are under investigation for the potent antimicrobial and cytotoxic activity. Terrestrial streptomycetes are known for being prolific producers of natural products and uninvestigated marine species may provide new scaffolds for drug discovery. For my thesis project, I selected strain *Pseudomonas aeruginosa* (PF-1-D) and identified the bioactive metabolites as detailed in Chapter 3.

In summary, future work on PF-74-A and 85-A strains derived from the Pacific Ocean fish microbiome will show if there is potential to isolate new metabolites. This survey discovered a high percentage of biological active extracts (40 %) when compared to terrestrial exploration.[26] Another aspect of interest will be the correlation between active strains and fish species, e.g. if certain fish species universally maintain bacterial species or select them, if the bioactivity data holds true against fish pathogens, if these fish are resistant to some of the agents we found, and/or what the function of these metabolites is, if not as host defense in the fish microbiome.

Chapter 3: CASE STUDY - BIOACTIVITY OF *PSEUDOMONAS* SP. PF-1-D

3.1 Introduction

Marine natural products (MNPs) have been shown to be an exemplary source of new antibiotics, especially against resistant pathogens that threaten human health.[4,26] Discovery of marine natural products exhibiting activity against resistant pathogens has steadily increased over the past 15 years.[4] In particular, marine microorganisms have continually provided unique MNPs with potent activity, such as mayamycin (**15**)[46] from the actinobacterium *Streptomyces* sp.[23] Inspired by the human microbiome project, researchers started to investigate the microbiome of marine organisms for natural products and unique bacteria. Sebastonic acid (**14**) was one of the first novel antibiotic harvested from the stomach microbiota of a Canadian Rock Cod caught near the coast of Santa Cruz.[34] Beyond sebastenoic acid's **(14**) powerful antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecium*, it provided further evidence that antibiotics can be originated from fish microbiome.[34] Here, we explore the Pacific fish microbiome and I studied in detail the metabolites from bacterial isolate PF-1-D. The strain was identified as *Pseudomonas aeruginosa* and its extract exhibited antibiotic activity against therapeutically relevant pathogens including methicillin-resistance *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. Metabolites were isolated using various chromatographic techniques, their structures elucidated by mass spectrometry and NMR methodology, and fractions/pure compounds were tested for bioactivity.

3.2 Experimental materials and methods

3.2.1 Identification of PF-1-D

For taxonomic analysis by 16S rRNA gene amplification, PF-1-D was grown in SYP-SW liquid culture for 5 days, the cells were collected by centrifugation at 14,000 rpm for 15 minutes. The cellular pellet was frozen in liquid N_2 and crushed using a micro-pellet pestle. The crushed cells were incubated with lysozyme at 37°C for 30 minutes followed by a 55°C incubation using 3.0 µL of proteinase K and 10% (w/v) sodium dodecyl sulfate (SDS) for 30 min. DNA was extracted in a 1:1 (v/v) phenol:chloroform solution by centrifugation at 12,000 rpm for 5 minutes followed by precipitation from the aqueous layer using 10%(v/v) 3M sodium acetate and isopropanol. The 16S rRNA region was amplified from the genomic DNA using the following primers: 8f (5`-AGAGTTTGATCMTGGCTCAG-3`) and 1513r (5`-ACGGCTACCTTGTTACGACTT-3`)[47], purchased from Integrated DNA Technologies, IDT. The PCR reaction mixture, totaling 50.0 µL, contained 20-40 ng of DNA template, 10 pmol of each of the previously listed primers, 25.0 µL of MangoMix™ (containing MangoTaq™ DNA polymerase, dNTPs, red and orange reference dyes and Mg^{2+}). The thermocycler program for amplification was as followed: 30 seconds at 94°C, 30 seconds at 55°C, and 72°C for 90 seconds for 30 cycles followed by a final extension step for 10 minutes at 72°C. PCR products were examined and purified by agarose gel electrophoresis using the PureLink™ quick gel extraction kit (purchased from Invitrogen by life technologies, Germany) according to the manufacturer's suggested protocol. Sequencing was carried out using an ABI 3730 capillary sequence machine at OSU's CGRB Core Facilities. Sequencing results were matched to existing bacterial genomes using two online databases (EzBioCloud 16S database[48] and NCBI BLAST[49] Database via Mega7[50])(http://www.ncbi.nlm.nih.gov/).

3.2.2 Growth and extraction methods

A marine nutrient minimal medium (SYP-SW)[44] containing soluble starch (10 g/L), yeast extract (4 g/L), peptone (2 g/L), and InstantOcean® (33.3 g/L), pH buffered to 7.0, was used for fermentation. For solid agar plates, 15 g/L agar was added to the growth media before sterilization. *P. aeruginosa* (PF-1-D) was inoculated onto fresh SYP-SW agar and grown for 3-5 days. A piece of agar growth was used to further inoculate 1 L SYP-SW liquid culture, kept constantly shaking at room temperature for 10-14 days. Cultures were clean streaked on SYP-SW agar plates prior to extraction to test for culture purity. After 10-14 days of growth, the pH of the culture was adjusted to 5.0-6.0. Notably, subtle acidification induced a pigmentation change in *Pseudomonas* sp. culture from green to pink.

Liquid culture was extracted with equal parts ethyl acetate followed by drying over $MgSO_4$ and then the organic extract was concentrated under reduced pressure. For culture growths of 2.5 L, extraction was performed using Amberlite XAD-7 absorber resin (Sigma Aldrich) followed by acetone extraction. Autoclaved, fresh XAD-7 resin was added to the culture after 10-14 days of growth to shake at room temperature for 24 hours. XAD-7 resin was collected by filtration through cheesecloth and soaked in acetone for 24 hours. Isolated resin in cheesecloth was then sonicated for 15 minutes with fresh acetone twice more to further extract organic compounds.

3.2.3 Fractionation methods

Organic extract was separated by polarity into 7 fractions by vacuum liquid column chromatography (VLCC) using normal phase silica gel and dichloromethane (DCM) and methanol (MeOH) in the following ratios: 99:1, 50:1, 30:1. 15:1, 9:1, 3:1, 0:1 respectively. First, the extract was dry loaded onto silica in a 1:10 ratio of extract:silica by mass. A 30 mL solid load

column was packed with 4 cm clean silica, dry loaded extract, and 2 cm of sand. 12 mL fractions at each solvent ratio was added to the column and eluted under vacuum into 20 mL vials. For C18 column chromatography separation, the extract was split into seven fractions using water and MeOH in the following ratios: 1:9, 1:5, 1:1, 3:1, 5:1, 9:1, 1:0 respectively. The extract was wet loaded onto a prepacked C18 cartridge (Sep-Pak). Fractions were analyzed by LCMS and tested in cell-based bioassays to guide isolation efforts.

3.2.4 Methods of isolation

All samples were filtered through a 0.45 μm nylon filter or centrifuged at 14,000 rpm for 5 min before LCMS and HPLC analysis. Analytical HPLC was performed using an Agilent 1100 using a Phenomenex kinetex C18 column, 5 μm × 150 mm × 4.6 mm. Preparative HPLC was performed to isolate compounds using an Agilent 1260 HPLC system using a Phenomenex Luna Prep 5u-C18(2), 100Å 250 mm x 21.2 mm column. Metabolites were analyzed using a gradient elution with ultra-pure H_2O and acetonitrile (ACN) with 0.05% formic acid in each solvent.

MY 12-62a **(16)** was isolated from VLCC fraction 5 (31 mg) using a Luna Prep 5u-C18(2) column and 50% ACN (50% water) isocratic method. 50 mgs of C18 fraction 3 on a Luna Prep 5u-C18(2) column with a 35% ACN isocratic elution yielded Phenazine-1-carboxylic acid **(17)**, Phenazine-1-carboxamide **(18)** and 1-hydroxyphenazine **(19)**. HQNO **(20)** and trans-Δ^1-NQNO **(21)** were isolated from C18 fraction 5, using a Phenomenex kinetex C18 column (5 μm × 150 mm × 4.6 mm) with a 52% ACN isocratic gradient. Direct isolation from agar extract on a Luna Prep 5u-C18(2) column and 20% ACN isocratic method yielded pigment pyocyanin **(22**). Isolated compounds were checked for purity using an appropriate isocratic method on LCMS and analytical thin layer chromatography (TLC). TLCs was performed on pre-coated silica gel 60 F254 plates (Eppendorf). TLC plates were visualized by UV (254 and 360 nm) and

by spraying with anisaldehyde solution followed by heating at 80 °C.

3.2.5 Structure elucidation

High resolution mass spectrometry data was collected on an Agilent 6230 TOF LC/MS system by direct inject. Solid phase infrared spectroscopy was run on a Nicolet 6700 FT-IR spectrometer (Thermo Scientific). ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and 2D NMR experiments including correlation spectroscopy (COSY), heteronuclear single-quantum correlation spectroscopy (HSQC), heteronuclear multiple-bond correlation spectroscopy (HMBC) were used to elucidate chemical structures. NMR experiments were conducted using either a 700 MHz or 500 MHz Bruker NMR instruments equipped with a 5 mm TCI cryoprobe or a 5 mm TXI probe, respectively, with the appropriate solvent signals used as an internal calibration standard. Pure compounds were dried from residual water by using a lyophilizer for 24 hours before being dissolved in deuterated solvents for NMR analysis. Pure compounds were dissolved in 600 µL of $CDCl_3$ or MeOD-d_4. Pure compounds of 1 mg or less were dissolved in 300 µL of deuterated solvent and placed into a Shigemi tube.

3.2.6 Bioactivity testing

Fractions and pure compounds were tested against methicillin-resistant *Staphylococcus aureus* (ATCC BAA-41) (MRSA) and human colon carcinoma (HCT-116) in cell-based bioassays. Samples were tested at 125 µg/mL for microbial assays and 10 µg/mL for carcinoma cell assays. Assay protocol and test conditions are detailed in section 2.2.

3.3 Results and discussion

The bacterium PF-1-D was isolated from the mucous of a juvenile pink surfperch (*Zalembius rosaceus*) off the coast of California. The specimen was swabbed by marine biologist Dr. Misty Paig-Tran and her team (California State University Fullerton). PF-1-D was identified

as a *Pseudomonas* sp. by 16S rRNA gene amplification. Seven known metabolites were isolated and elucidated resulting in the identification of three potent antibiotics and one moderate cytotoxic compound **(Figure 3.1).**

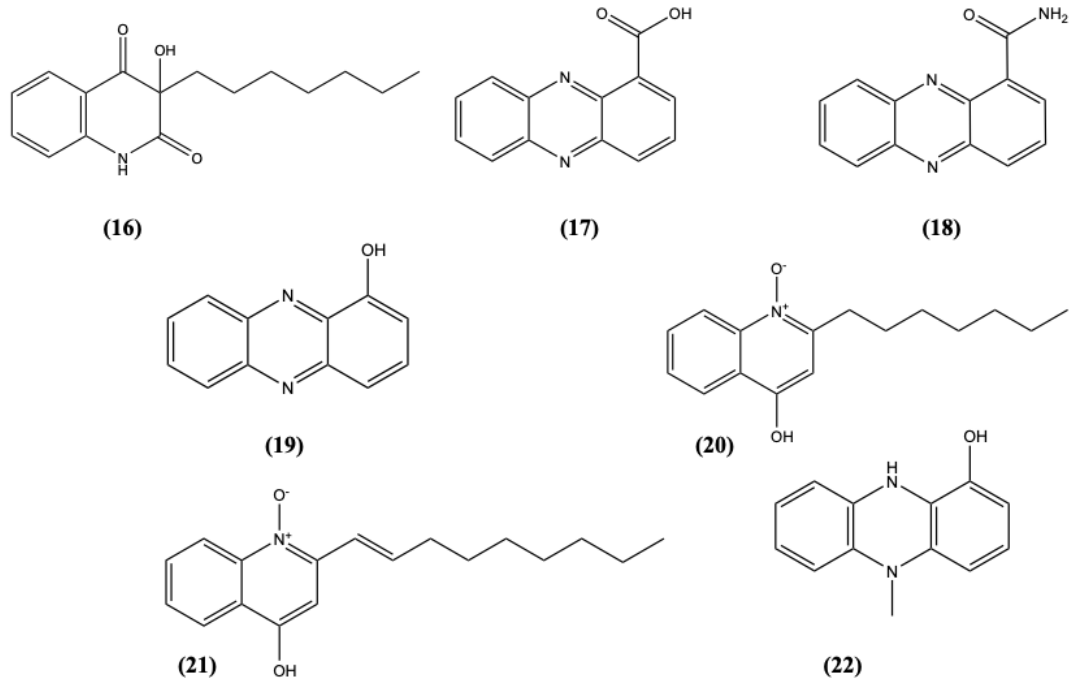


Figure 3.1 Structures for compounds **16-22** isolated from *Pseudomonas aeruginosa* (PF-1-D).

One 1 L culture was extracted with ethyl acetate to obtain an extract. The organic extract was further separated by polarity into 7 fractions by vacuum liquid column chromatography (VLCC). VLCC fractions were tested in single-dose bioassays and tested for activity against methicillin-resistant *Staphylococcus aureus* (ATCC BAA-41) (MRSA), and human colon carcinoma (HCT-116) (**Figure 3.2**). Fractions two, three, four, six and seven exhibited potent activity against MRSA. Fraction 5 was the only VLCC fraction active against colon carcinoma cells. Inhibition of MRSA by fraction 5 was only moderate with about 40% cell survival. Quantities for fractions 2, 3, 4, 6 and 7 were too small for further isolation efforts, and no chemical entities could be easily identified. With ample material for isolation, cytotoxic

metabolites from VLCC fraction 5 were prioritized to be analyzed in more detail.

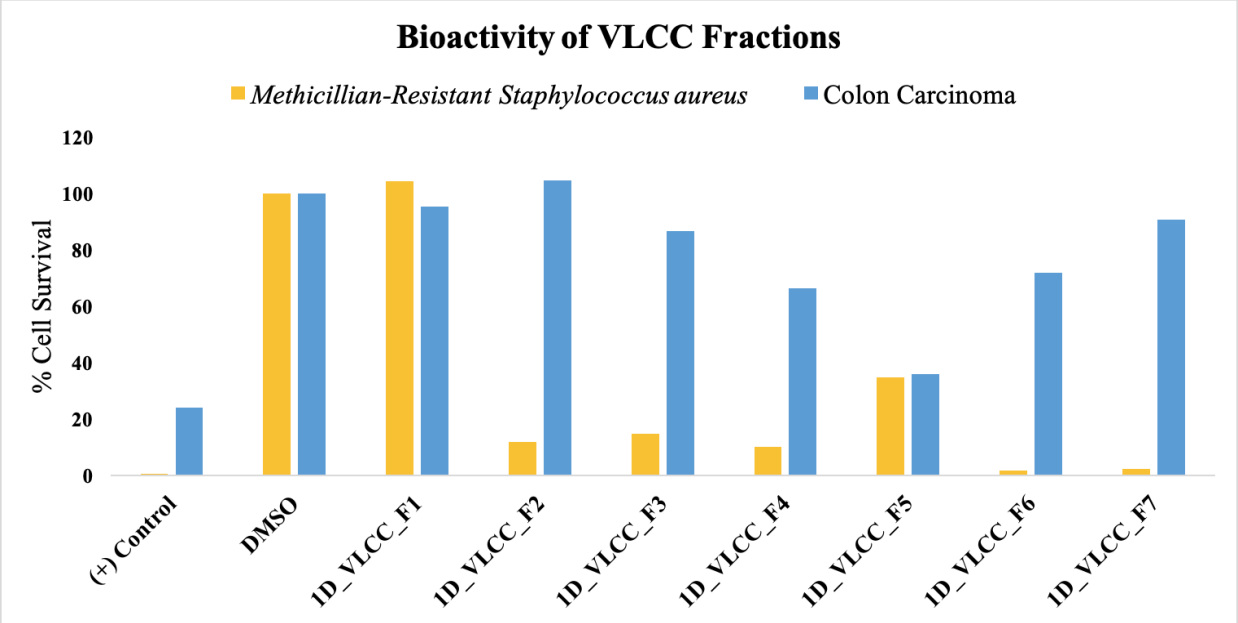


Figure 3.2 Inhibition of multidrug-resistant *S. aureus* (ATCC BAA-44) and colon cancer (HCT-116) by *Pseudomonas sp.* fractions eluted by VLCC separation. Fractions Tested at 125 µg/mL.

Compound 3-heptyl-3-hydroxyquinoline-2,4(*1H,3H*)-dione (**16**) was isolated with a yield of 3 mg/L. The molecular formula was assigned as $C_{16}H_{21}NO_3$, based on an m/z value of 276.2 for $[M+H]^+$ and an m/z value of 573.2923 for $[2M+Na]^+$; calc. for $C_{32}H_{42}N_2NaO_6+$, 573.294; Δppm = 2.1. UV absorbance and infrared spectra matched published literature values for **16**.[51] ^1H and ^{13}C NMR in $CDCl_3$ matched literature values published by Kitamura in 1986 (Appendix X).[52] COSY, HSQC, and HMBC spectra in $CDCl_3$ defined key spin systems and 2D correlations, confirming the positions of key functional groups and heteroatoms. The compound was first isolated from *Pseudomonas aeruginosa* and made synthetically by Nuenhaus et. al. in 1978.[51] In 1986, **16** was named MY 12-62a by Kitamura et. al. and described as a nonselective lipoxygenase inhibitor isolated from *Pseudomonas methanica.*[52] Later, **16** was reported again from a *Pseudomonas aeruginosa* associated with the sponge *Suberea creba.*[53] Here, **16**

exhibited moderate cytotoxicity against colon carcinoma, an unreported bioactivity for this compound.

A second liquid culture of *P. aeruginosa* was grown (5 L) to allow for addition material to isolate from. Extraction was assisted by XAD-7 absorptive resin and acetone solvent. The organic extract was further separated by polarity into 6 fractions by C18 column chromatography. Analysis by LCMS guided the separation efforts to purify metabolites via C18 column chromatography (**Figure 3.3**). Rough polarity fractions from C18 were tested in single-dose bioassays for inhibition against methicillin-resistant *Staphylococcus aureus* (ATCC BAA-41) (MRSA) and human colon carcinoma (HCT-116) (**Figure 3.4**). Fraction 3 showed moderate inhibition of MRSA while fractions 4, 5, and 6 showed potent inhibition. The most cytotoxic fraction was C18 fraction 5 with only ~3% cell survival. C18 fractions 4, 5, and 6 also showed moderate cytotoxicity. Ample material and strong to moderate assay activity, fractions 3 and 5 was chosen for further analysis.

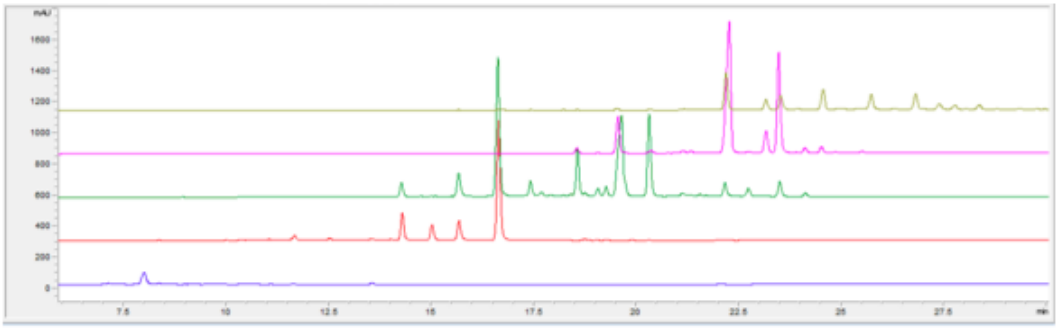


Figure 3.3 254 nm LCMS chromatogram of C18 fractions: 1, 2 (blue), 3 (red), 4 (green), 5 (magenta), and 6 (yellow).

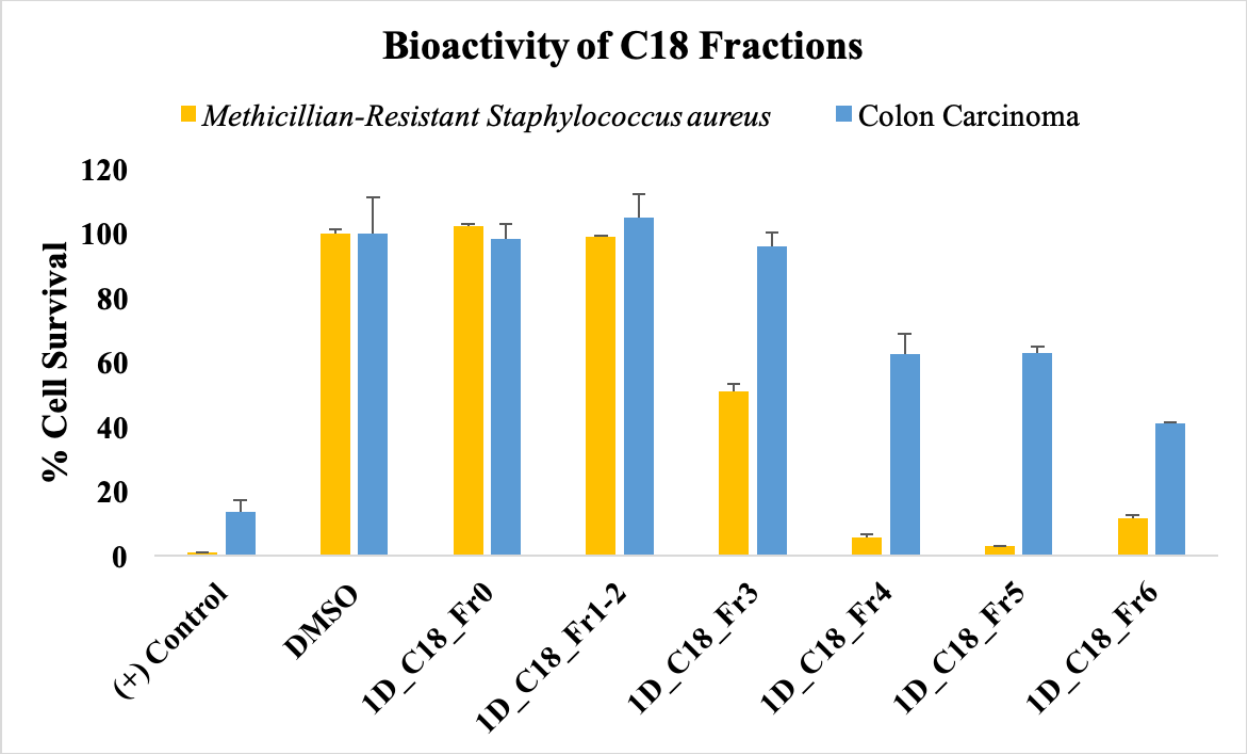


Figure 3.4 Inhibition of multidrug-resistant *S. aureus* (ATCC BAA-44) and colon cancer (HCT-116) by *Pseudomonas sp.* fractions eluted from C18 Sep-Pak separation. Fractions tested at 125 µg/mL.

Phenazine-1-carboxylic acid **(17)** and phenazine-1-carboxamide **(18)** were obtained as yellow solids. The structures of **17** and **18** were identified based on UV, LRMS, and spectroscopic data was closely matching the literature values for these compounds known from *Pseudomonas* sp.[54-57] (**Appendix**). Two distinct ^{1}H signals for each of the amide protons were observed in the ^{1}H NMR for **18** at 9.75 ppm and 8.14 ppm which is only reported in some isolation studies.[55-57 **18** was found to be a potent inhibitor of MRSA, which agreed with previously reported activity.[58] A minimum inhibitory concentration (MIC) value of 250 µg/mL against MRSA was previously found by Cardoza et. al. for **18**.[58]

A third phenazine derivative, 1-hydroxyphenazine **(19),** was isolated in minute quantity of less than 0.5 mg but the UV chromatogram clearly matched literature values[59]. An LRMS m/z value of 197.1 for $[M+H]^{+}$ matched the structure of **19**. **19** is a known metabolite produced

by *Pseudomonas* sp.[59,60] While the biological origin, UV, and molecular weight data shown herein provide strong evidence for the isolation of **19**, without more material and additional spectroscopic evidence the identity of this metabolite cannot be established. However, bioactivity-guided fractionation using MRSA and HCT-116 indicated that 1-hydroxyphenazine (**19)** was not the active component.

2-heptyl-4-hydroxyquinoline n-oxide (HQNO) (**20**) was isolated as a white power. The chemical formula was assigned as $C_{16}H_{21}NO_2$, based on the m/z value of 519.3213 for $[2M+H]^+$; calc. for $C_{32}H_{43}N_2O_4^+$, 519.322; Δppm = 0.84. UV maxima of 235, 325 and 340 nm for **20** matched known literature values.[61] ^1H NMR and ^{13}C NMR data recorded in MeOD-d_4 matched literature values[62] **(Appendix)**. Production of **20** was previously reported for *Pseudomonas aeruginosa* along with its known antibiotic activity against MRSA.[63] Cell survival was 19 % for **20.** Many quinolones, including **20** are known cytochrome inhibitors that specifically target the bc_1 complex in the electron transport chain.[64,65]

Trans-Δ^1-2-(non-1-enyl)-4-quinoline N-oxide (trans-Δ^1-NQNO) **(21)** was isolated as a white solid. A chemical formula of $C_{18}H_{23}NO_2$ was proposed from an m/z value of 569.3403 of $[2M-H]^-$; calc. for $C_{36}H_{45}N_2O_4$-, 569.338; Δppm = 3.2. HRMS and NMR spectroscopic data agreed with literature values[64] (**Appendix)**. Previous research articles on metabolites from *P. aeruginosa* report on an unsaturated quinolone derivative resembling **21** that was first synthesized with reported spectroscopic data by Szamosvari et. al in 2017.[64] Cell survival of MRSA for **21** 7% revealed that **21** is as stronger inhibitor compared to **20** in our assays. **21** has reported MIC values of 10-25 μg/mL against several strains of *S. aureus*, including MRSA.[64] The potent activity of **21** suggests the rigidity and structure of the trans-unsaturated alkyl chain is a more effective inhibitor, mimicking the native ligand in the electron transport chain.[64]

Pyocyanin **(22)** was isolated from agar cultures and extracted with chloroform. An m/z value of 211.0873 for $[M+H]^+$ by HRMS confirmed a formula of $C_{13}H_{10}N_2O$; calc. for $C_{13}H_{11}N_2O+$, 211.087; Δppm = 3.3. HRMS and UV maxima matched literature values.[59] Liquid and agar cultures of PF-1-D exhibited a blue-green color that is attributed to pigment **22** and known from *P. aeruginosa*[66,67] **(Figure 3.11)**. Acidification of the culture induced a noticeable pigmentation change from blue-green to pink, a reaction known for **22**[66,67] **(Figure 3.11)** . Pyocyanin **(22)** has been reported as a virulence factor known to *Pseudomonas* sp.[66] and Szamosvari et. al reported an MIC of 50 µg/mL against *S. aureus*.[64]

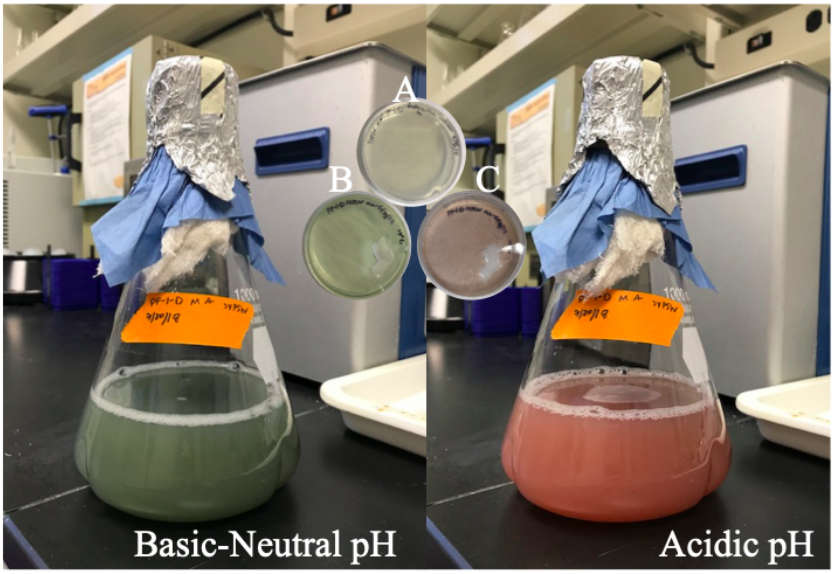


Figure 3.5 Liquid and agar cultures of *Pseudomonas sp.* PF-1-D undergo pigmentation change under acidic conditions due to production of pigment pyocyanin (22).[66,67]

3.4 Conclusion

Bioactivity guided fraction of the *Pseudomonas* sp. PF-1-D extract lead to isolation of seven known metabolites. Dereplication was accomplished using UV, NMR, IR, and HRMS analysis. Phenazine-1-carboxamide **(18)** was found to be responsible for the activity against MRSA observed in fraction 3. 2-heptyl-4-hydroxyquinoline n-oxide (HQNO) **(20)** and trans-Δ^1-2-(non-1-enyl)-4-quinoline N-oxide **(21)** exhibited strong anti-MRSA activity when tested at 125

μg/mL with minimal cytotoxic activity. Multiple chemical entities in fraction 5 matched the UV absorbance patterns of **20** and **21** with varying masses and increasing retention times. Many hydroxyalkyl-quinolines derivatives are known to be signaling molecules and/or virulence factors produced by *P. aeruginosa*.[68] However, antimicrobial activity has been found to vary significantly among derivatives. N-oxides are found to be more potent inhibitors with more activity coming from unsaturated N-oxide derivatives. Saturated NQNO was found to have an MIC of 200 μM against *S. aureus* compared to an MIC of 10-12 μM for the unsaturated derivative.[64] Unsaturated trans-NQNO conformational isomers also vary in activity.[64] Comparatively, the cis-isomer showed no activity at 200 μM.[64] Differences in the activity of the NQNO derivatives indicates the importance of the trans configured unsaturation. Competitive binding strength in the bc_1 complex of the electron transport chain is greatly affected by changes to the carbon chain on hydroxyalkyl-quinolines.[64,65] **20** and **21** showed strong inhibition of *S. aureus* and MRSA which could serve as a starting point for new, potent antibiotics. Bottcher et. al. reported **21** as the most potent 2,4-hydroxyalkyl quinolone from *P. aeruginosa.*[69] C18 fraction 6 exhibited strong inhibition against MRSA, but metabolites have yet to be isolated and characterized. UV chromatograms reveal metabolites belong to the class of quinolines related to **20** and **21** This research shows there are ample antibiotics produced by bacteria in fish mucous and exploration of the microbiome can identify new active metabolites to fight infectious diseases and cancer.

CHAPTER 4: CONCLUSION

4.1 Conclusion

From the external mucous of Pacific Ocean juvenile fish, 47 unique bacterial strains were isolated, grown, and extracted. The organic extracts of each of the 47 bacterial strains were tested against a microbial pathogen panel (*Staphylococcus aureus* (ATCC 25923), methicillin- resistant *S. aureus* (ATCC BAA-41), *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 90027), amphotericin B-resistant *C. albicans* (ATCC 200955), and fluconazole-resistant *C. albicans* (ATCC 10231) and human colon carcinoma cell line (HCT-116). Approximately 40% of the organic extracts were active in at least one of the antimicrobial and colon carcinoma panel, highlighting the potential of the fish external microbiome as a source of anti-infective compounds. 1-D, 37-A, 74-A, and 85-A were active against MRSA and colon cancer. 62-A was active against fluconazole-resistant *Candida albicans* and colon cancer. Future work aims to investigate the chemical ecology of selected bacteria in their associated fish source. Whole fish with intact mucous will be tested for either excreted metabolites from the active bacteria from initial isolation, or 16S sequencing for the DNA barcode of the bacteria.

From the initial screening of 47 unique organic extracts, one extract from the bacterium *Pseudomonas* sp. (PF-1-D) was bioactive against MRSA and HCT-116 colon carcinoma led to isolation of seven known metabolites. Dereplication was accomplished using spectroscopic techniques, including nuclear magnetic resonance (NMR) and liquid chromatography mass spectrometry (LCMS), and literature comparisons. Phenazine-1-carboxamide was found to be one of the potent inhibitors of MRSA from the extract. The compounds HQNO **(20)** and trans-Δ^1-NQNO **(21)** also exhibited potent anti-MRSA activity. Exploration of metabolites from *Pseudomonas* sp.as a specific case study illustrated the use of a unique source for natural products with potent biological activity. Our results have shown that there are several active metabolites that remain unelucidated in *Pseudomonas* sp.

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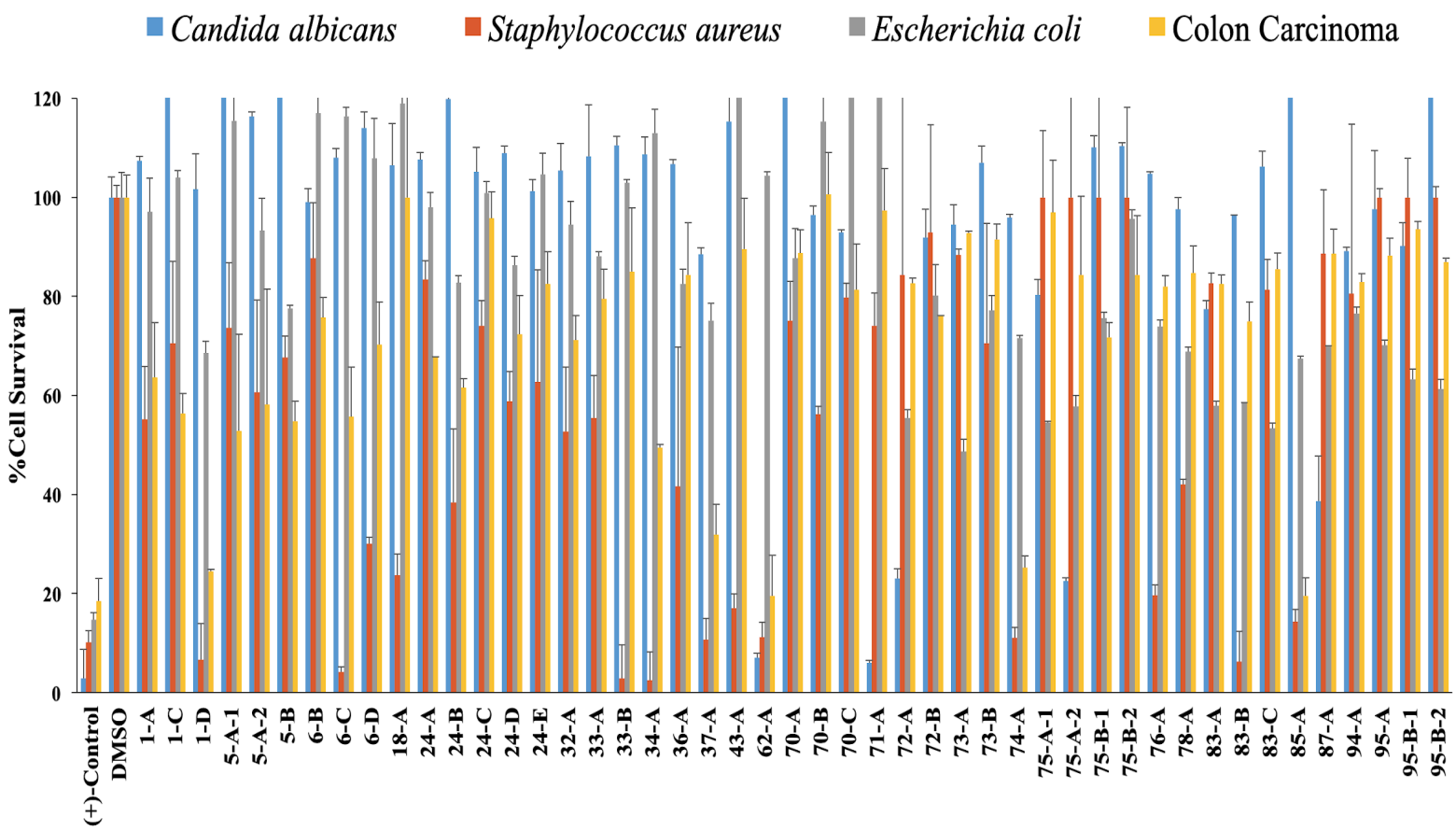
APPENDICES

Appendix 1 Fish species, swabs assignments and internal IDs

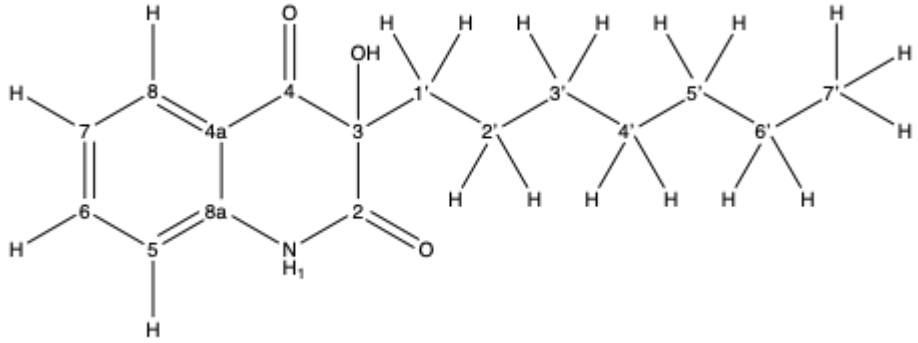
Lab Identifi	Fish Name:	Species:	Genus:
PF_1	Pink Surfperch	Zalembius	rasaceus
PF_2	Tongue Fish, T1	Symphurus	atricauda
PF_3	Tongue Fish, T2	Symphurus	atricauda
PF_4	Tongue Fish, T3	Symphurus	atricauda
PF_5	White Croaker	Genyonemus	lineatus
PF_6	Poacher	Agonidae	sp.
PF_7	Hornyhead Tur Bot, HH1	Pleuronichthys	verticalis
PF_8	Hornyhead Tur Bot, HH2	Pleuronichthys	verticalis
PF_9	Hornyhead Tur Bot, HH3	Pleuronichthys	verticalis
PF_10	Hornyhead Tur Bot, HH4	Pleuronichthys	verticalis
PF_11	Longfin Sanddab, LF 1	Citharichthys	xanthostign
PF_12	Longfin Sanddab, LF 2	Citharichthys	xanthostign
PF_13	Longfin Sanddab, LF 4	Citharichthys	xanthostign
PF_14	California Halibut	Paralichthys	californicus
PF_15	Dover Sole	Microstomus	pacificus
PF_16	Bigmouth Sole	Hippoglossina	stomata
PF_17	English Sole, ES 1	Parophrys	vetulus
PF_18	English Sole, ES 2	Parophrys	vetulus
PF_19	English Sole, ES 3	Parophrys	vetulus
PF_20	Pacific Sanddab	Citharichthys	stigmaeus
PF_21	Spotted Turbot, ST 1	Pleuronichthys	ritteri
PF_22	Spotted Turbot, ST 2	Pleuronichthys	ritteri
PF_23	Longspin Combfish, CF1	Zanniolepis	latipinnis
PF_24	Longspin Combfish, CF2	Zanniolepis	latipinnis
PF_25	Longspin Combfish, CF3	Zanniolepis	latipinnis
PF_26	Longspin Combfish, CF4	Zanniolepis	latipinnis
PF_27	Longspin Combfish, CF5	Zanniolepis	latipinnis
PF_28	Lizardfish, L1	Synodontidae	sp.
PF_29	Lizardfish, L2	Synodontidae	sp.
PF_30	Lizardfish, L3	Synodontidae	sp.
PF_31	Lizardfish, L4	Synodontidae	sp.
PF_32	Lizardfish, L5	Synodontidae	sp.
PF_33	Skate, S1	Rajidae	sp.
PF_34	Skate, S2	Rajidae	sp.
PF_35	Octopus, O1	Cephalopoda	sp.
PF_36	Octopus, O2	Cephalopoda	sp.
PF_37	Midshipman, M1	Porichthys	spp.
PF_38	Midshipman, M2	Porichthys	spp.
PF_39	Midshipman, M3	Porichthys	spp.
PF_40	Midshipman, M4	Porichthys	spp.
PF_41	Midshipman, M5	Porichthys	spp.
PF_42	Midshipman, M6	Porichthys	spp.
PF_43	Grass Rockfish	Sebastes	rastrelliger
PF_44	Halfbanded Rockfish, HR1	Sebastes	semicincus
PF_45	Halfbanded Rockfish, HR2	Sebastes	semicincus
PF_46	Northern Blue Lanternfish	Tarletonbeania	crenularis
PF_47	Northern Blue Lanternfish	Tarletonbeania	crenularis
PF_48	Crested Bigscale	Poromitra	crassiceps
PF_49	Crested Bigscale	Poromitra	crassiceps
PF_50	California Smoothtongue	Leuroglossus	stilbius
PF_51	California Smoothtongue	Leuroglossus	stilbius
PF_52	Blackbelly Dragonfish	Stomias	atriventer
PF_53	Blackbelly Dragonfish	Stomias	atriventer
PF_54	Benttooth Bristlemouth	Cyclothone	acclinidens
PF_55	Benttooth Bristlemouth	Cyclothone	acclinidens
PF_56	Broadfin lanternfish	Nonnobrachium	ritteri
PF_57	Broadfin lanternfish	Nonnobrachium	ritteri
PF_58	Dollar Hatchetfish	Sternoptx	diaphana
PF_59	Dollar Hatchetfish	Sternoptx	diaphana
PF_60	Snipe Eel	Nemichthys	scolopaceus
PF_61	Snipe Eel	Nemichthys	scolopaceus
PF_62	Midwater Eelpout	Melanistigma	pammelas
PF_63	Midwater Eelpout	Melanistigma	pammelas
PF_64	Myctophids		
PF_65	Myctophids		
PF_66	Myctophids		
PF_67	Myctophids		
PF_68	Myctophids		
PF_69	Myctophids		
PF_70	Bristlemouth		
PF_71	Bristlemouth		
PF_72	Bristlemouth		
PF_73	Bristlemouth		
PF_74	Eel Pout		
PF_75	Eel Pout		
PF_76	Eel Pout		
PF_77	Eel Pout		
PF_78	Big Scale		
PF_79	Big Scale		
PF_80	Big Scale		
PF_81	Big Scale		
PF_82	Myctophids		
PF_83	Myctophids		

Pacific Fish Total Bioassay Results



Appendix 3 Spectroscopic data for MY 12-62a **(16)**

Structure and positions for MY 12-62a **(16)**



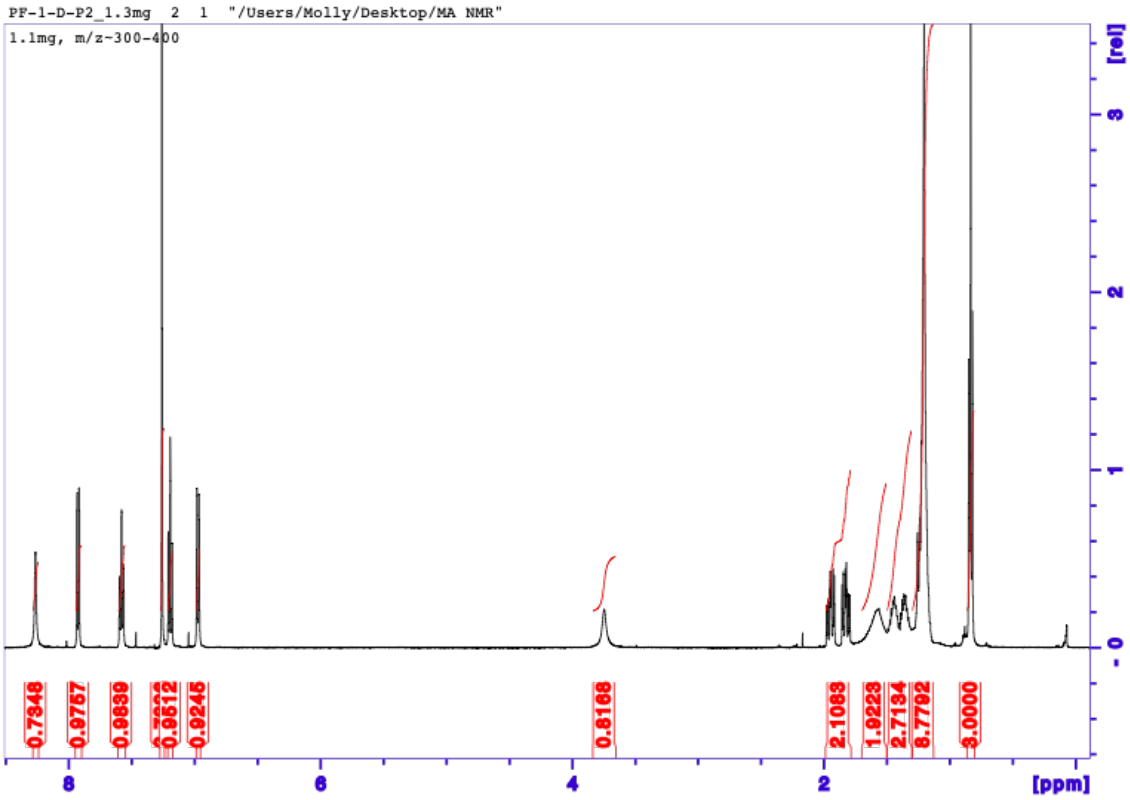
^{1}H (500MHz) and ^{13}C NMR (700MHz) in $CDCl_3$

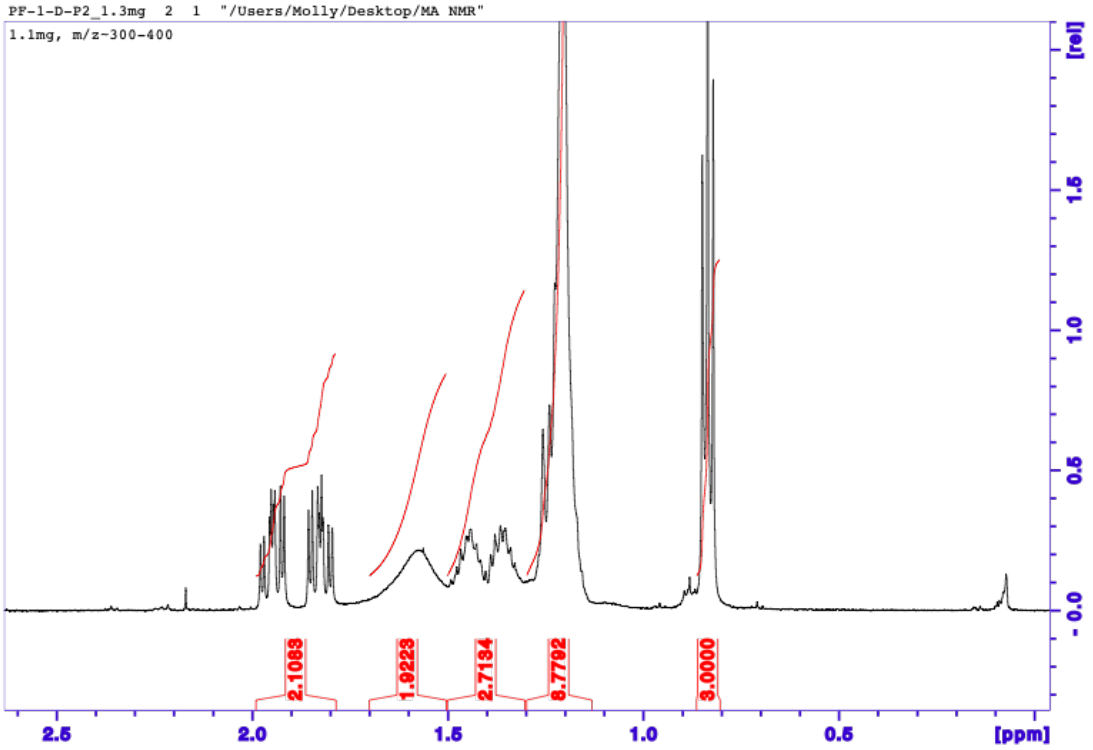
Position	Experimental δC, mult., J(Hz)	Experimental δH, mult., J(Hz)	Literature δC, mult., J(Hz)	Literature δH, mult., J(Hz)
2	172.8		173.7	
3	83.1		82.7	
4	194.9		195.9	
4a	119.1		119.2	
5	128.2	7.93, dd, J = 7.89, 1.31	127.7	7.91, dd, J = 7.8, 1.5
6	124.2	7.19, td, J = 7.61, 1.44	123.8	7.18, dd, J = 7.5, 6.8
7	136.6	7.58, t, J = 7.57	136.4	7.59, ddd, J = 7.8, 6.8, 1.5
8	116.8	6.97, d, J = 8.12	116.6	7.07, d, J = 7.8
8a	139.9		140.6	
1'	41	1.89, m	41.0	1.8, m
2'	31.4	1.41, m	31.6	1.2, m
3'	28.8	1.19, m	29.3	1.2, m

4'		1.12, m	28.9	1.2, m
5'	22.5	1.235, m	22.8	1.2, m
6'	22.5	1.235, m	22.5	1.2, m
7'	14	.836, t, J = 6.77	14.0	.83, t
3-OH		3.747, br s)		3.83, br s
1-NH		8.266, br s)		9.30, br s

Spectra for My 12-62A **(16)**

^{1}H NMR Spectra

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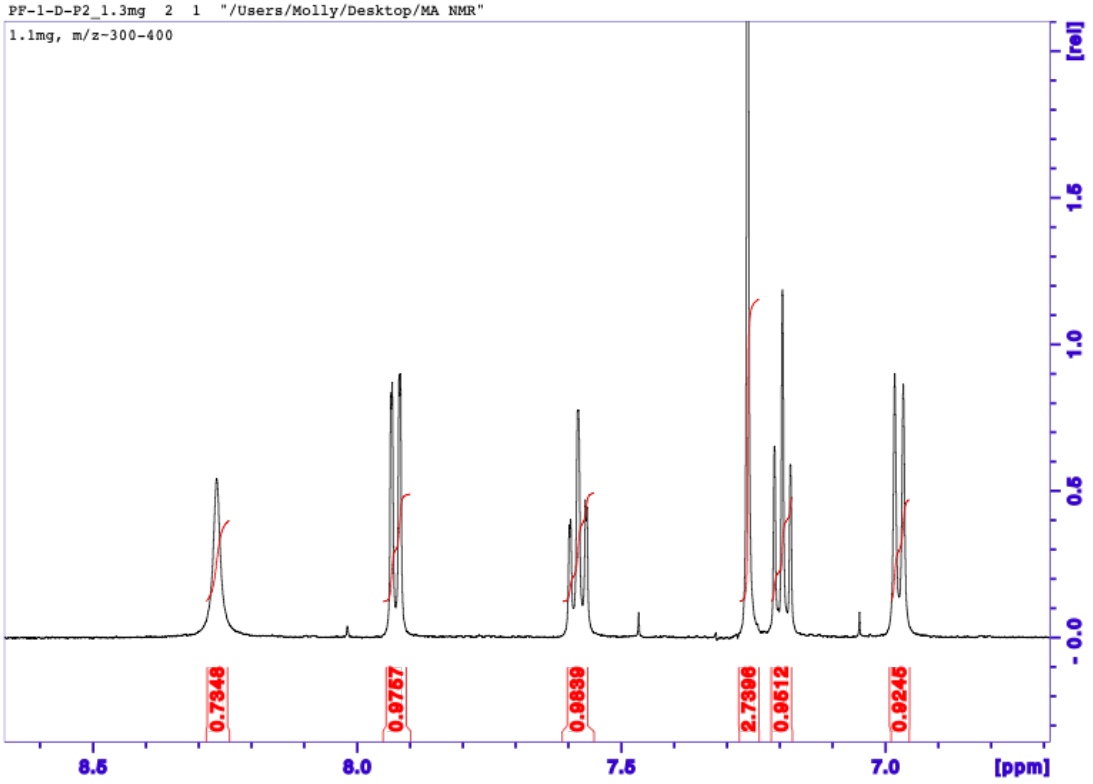
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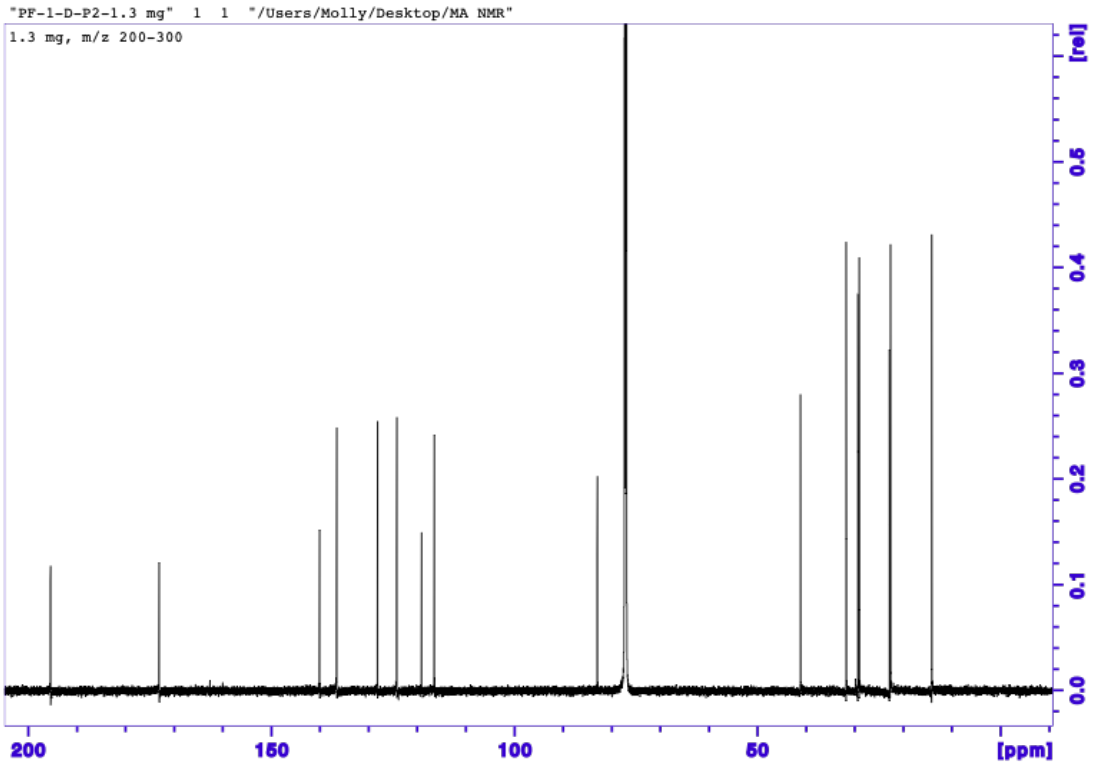
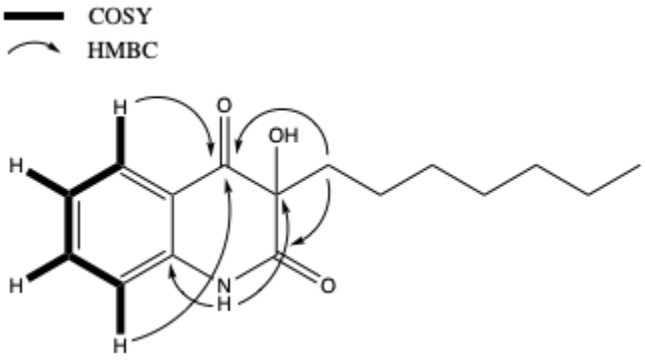
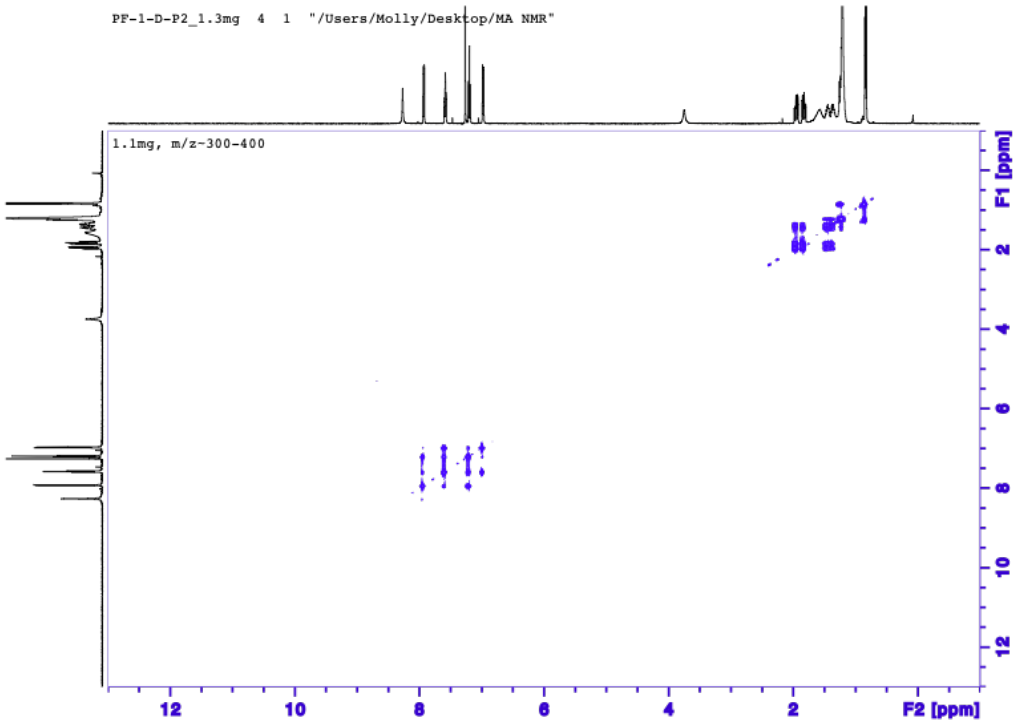
^{13}C NMR spectra



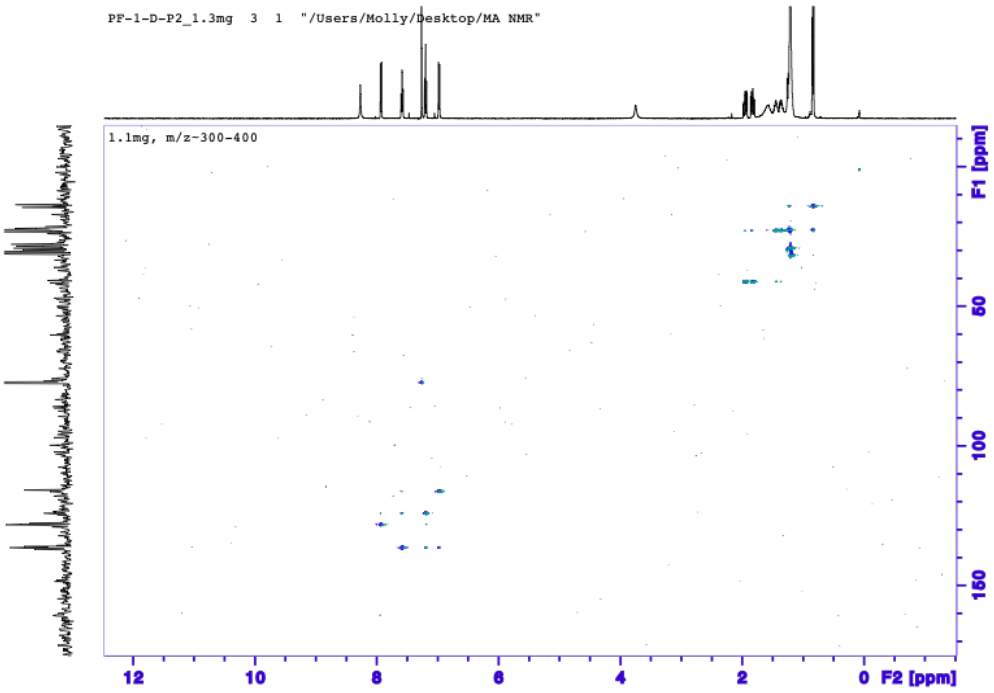
Key COSY and HMBC correlations



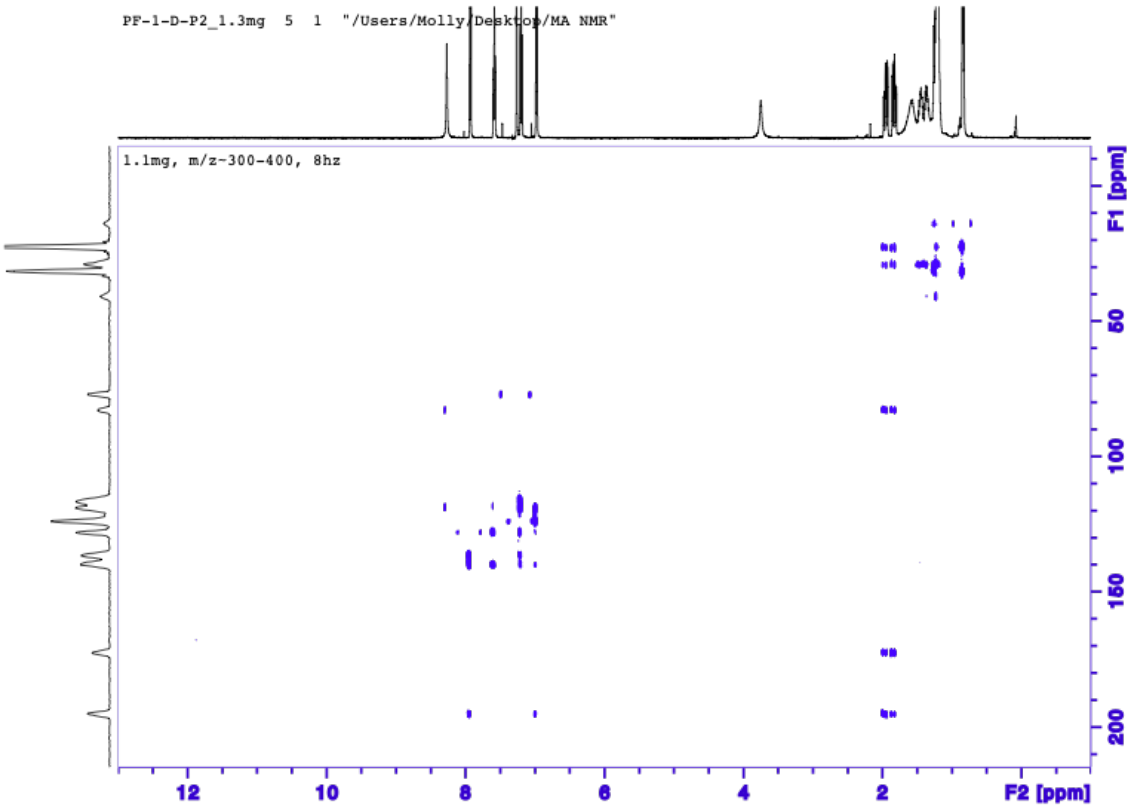
COSY (500MHz. $CDCl_3$)



HSQC (500MHz. $CDCl_3$)



HMBC (500MHz. $CDCl_3$)



Appendix 4 Spectroscopic data for phenazine-1-carboxylic acid **(17)**

^{1}H and ^{13}C NMR spectra (700 MHz; $CDCl_3$)

Experimental δH	Literature δH	Experimental δC	Literature δC
15.58	15.62	166.0	166.1
9.01 dd	9.01 dd	144.14	143.72
8.57 dd	8.42 dd	143.42	143.36
8.38 dd	8.28 m	140.13	141.73
8.32 dd	8.23 m	139.87	141.03
8.09-7.98 m	7.97 m	137.49	136.16
8.09-7.98 m	7.9 m	135.16	134.53
8.09-7.98 m	7.9 m	133.25	131.95
		131.76	131.27
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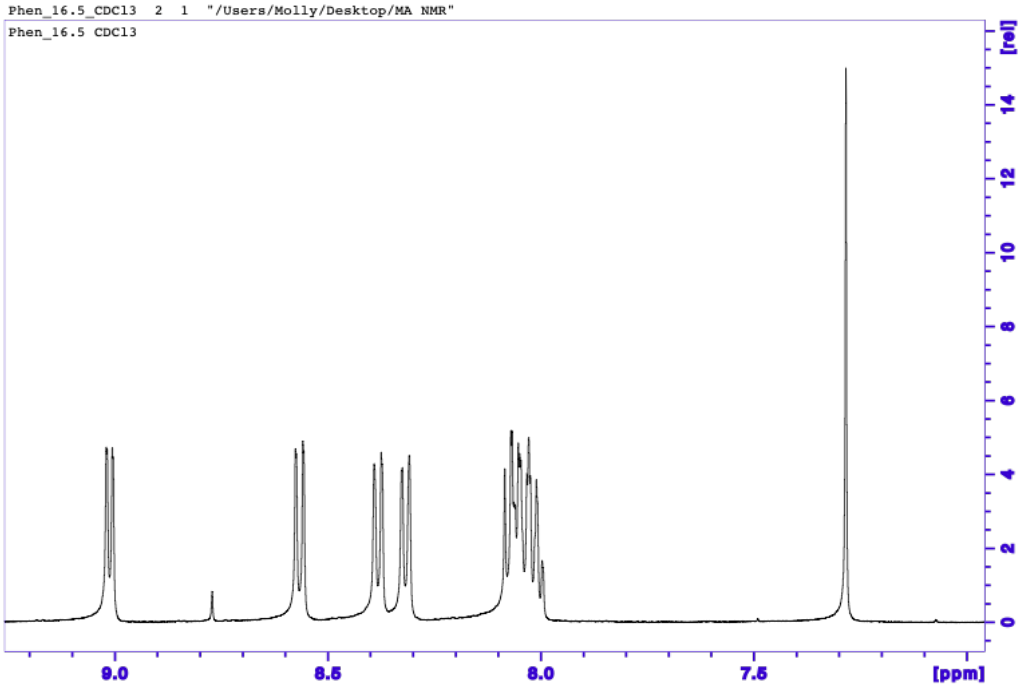
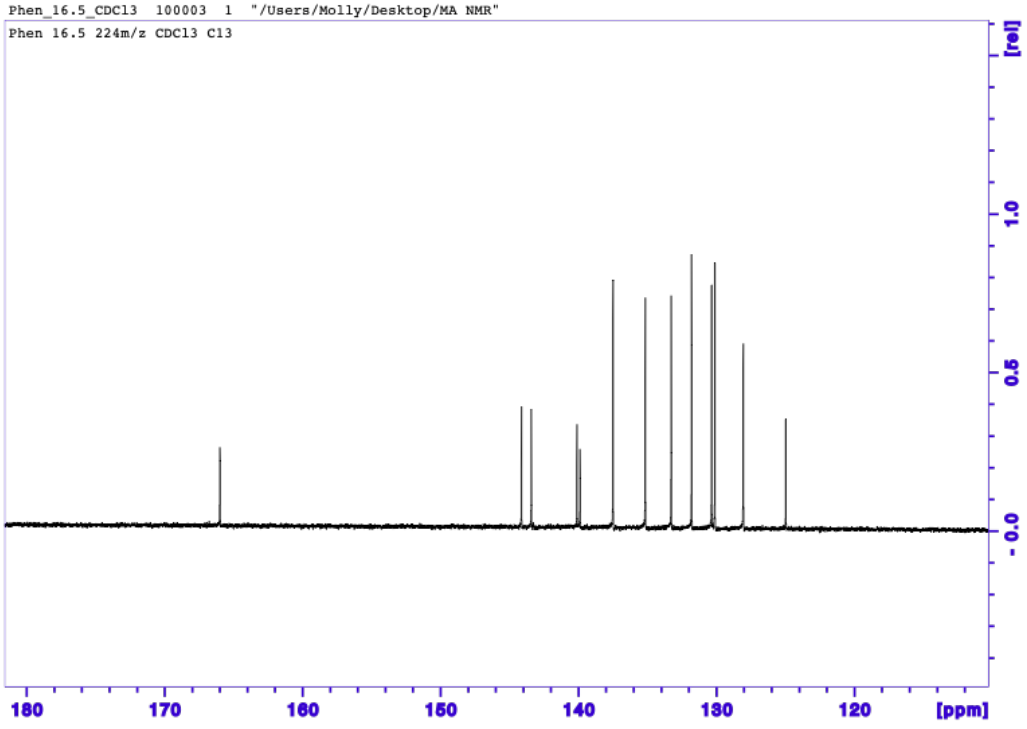
		130.12	130.09
		128.02	129.97
		124.96	129.31

^{1}H NMR Spectra



^{13}C NMR Spectra

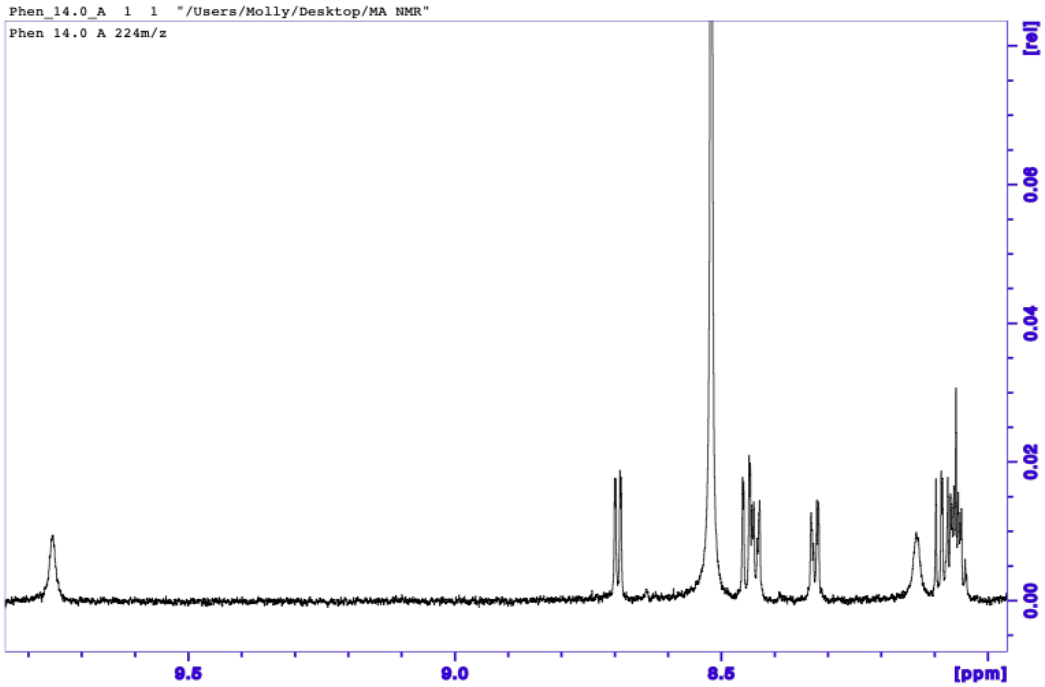


Appendix 5 Spectroscopic data for phenazine-1-carboxamide **(18)**

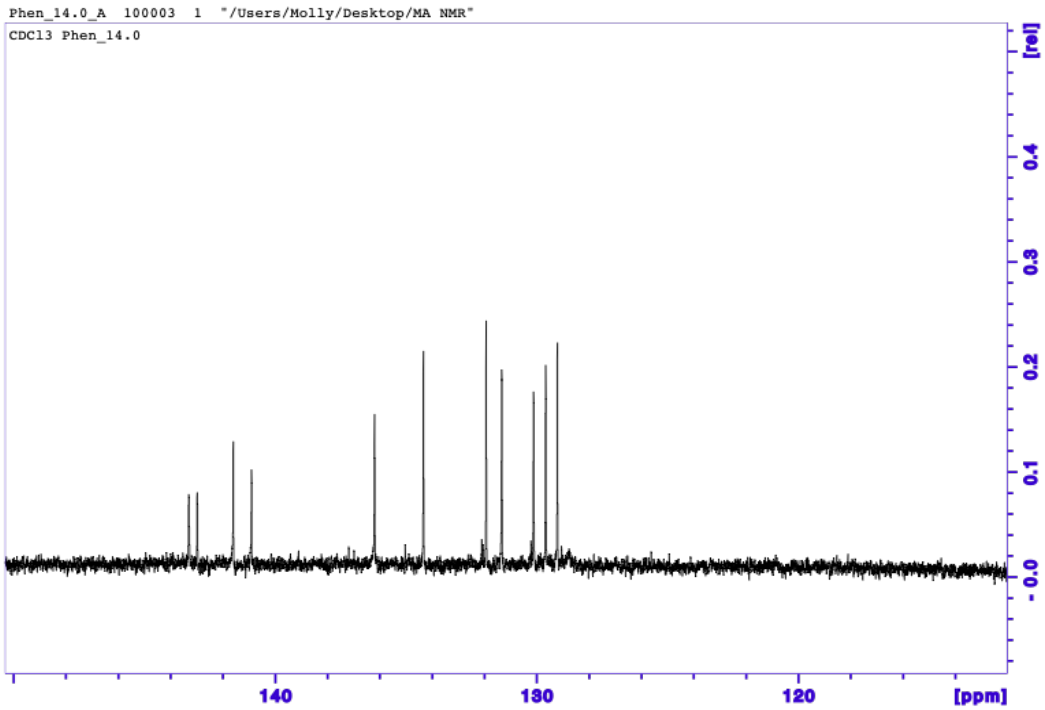
^{1}H and ^{13}C NMR spectra (700 MHz; $CDCl_3$)

Experimental δH	Literature δH	Experimental δC	Literature δC
8.69 dd	9.02 dd	-	166.54
8.46 dd	8.44 dd	143.31	143.56
8.44 dd	8.30 ddd	142.98	143.20
8.32 d	8.24 ddd	141.59	141.55
8.08 dd	7.98 dd	140.93	140.85
8.06 m	7.94 ddd	136.23	136.0
8.06 m	7.92 ddd	134.36	134.35
9.75 (N-H)	10.72	131.94	131.75
8.14 (N-H)	6.32	131.33	131.07
		130.09	129.90
		129.65	129.81
		129.21	129.12
			128.12

^{1}H NMR Spectra

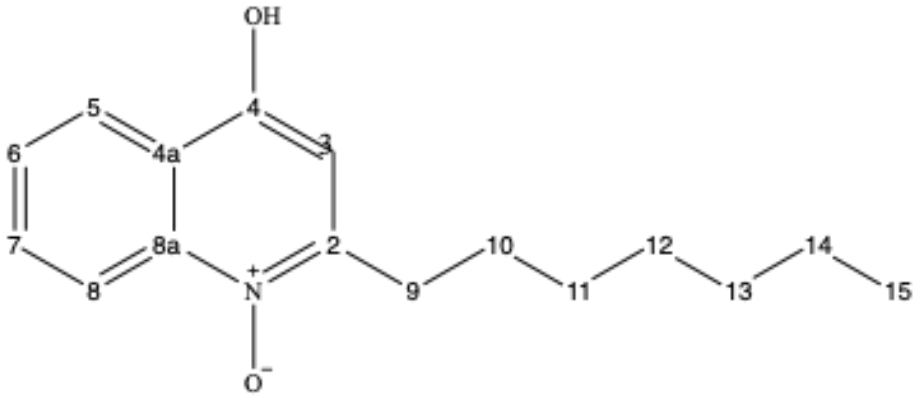


^{13}C NMR Spectra



Appendix 6 Spectroscopic data for HQNO **(20)**

Structures and positions for **20**

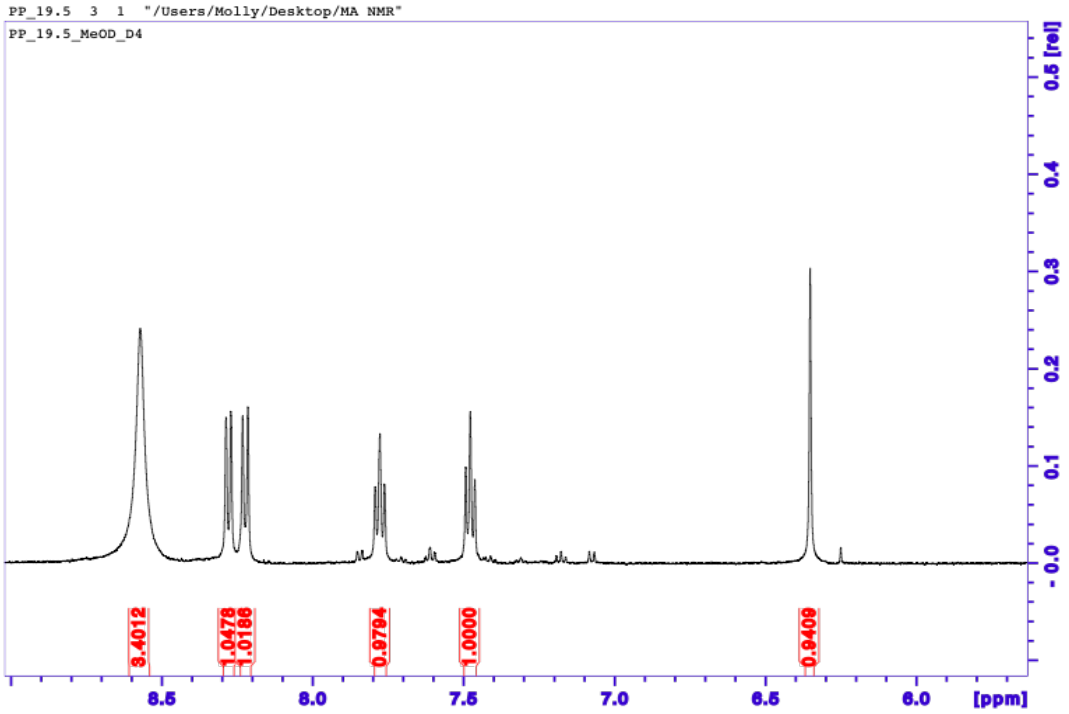


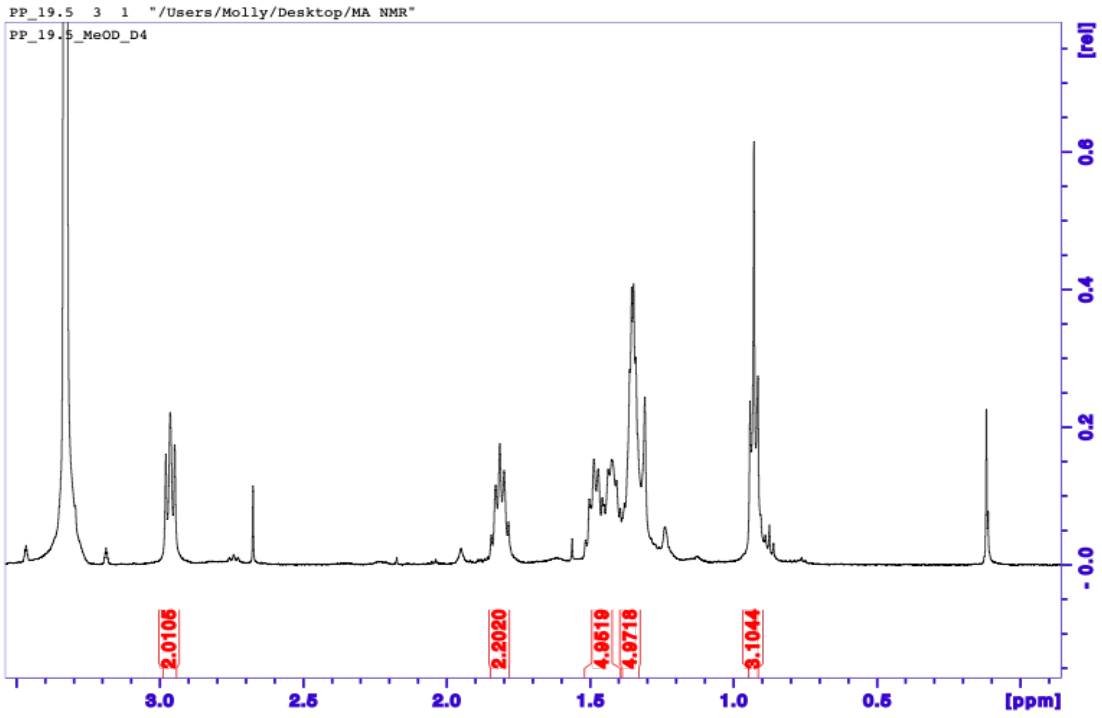
^{1}H (500MHz) and ^{13}C NMR spectra (700 MHz) in MeOD-d_4

Position	Experimental δC, mult.	Experimental δH, mult.	Literature δC, mult.,	Literature δH, mult.
2	154.5		157.1	
3	106.07	6.35 s	108.8	6.21 s
4	-		180.6	
4a	124.77	126.0	125.5	
5	124.3	8.28 dd	125.5	8.2 dd
6	124.05	7.78 t	125.0	7.65 t
7	131.47	7.48 t	133.3	7.36 t
8	116.3	8.22 dd	199.1	8.08 d
8a	141.0		141.6	
9	31.6	2.68 t	35	2.0 m

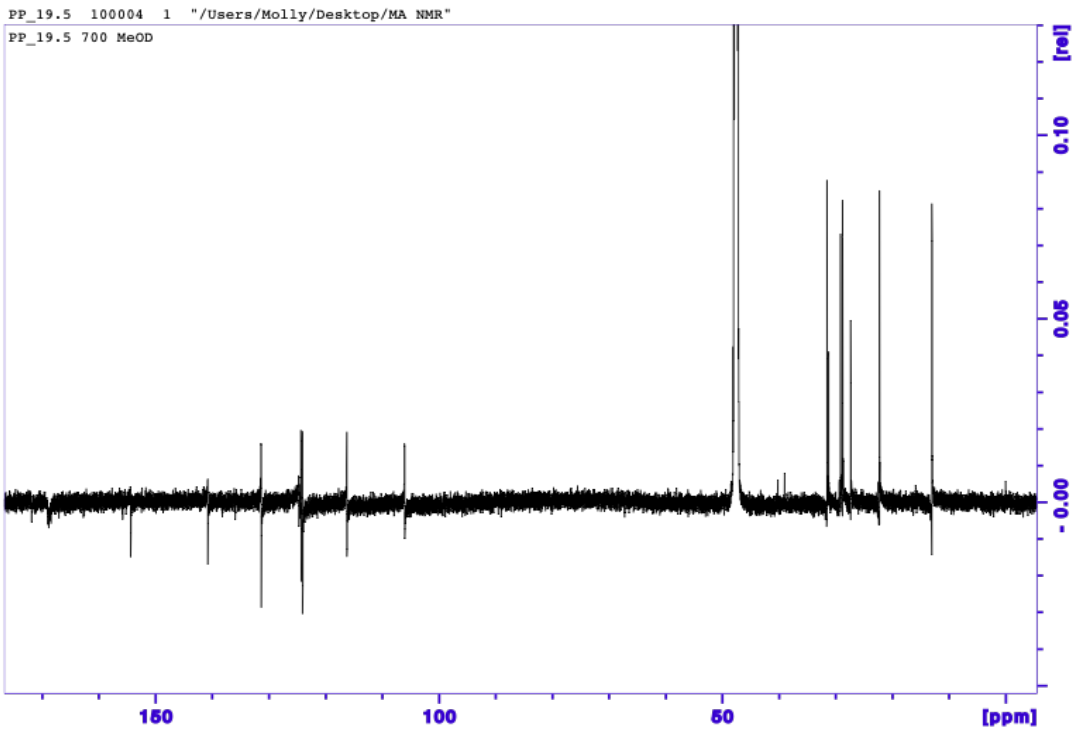
10-11	31.3, 29.2	1.73 m	32.8, 30.2	1.5 m
12-14	28.7, 27.35, 233.3	1.33 m	30.1, 30.0, 23.6	1.35 m
15	13.0	.91 t	14.4	.87 t

^{1}H NMR Spectra



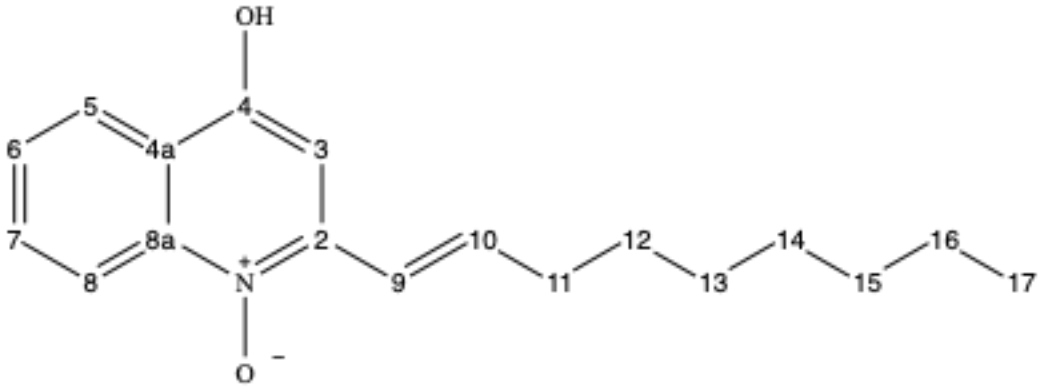


^{13}C NMR Spectra



Appendix 7 Spectroscopic data for trans-Δ^1-NQNO **(21)**

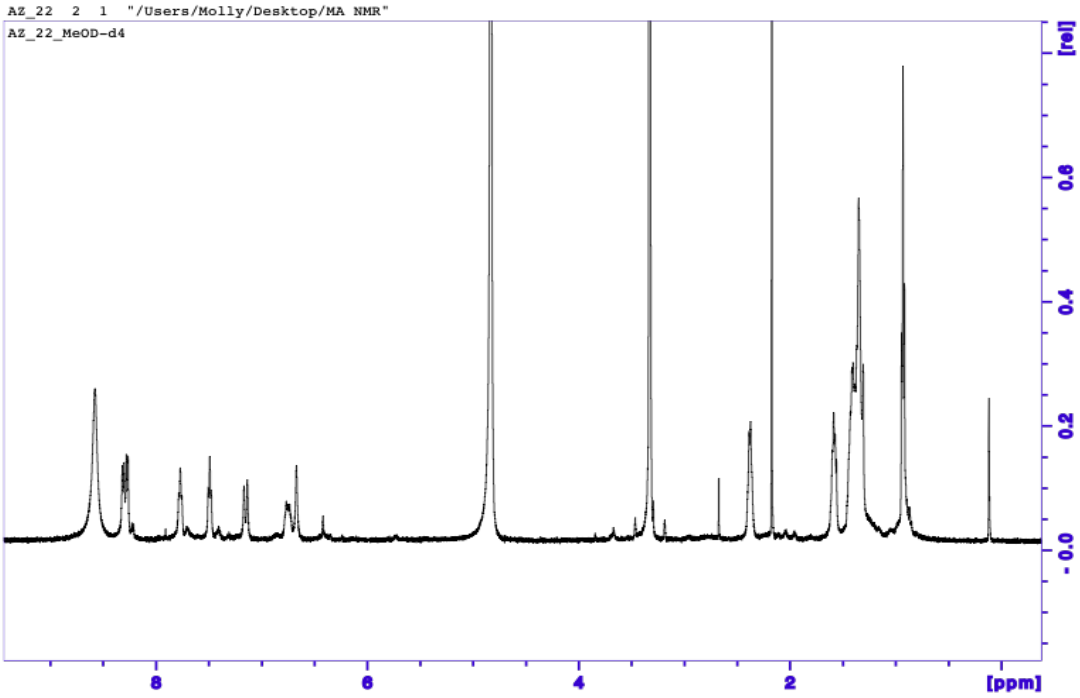
Structure and positions for **21**

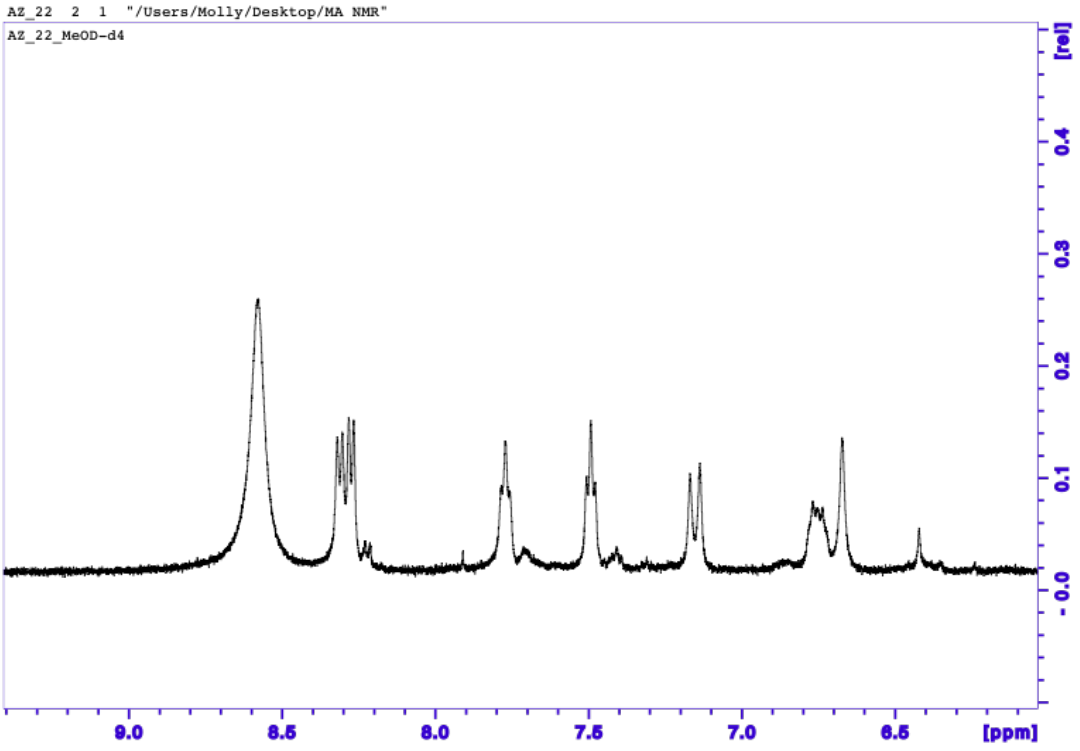


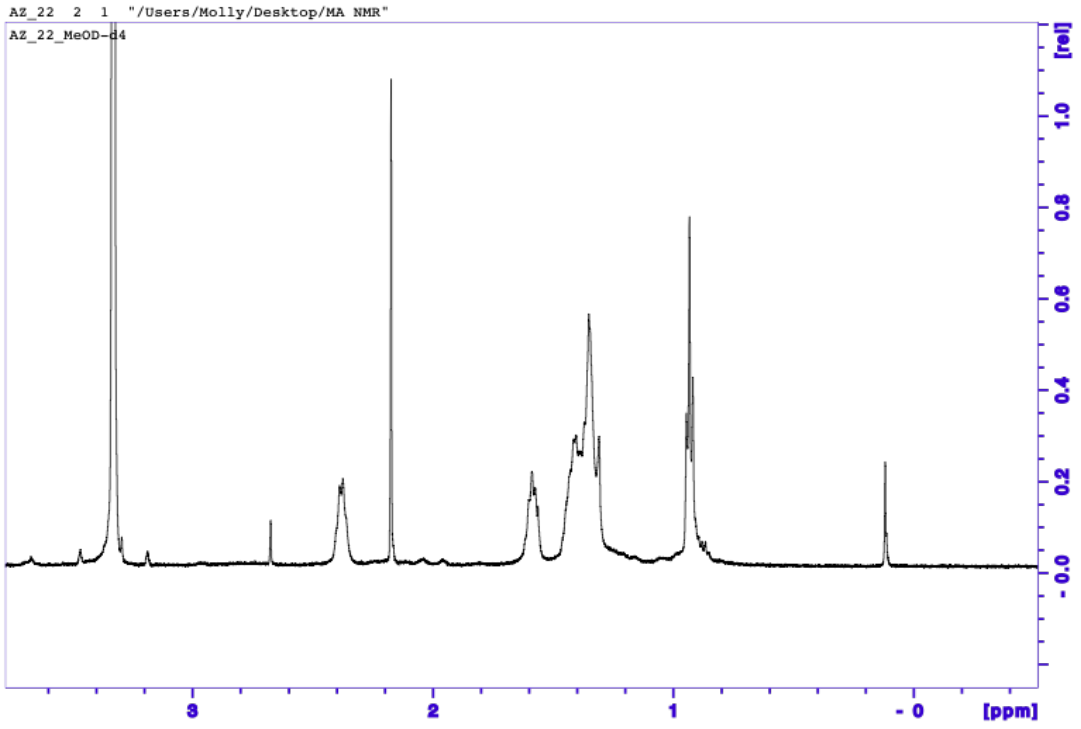
^{1}H (500MHz) and ^{13}C NMR spectra (700 MHz) in MeOD-d_4

Position	Experimental δC, mult., J(Hz)	Experimental δH, mult., J(Hz)	Literature δC, mult., J(Hz)	Literature δH, mult., J(Hz)
2	148.7	-	150.6	-
3	101.9	6.68 s	103.2	6.71 s
4	-	-	169.7*	-
4a	124.2	-	125.0	-
5	124.6	8.31, d, J = 8.56	125.5	8.26, dd, J = 8.2, 1.4
6	124.6	7.49, t, J = 6.88	126.7	7.55, m
7	131.3	7.77, t, J = 7.13	133.7	7.83, m
8	117.1	8.27, d, J = 8.13	117.7	8.21, d, J = 8.7
8a	140.7	-	141.8	-
9	121.2	7.14, d, J = 16.1	121.5	7.04, dt, J = 16, 1.6
10	141.5	6.75, m, J = 16	144.6	6.70, dt, J = 16, 6.9
11	33.14	2.38, m	34.5	2.38, m
12	28.4	1.59, m	29.7	1.57, m
13-16 (8H)	22.3, 28.9. 31.62	1.44 - 1.3, m	23.7, 30.2, 30.3, 33.0	1.26 – 1.29, m
17	13.1	.93, t, J = 6.72	14.4	.92, m

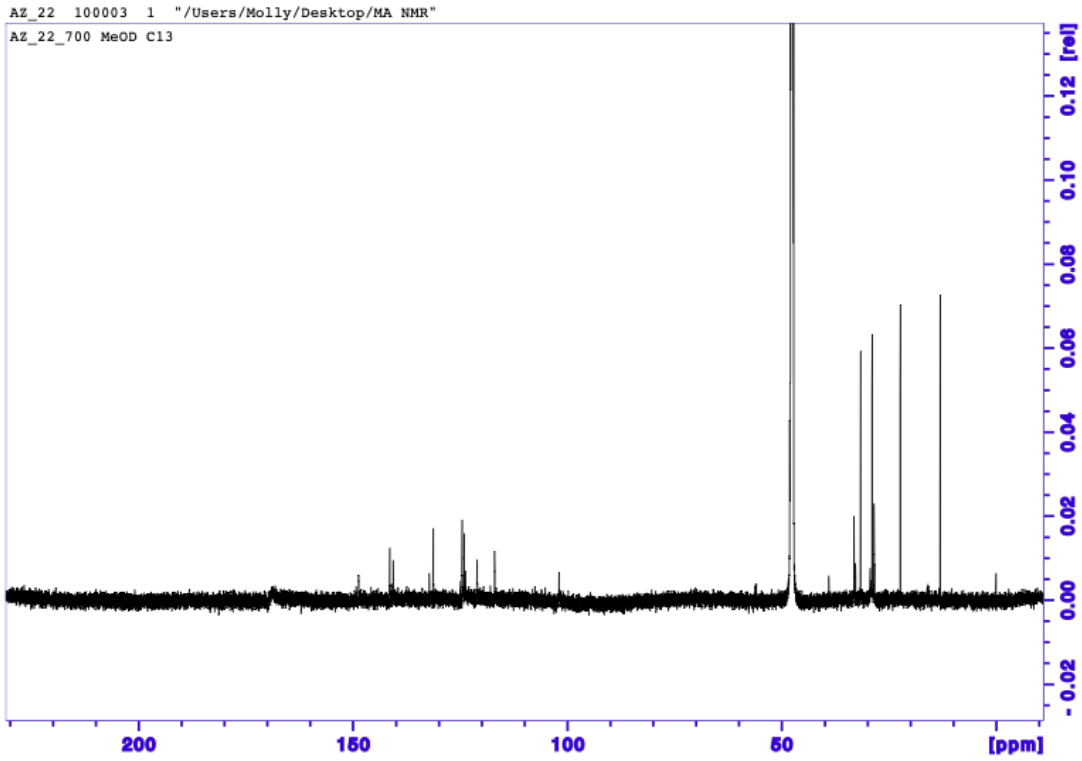
^{1}H NMR spectra

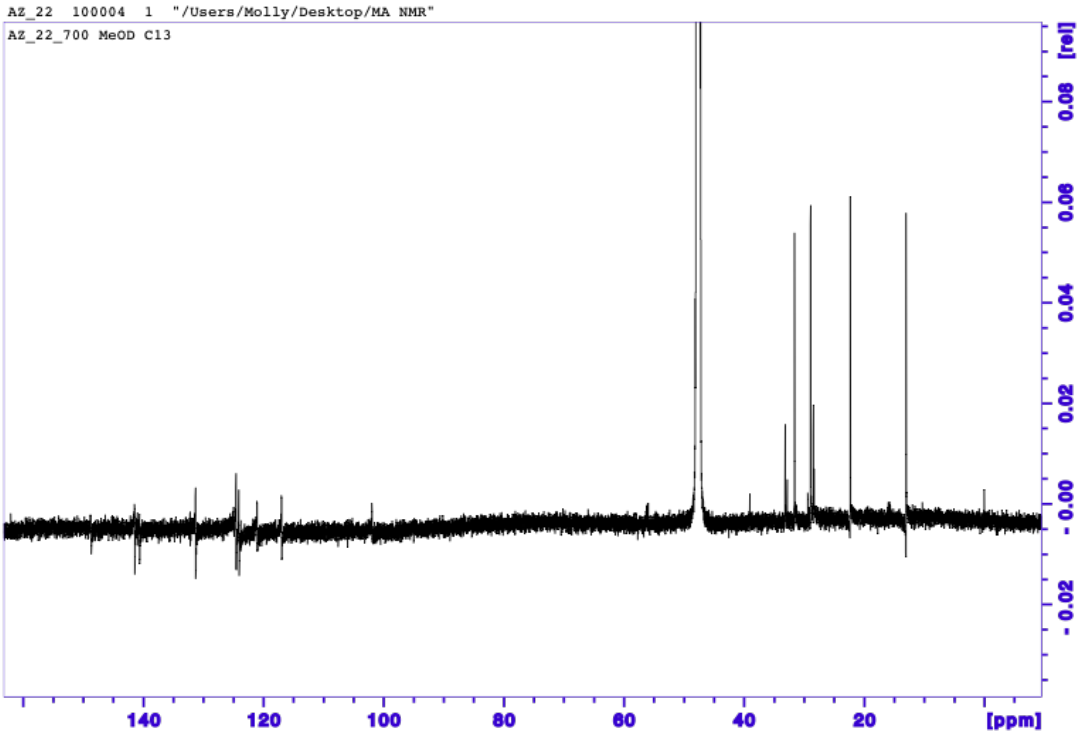






^{13}C NMR spectra

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AZ_22_700 MeOD C13
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0.04
0.02
- 0.00
- 0.02
140
120
100
80
60
40
20
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