Natural Product Prefractionation Library

Development and Evaluation of a Microbial Natural Product Prefractionation Library

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Lay Abstract

To combat the growing antibiotic resistance crisis, new strategies for drug discovery must be investigated and implemented. Natural products from bacteria and fungi have long been a source of critically important drugs. Prior research suggests that looking for vital natural products in fraction libraries can be more productive than screening crude extracts. Here the development and assessment of a fractionation library suitable for an academic lab's ingrained limitations are described. Assessing the library indicates an increased hit rate on screening fractions compared to crude extract. Furthermore, pursuing these hits may have revealed a novel antifungal lipopeptide.

Abstract

Ongoing antibiotic drug discovery is vital as antimicrobial resistance continues to be a significant issue faced in the clinic. Natural products have long been a highly productive source to mine for new antimicrobials. While it has been challenging to discover new and unique antimicrobial natural products, numerous drugs have been derived from studying how natural products function as secondary metabolites. Previous studies suggested that screening natural product extract fraction libraries for antimicrobials can be more productive than screening crude extracts alone. These studies from large industrial enterprises are generally not directly portable to an academic setting due to significant infrastructure costs. We developed a screening platform consisting of low pressure reversed-phase chromatographic separation of methanolic extracts of bacteria and fungi to generate a prefractionated natural product library. This platform is suitable for academic labs to screen for antimicrobial compounds. A large growth inhibitor screen against multiple pathogens and lab strains of microbes was conducted to assess the validity of the advantages of screening fraction libraries versus crude extract libraries and to search for potential new drug-like compounds. Hits were investigated for reproducibility, isolated, and purified. One compound was discovered in an antifungal screen which may be a novel lipopeptide.

Acknowledgements

Although I have been in the Wright lab for quite some time now, it all seemed to go so quickly. Completing a graduate degree during a pandemic certainly isn't the easiest thing in the world. This global upheaval has certainly made me realize how thankful I am for the position I was allowed to be in during my time in the Wright lab.

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To my good friends Derek Picketts and Rachel Hayton, thank you so much for including me in your so very small covid-era wedding party last autumn, since 1st year undergrad, I have had the pleasure of enjoying your friendship. To my brothers, Chris and Brendan, whether it was joining me for a round on the golf course or organizing a fantasy football league, thank you for being such great brothers. To my parents, Tim and Dayna, I can't thank you enough for your unending support and love, even if your eyes went a little blank when I tried explaining what I was working on. And finally, to my grandparents, Ann and Michael Pallant, who I became very close to while living so close during this time. It was tough this summer when we lost Grandad, but I promise I'll keep visiting you, Granny, even if it's just for a spot of tea and helping you figure out how to work your iPhone.

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List of Abbreviations

ATCC	American Type Culture Collection
AMR	Antimicrobial resistance
caMHB/A	Cation adjusted Mueller-Hinton II broth/agar
CMCB	Centre for Microbial Chemical Biology
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
HPLC	High-performance liquid chromatography
HEK cells	Human embryonic kidney cells
IIDR	Institute for Infectious Disease Research
MBL	Metallo-β-lactamase
MeCN	Acetonitrile
MS2	Molecular simulation program (open source)
NDM1	New Delhi metallo-β-lactamase 1
NPL	Natural product library
NMR	Nuclear magnetic resonance
$OD_{600/625}$	optical density measured at a wavelength of 600/625 nanometers
PBS	Phosphate-buffered saline
ppSPE	Positive pressure solid phase extraction
PFL	Prefractionated library
Psi	Pounds per square inch
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RP-HPLC	Reverse-phased high-performance liquid chromatography
SAM	Streptomyces Antibiotic Activity Media
FDA	The United States Food and Drug Administration
NCI	US National Cancer Institute
WAC	Wright Actinomyces Collection
YPD	Yeast extract peptone dextrose

Declaration of Academic Achievement

Daniel Pallant performed the majority of the experiments, data collection, and analyses presented in this thesis. The report was also prepared by Daniel.

Supervisor Dr. Gerry Wright and committee members Dr. Lori Burrows and Dr. Jakob Magolan, and collaborator Dr. Jarrod Johnson provided guidance and direction throughout the project. Other members of the Wright lab provided advice and support at different times.

Dr. Jarrod Johnson and Daniel Pallant collaborated to develop a protocol to fractionate natural product extracts to form the library

Linda Ejim contributed to the expansion of the pre-fractionated library after the protocol had been developed.

Dr. Kalinka Koteva, Dr. Xuefei Chen, and others in the lab have assisted in following up on active hits after the high-throughput screening

Chapter One – Introduction

1.1 Natural products: A productive source of antibiotics and other medicines

For over 80 years, natural products have been the most prolific source of novel antibiotics, anticancer agents, antiparasitic molecules, and modulators of metabolism, many of which find use as drugs and powerful probes of biology.[1] Natural products are specialized metabolites produced by living organisms, such as plants, bacteria or fungi, that have the potential to be bioactive.[1] These genetically encoded molecules are the source and inspiration for many drugs for human and animal use **(Figure 1-1)**.

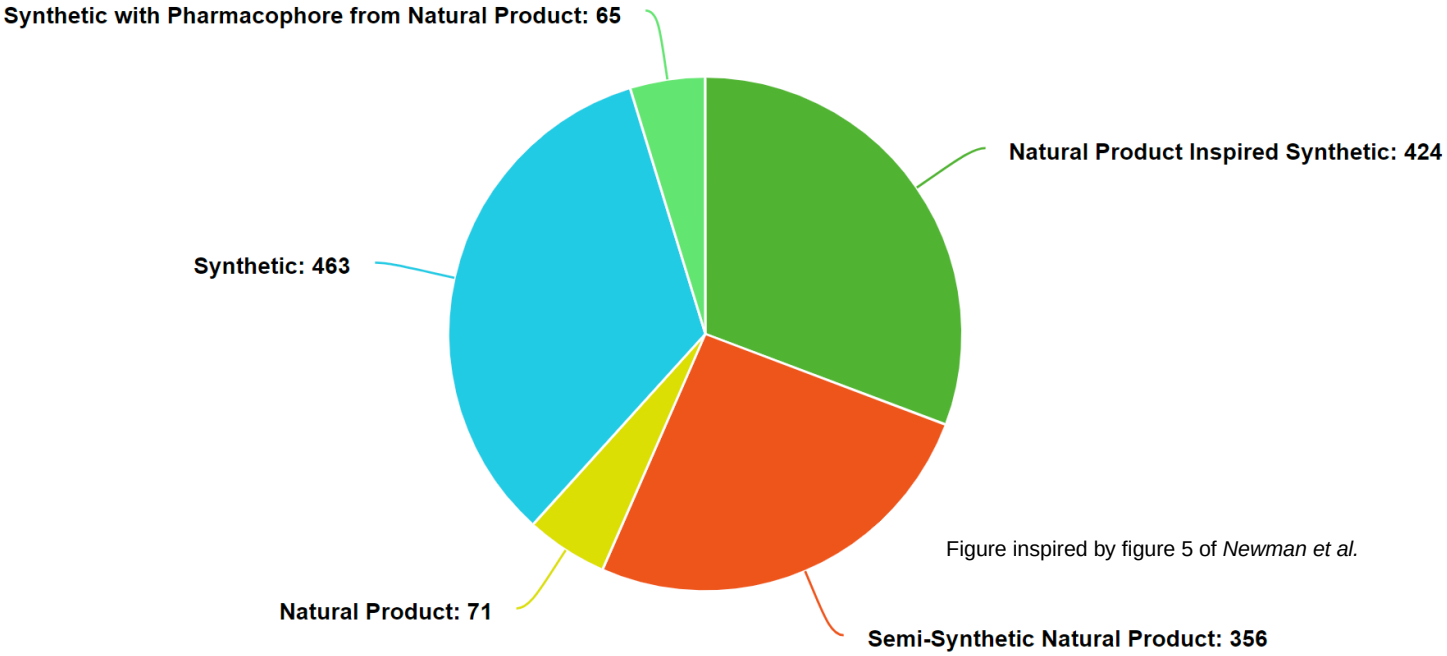


Figure inspired by figure 5 of *Newman et al.*

Figure 1-1. Sources of small molecule FDA and similar organization approved drugs from January 1981 to September 2019, 66.4% of these drugs have been fully or partially derived from natural products.[2]

As society is entering an era of greater antibiotic resistance, we need to continue to search this reserve for next-generation leads for antimicrobial compounds. After the

golden era of antibiotic discovery, the drug discovery in the private sector moved away from investigating natural products for antibiotics.[3] Antimicrobials are incredibly expensive to discover, with a lower economic incentive due to low cost of doses and short treatment lengths than other therapeutic drugs such as those used to control cholesterol or asthma.[3] Their higher development cost offers a better return on investment for drug companies due to long-term use and higher economic yields. In the mid-90s, hope rose that, with the advent of genome sequencing, drugs could be more effectively targeted and screened. There were hundreds of genes present in these newly sequenced bacterial genomes that looked promising.[4] Some productive compounds came out of this approach, including those that targeted peptide deformylase (PDF) and phenylalanyl-tRNA synthetase (PheRS).[4] The whole-cell screening approach decreased due the lack of dereplication requirement and low chemical diversity from traditional screening practices.[4] Approaching drug discovery from a genetic perspective, the private sector became more interested in synthetic compounds than natural products. Companies wanted to screen large synthetically created chemical libraries for new drugs. They were hopeful that perhaps they would discover more effective compounds that did not appear in nature. By screening these fully synthetic libraries, they would be able to skip the step of purification and identification of the active component seen in natural product drug discovery. This method has been successful in finding potential targets, by screening for drugs such as acetylcholinesterase to treat Alzheimer's disease and Hsp90 to treat cancer.[5] Still, the overall output has been lower than projected. This strategy's actual output of successful drugs has been less than expected from these large synthetic chemical library

screenings. Biochemical screens can identify chemicals of genetically validated targets but often hit roadblocks when whole-cell assays are performed.[6] This failure of the synthetic compounds *in vitro* could be due to membrane permeability issues not seen during *in silico* chemical assessment.[6] Large chemical libraries that drug companies are accustomed to screening were created for human rather than microbial targets.[3]

Recent antimicrobial screening technique advances can pave the way for previously missed novel antimicrobial agents. New strategies and technologies can increase the throughput of screening, reduce cost per assay, and minimize time requirements to identify new agents. Advances in inhibition of resistance, drug combinations not previously examined, new ways to target pathogens, and natural products screening can once again lead the way for drug discovery.[3] Instead of simply attempting to find another broad-spectrum antibiotic that seems like trying to find a needle in a haystack, attempts should be made to investigate more narrow-spectrum therapeutics to target the specific AMR strains seen in the clinics.[3]

The world is currently experiencing an antibiotic resistance (AMR) crisis. AMR is a significant public health problem that will continue to grow as resistance becomes more prevalent in the clinic.[7,8] Resistance is a natural phenomenon, but the increased prevalence of resistance in once drug-susceptible pathogenic bacteria has been attributed to the overuse and misuse of antibiotics in hospital and agricultural settings.[9] The loss of efficacy of existing antibiotics is spurring researchers and clinicians worldwide to discover and develop new antibiotics and alternatives. However, the current rate at which new antibiotic drugs are coming to market is drastically reduced compared to historic levels.[10,11] This market failure by the pharmaceutical industry is

concerning as the entire system by which drugs are brought to the clinic and farm is unable to meet the demands of a world currently facing an antimicrobial resistance crisis. Already in 2017, there were estimated to be 23,000 deaths in the US alone that were due to a resistant strain of bacteria.[12] Also in 2017, it was estimated that by 2050 there could be 10 million deaths per year due to resistance.[12] In Canada, one study found that in 2018, over 1400 deaths could be attributed to resistant infections.[13] By 2050, the loss of life due to resistance to current treatments could be as high as 396,000.[13] In the context of the AMR threat, continued research and innovation in this area is important. It makes sense that a return to natural products is warranted, as their potential to produce new and discoverable compounds is high.

1.2 Natural product success stories in medicine

Natural products have had many uses throughout history; from January 1981 to September 2019, 66.4% of FDA and similar organization approved drugs have come out of natural product research **(Figure 1-1)**. Furthermore, less than ten percent of the earth's biodiversity has been explored for the production of novel natural products.[14] Medical uses are vast and include, but are not limited to, anticancer, antibiotics, and antifungals (**Figure 1-2)**.[15]

One of the most effective natural products used as an anticancer drug, paclitaxel (Taxol), is derived from the yew tree.[16] It was the first of a new class of microtubule-stabilizing antitumor agents.[17] Paclitaxel targets tubulin to induce apoptosis by means of blocking the metaphase spindle configuration.[18] This compound can target cancer cells and has shown benefits in treating various cancers such as ovarian, breast, lung,

bladder, prostate, melanoma, and esophageal. This natural product comes from the Pacific yew tree, *Taxus brevifolia,* and was believed to be derived from its bark.[19] However, the compound is now believed to come from an endophytic fungus, *Taxomyces andreanae*, residing within the bark.[20]

Another anticancer natural product is daunorubicin, isolated from the bacterium *Streptomyces peucetius*.[21] The original producing strain was isolated from an Italian soil sample in the 1950s, and the red-pigmented daunaorubicin was found to have anticancer properties.[22] Daunorubicin exhibits antitumor activity across multiple cancer cell lines.[23] The biosynthesis and overproduction of daunorubicin has been categorized in genetically modified strains of *S. peucetius*.[24] This anthracycline was found to be effective in treating acute leukemia and lymphoma.[25] Soil is a major source of natural product-producing bacteria and fungi.

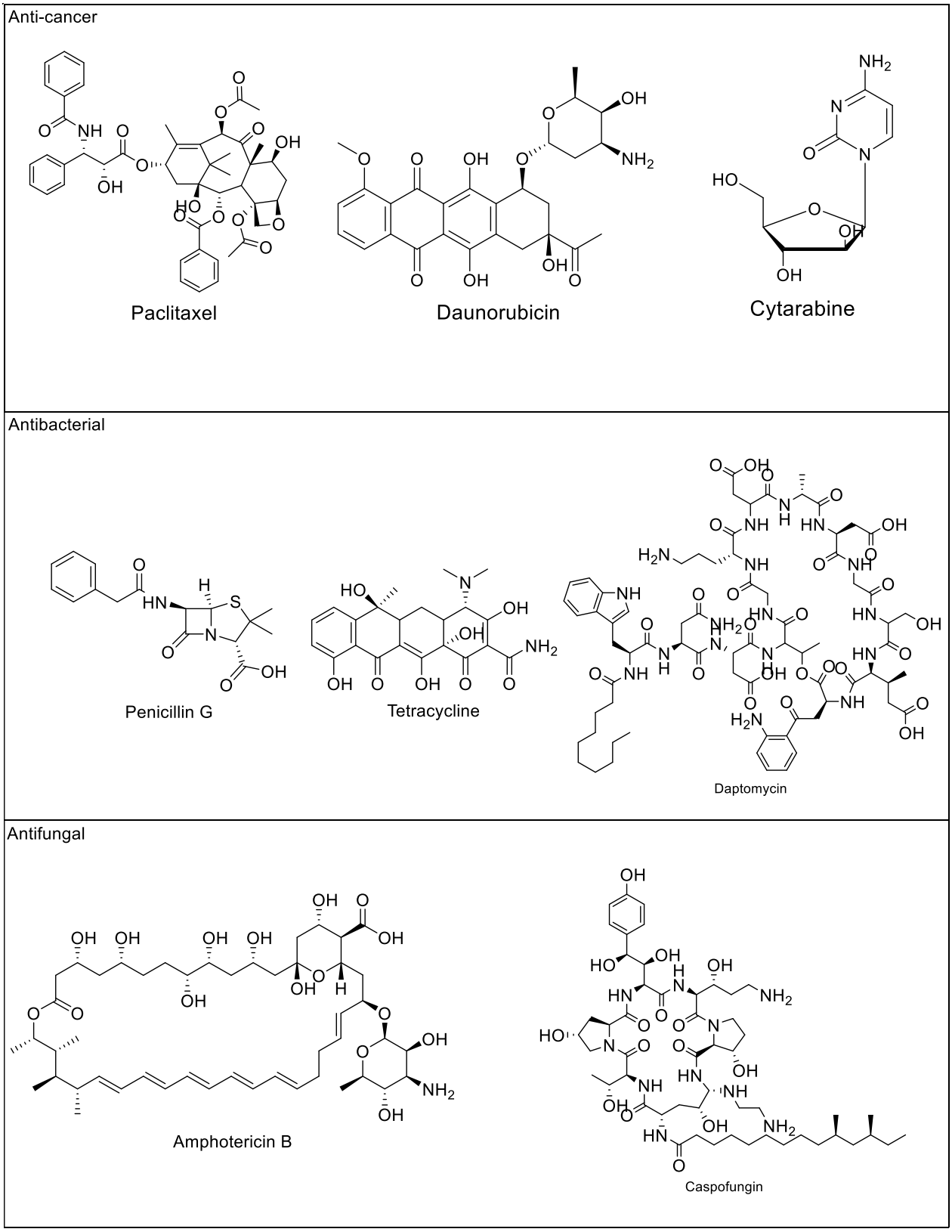


Figure 1-2. Important medically used drugs discovered from natural products.

Cytarabine is another natural product anticancer agent and functions by blocking the activity of DNA polymerase. It was first isolated from the Caribbean sponge *Cryptotheca crypta*.[26] C-nucleosides were isolated from the sponge, and this provided the basis for the synthesis of the cytarabine phosphoramidate prodrug 1.[26,27] *In vitro*, cytarabine induces cell death of human leukemia cell lines, which suggested that there would be clinical potential in treating acute myeloid leukemia.[28] Cytarabine is now a routine treatment alongside other cancer drugs in leukemia and lymphoma patients. Natural products have led to advancements in the cancer chemotherapy medical field and have proven to be a productive source for further advancement and continued research in drug discovery.

At St. Mary's Hospital in London, 1928, Alexander Fleming's discovery of the β-lactam antibiotic penicillin is one of the most well-known natural product success stories and one that launched the antibiotic revolution.[29] This discovery came when Fleming returned from a vacation and noticed a zone of growth inhibition on a Petri dish containing *Staphylococci* created by a contaminant mold belonging to the *Penicillium* genus.[30] β-lactams are the most widely used class of clinical antibiotics and function by inhibiting bacterial cell wall biosynthesis.[31] Production of penicillin exploded in 1941 as a consequence of intense efforts in industry and government, and by 1943 the US was able to manufacture enough of the antibiotic to supply the whole Allied Armed Forces during WWII.[30] This discovery and successful drug development on an industrial scale kicked off what is known as the "Golden Era" of antibiotic discovery.

Tetracyclines are produced from bacterial cultures of *Streptomyces* and other actinobacteria. Discovered in 1948, the first tetracycline antibiotic, chlortetracycline, is a

broad-spectrum antibiotic that is effective against both Gram-positive and Gram-negative bacteria.[32] Typically, tetracyclines bind to the ribosome and block bacterial growth by inhibiting protein synthesis. Tetracyclines block the A-site, preventing new aminoacyl tRNAs from binding.[33] However, there are atypical tetracyclines that do not directly inhibit this synthesis at the ribosome. Tetracyclines are commonly used to treat many bacterial infections and sexually transmitted diseases, such as chlamydia and syphilis. Some tetracyclines are even useful against eukaryote parasite infections such as *Giardia lamlia*.[34,35] Tetracycline can also be used for other diarrhea-inducing pathogens like *Vibrio cholerae*.[36] Unfortunately, as with many antibiotics, resistance was discovered shortly after its use began.[32]

Daptomycin is a cyclic lipopeptide discovered in the early 1980s by researchers at Eli Lilly. However, it was not approved by the US FDA for clinical use until 2003, making it one of the more recent antibiotics to enter clinical use and one of the last new chemical scaffolds to become an approved drug.[37] Daptomycin is produced by a strain of *Streptomyces roseosporus.*[38] This drug is often viewed as a last-resort antibiotic to treat infections caused by Gram-positive pathogens resistant to standard antibiotic treatment.[39] Pathogens resistant to methicillin, vancomycin, and linezolid have been treated successfully with daptomycin.[37] Daptomycin targets Gram positive bacteria by disrupting cell membrane functions. This disruption occurs through the formation of oligomeric pores in the membrane, which induce rapid depolarization and cell death.[40] Although still rare, there are increasing instances of resistance to daptomycin observed in the clinic.[41] This reality makes it all the more important to discover new antibiotics and new ways to combat resistance in bacterial pathogens.

Drug discovery through research into natural products has also led to important antifungal breakthroughs.[42] Amphotericin B was first isolated from a culture of *Streptomyces nodosus* in 1955.[43] It has broad antimycotic activity but is no longer used widely in the clinic due to nephrotoxicity causing kidney failure. In a liposomal formulation, amphotericin has broad application to treat fungal infections such as *Candida albicans* or *Aspergillus fumigatus*, demonstrating the importance of drug delivery methods in efficacy.[44] Amphotericin B works by binding to ergosterol, the principal sterol component of the fungal cell membrane. The drug forms pores in the cell membrane, which in turn causes ion leakage and cell death.[45]

Caspofungin is a semi-synthetic natural product antifungal drug that is a member of the Echinocandin family.[46] Echinocandins were first discovered from a strain of *Papularia sphaerosperma* by researchers searching for a drug candidate that was less toxic than amphotericin B, the current standard of treatment.[47] Echinocandins in clinical use are semi-synthetic lipopeptides designed to treat serious *Candida* and *Aspergillus* infections. For example, caspofungin is derived from pneumocandin B_0, which is produced by *Glarea lozoyensis.*[48] Echinocandins non-competitively inhibit (1,3) beta-d-glucan synthase. This enzyme is vital for the formation of glucan, an essential component of many fungal cell walls.[48] Natural products remain an essential source for the continued antifungal discovery, and indeed, our lab identified a new antifungal macrocycle, ibomycin.[49] It's clear that natural products are important sources of clinically relevant anticancer, antibiotic, and antifungal drugs.

1.3 Natural product extracts and fractionation

Since the Golden Era of antibiotics, the standard protocol for natural product discovery has been relatively straightforward. Selman Waksman was one of the pioneers of this approach in the 1940s, used to discover new molecules of interest. The Waksman platform, as it became known, was a systematic screening strategy using soil-derived microbes.[50] Extracts of these soil bacteria, prepared using organic alcohols such as *n*-butanol or methanol, were assayed for zones of inhibition against a target microorganism. Using this systematic platform, Waksman discovered many antibacterial and antifungal agents, including streptomycin, the first effective treatment against *Mycobacterium tuberculosis* for which he was awarded a Nobel Prize.[50] This process was successfully applied over the next decades resulting in the discovery of many of the broad-spectrum antimicrobials we know today.

Unfortunately, the discovery rate of novel drug candidates soon diminished through the regular rediscovery of known molecules, resulting in fewer new drugs consequently, a new method of drug discovery needed to be implemented. The pharmaceutical industry began modifying known natural products semi-synthetically. These active analogs produced more effective cures against resistance.[51] Fully synthetic compounds, such as fluoroquinolones, were also developed at this time. As fewer natural products were found and the effort and cost of discovering increased, many pharmaceutical companies left the field.

There is a belief that actinomycetes are potentially overmined and that there may not be many natural products left to discover from these sources. However, their large genome size and many uncharacterized biosynthetic gene clusters indicate that there

are likely more drug-like compounds left to discover.[52] At the same time, it could be fruitful to look more to the fungi, the original source of penicillin, for new natural products. The difficulty that now exists in finding the next novel natural product antibiotic likely cannot be overcome by simply applying traditional screening and Waksman approaches. Combining synthesis and new screening technologies could significantly advance the field.

Building on the optimization of past antimicrobial discovery processes and platforms, natural product extract fractionation may offer a step forward to new drug discovery.[53] Fractionation is a separation process where a mixture, such as crude extract, is separated across a phase transition (**Figure 1-3)**.[54] Partially purified natural product fractions can generate a much higher hit rate than crude extracts alone. The Wyeth group reported on a 20,000 microorganisms crude extract library developed from 1996 to 2002.[55] This group fractionated their library by reversed-phase high-performance liquid chromatography (RP-HPLC). Nine high throughput screens were run using the fraction library and compared to the parent crude extract. Primary hits from 1882 unique cultures were found, and 79.9% of these hits were from fractions only.[55] Another group, Merlion Pharmaceutical, fractionated 120,000 samples and found similar results over their 11 enzyme/protein-based assays. Eighty percent of the 1700 active fractions lacked activity in the crude extract sample run in parallel.[56]

Table 1-1. Previous studies show fractionation prior to high throughput screening assay has advantages in increasing hit rate.[55–57]

Group	Fractionation Method	Number of Fractions	Type of Screening Assay	Hit Rate
Wyeth	RP-HPLC	200,000	misc A misc B misc C ion channel A ion channel B ion channel C kinase A kinase B kinase C	- 2750 primary hits from 9 screens - 79.9% of the active strains showed activity only in the fractions
Merlion Pharmaceutical	RP-HPLC	120,000	enzyme/protein-based assays	- 1700 active fractions from 11 screens - 80% of the active strains showed activity only in the fractions.
NCI Program for Natural Product Discovery	Solid Phase Extraction	>1,000,000	NCI-60 Human Tumor Cell Lines Screen	77.3 increase (preliminary data)[57]

In crude extracts, metabolites can be diluted, high viscosity can lead to problems in high throughput screening, and fractions are easier to follow up in compound

purification.[54] Other groups, including Sequinoa Sciences Ireland Lab, Guy and Yan Group, RIKEN, Quinn Lab, Watnick Lab, Biomedicinal Information Research Center, and the Linington Lab, have seen a similar improvement to their libraries from pre-fractionating.[15] Viscosity can cause problems during high throughput screens, resulting in pipette tips not loading the sample of interest. Consequently, the samples may not be sufficiently concentrated for a hit to be discovered. Furthermore, dealing with highly viscous extracts during follow-up research and discovery can cause delays and lead to missing potentially interesting compounds.

A fraction library was made public by the US National Cancer Institute's (NCI) Natural Product Repository. It contains over 1 million fractions from over 230,000 unique extracts from natural sources, including microbes, plants, and marine samplings.[57] Unlike the other platforms, which were fractionated by RP-HPLC, this collection was fractionated by a customized positive pressure solid phase extraction workstation (ppSPE), Using HLB with C4 columns for aqueous extracts, and C8 columns for organic extracts.[57] Using these protocols, another group suggested that water-soluble compounds such as new glutamic acid derivatives solitumine A, solitumine B, and solitumidines A–D could be discovered in fractions.[58] These new libraries and discoveries show the importance of finding new ways of screening for natural products **(Table 1-1)**.

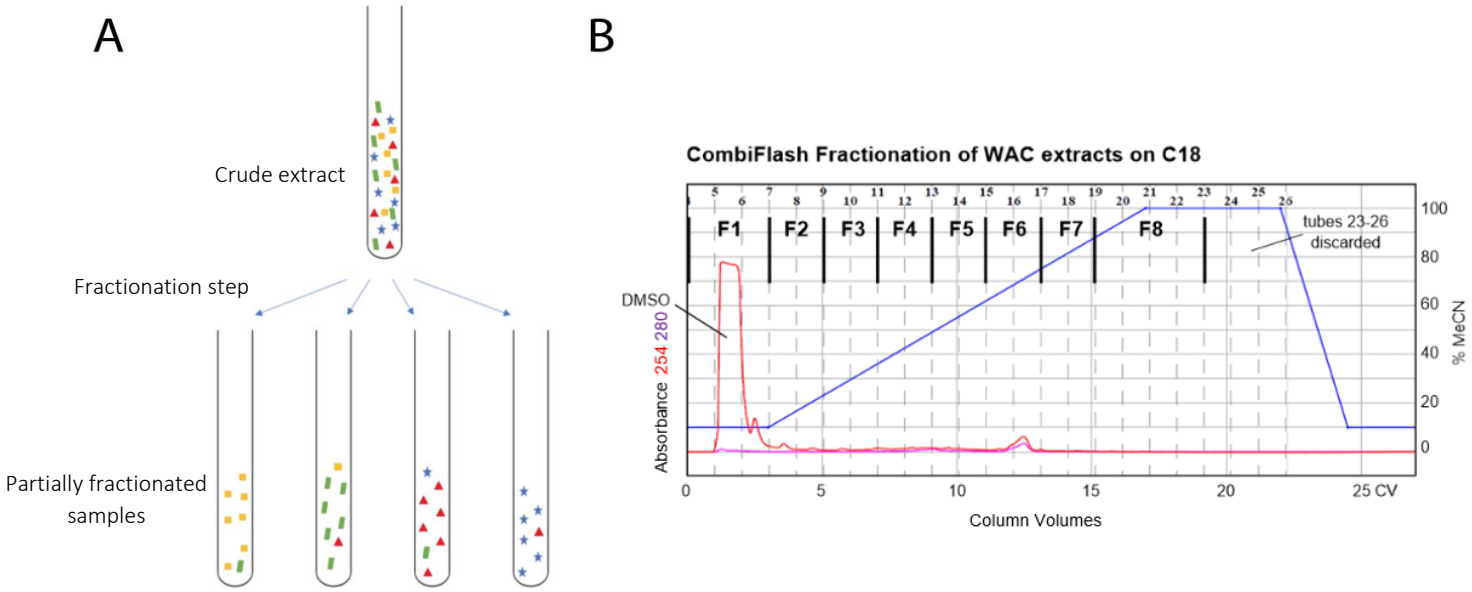


Figure 1-3. Fractionation by medium-performance liquid chromatography on a Combiflash is not as precise as HPLC (A) Diagram of the principle of fractionation to separate fractions by hydrophobicity on a Combiflash (B) Reversed-phase chromatography with a C18 column separates fractions in the creation of the PFL – absorbance read at both 254 nm and 280 nm.

1.4 Objectives

The literature shows that fractionation of natural product extracts prior to screening can positively identify bioactive compounds.[55,56] However, these papers have emerged from the commercial landscape, involving high investment and workforce to produce the very costly fractions. The time and cost associated with HPLC and the

limited number of instruments make such methods challenging in resource-limited labs. For example, in an academic lab, a protocol must be developed that is both more cost-effective and more time-efficient to require less labor input.

This research aims to establish a protocol for a cost-effective fraction library of microbial natural product extracts and to evaluate it in a series of screens for bioactivity. Inspired by the previous groups' work, we based our fractionated library on reversed-phase chromatography using C18 columns. However, the fractionation was performed using a medium pressure automated flash chromatography and fraction collection system that allows the user to customize flowrates, peak separation, and UV detection. This fractionation setup allows the user to produce far more fractions per hour than a standard HPLC. HPLC pressure limits are in the ~6000 psi range and uses expensive columns. Medium pressure is less than 200 psi an uses plastic columns. The cost saving advantage comes from not having to buy HPLC columns which are much more expensive than plastic columns. The medium pressure system used in this research was a Combiflash. Therefore, we predicted that use of this system would allow an academic lab to efficiently produce a small fraction library for drug discovery. However, a disadvantage is that the approach loses the high resolution of HPLC; consequently, we term our approach a prefractionation library (PFL).

The PFL can be used for drug discovery by the Wright lab and the whole of the Institute for Infectious Disease Research (IIDR). Many of the member labs in the IIDR investigate antibiotic resistance and could make use of this screening library. The PFL does not need to be limited to application in antibiotic resistance. The IIDR covers many disciplines that could make use of a partially fractionated library of natural products.

Finding new treatments and preventions to control infections is a core value of the IIDR. Some examples of how the IIDR could use the PFL include searching for antivirals in a post-covid world, new antimalarials, and both antifungal and antibacterial drugs, as well as anticancer agents. Any lab in the IIDR that has traditionally searched crude extracts for new compounds in their discipline of interest should be inclined to screen the PFL for their target.

Once the library was created, the hypothesis that fractions can produce a higher hit rate than that of crude extracts was tested. A series of biological assays in the form of high throughput screens against various pathogens and lab strains were conducted. To test mammalian cell toxicity, we targeted the ESKAPE pathogens (six pathogens that show widespread clinical antibiotic resistance), *E. coli* lab strains, fungal pathogens, and human HEK-293 cells.

Chapter Two – Material and Methods

2.1 Natural Product Extract Purification and Fractionation

Using an autoclaved sterile wooden applicator, strains were streaked from the Wright Actinomyces Collection (WAC) on a Bennett's media agar plate (10 g potato starch, 2 g casamino acids, 1.8 g yeast extract, 2 mL Czapek mineral mix (10 g KCl, 10 g $MgSO_4 \cdot 7H_2O$, 12 g $NaNO_3$, 0.2 g $FeSO_4 \cdot 7H_2O$, 200 µL concentrated HCl, ddH_20 to 100 mL, filter sterilize), ddH_2O to 1 L, pH to 6.80) and grown for 6 days at 30 ^oC to check for purity. Strains were then inoculated in 3 mL of Streptomyces Antibiotic Activity Media (SAM) (15 g glucose, 15 g Soytone (soya peptone), 5 g NaCl, 1 g yeast extract. 1g $CaCO_3$, 2.5 mL glycerol, ddH_2O to 1 L, pH to 6.80) and grown from 6 days at 30 ^oC; shaking at 250 rpm. The strains were next checked by plating to ensure a pure culture and streaked from the SAM stock for growth on Bennett's agar at 30 ^oC for six days. These cultures were then pressed through an empty 50 mL syringe using a mechanical 'can crusher' **(Figure S-1)** and extracted with 10 ml methanol overnight, shaking at 250 rpm. The methanol extract was then passed through a KenAG milk filter (model number D110 - B01J2I8CO6) to remove the solid component and funneled into 16x100 mm disposable borosilicate glass tubes. The extracts were evaporated overnight in a Genevac centrifugal evaporator (SP Scientific) and resuspended in dimethyl sulfoxide (DMSO). The crude extracts were combined and transferred to two 1.5 mL centrifuge tubes. For fungal cultures, the same protocol was used, but the media was used was yeast extract peptone dextrose (YPD) medium (10 g yeast extract, 20 g peptone, 20 g dextrose, ddH_2O to 1 L).

2.2 Conditioned Media

Liquid Bennett's media (5 mL in a 50 ml Falcon tube) was inoculated with a single colony. Three sterile 3 mm glass beads were added to prevent clumping. To culture the bacteria, the tubes were shaken at 250 rpm and 30 ^{o}C for 6 days with loose caps. After incubation, the conditioned medium was filtered through 0.22 µm polyethersulfone filters (Millex-GP) and added to the library plates.

2.3 Library Creation

To fractionate the crude extracts, reversed-phased chromatography on a Teledyne NextGen CombiFlash was used to separate compounds by hydrophobicity. Solution A was made up of HPLC grade water, and 0.1% formic acid, and Solution B was made of HPLC grade acetonitrile and 0.1% formic acid. The crude extract was applied to a 15 g C18 Gold column (RediSep Rf Gold C18 High Performance Columns #69-2203-334). The fractions were collected over an 11 minute run by raising solution B from 10% to 100%. Reversed-phased chromatography was performed at room temperature at a flow rate of 33 mL/min. After the CombiFlash run, the first 24 fractions were evaporated overnight in a Genevac. Genevac runs were performed overnight with the standard HPLC fraction protocols, which are designed for methanol/water and acetonitrile/water combinations, with the adjustment that the temperature did not exceed 30 ^{o}C, to protect any potentially temperature sensitive natural products. To accommodate storage in 96 well format, the fractions were resuspended in DMSO and combined as described below to form 8 total fractions for a total of 750 µl DMSO per fraction. Then 250 µl DMSO was added to the first 3 fractions, and then combined. Next 375 µl of DMSO was added to fractions 4 through 15, and 2 vials were combined

respectively (e.g., 4 and 5, 6 and 7, etc.). Finally 100 µl DMSO was then added to fractions 16-24 to form fraction 8 of the PFL as at this stage the acetonitrile is 100% on the column. The fractions were added along with the crude extract and conditioned media to 96-well Costar polystyrene plates. Next 105 µl were added to each well to generate 6 daughter plates and the remainder in 'deep-well' 1 mL 96-well Costar polystyrene plates. Plates are stored at -30 ^{o}C.

2.4 Cell-based High Throughput Screens

High throughput screens were performed against the ESKAPE pathogens; *Enterobacter aerogenes* ATCC 13048, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 33495*, Acinetobacter baumannii* ATCC 17978*, Pseudomonas aeruginosa* PAO1*, Enterococcus faecium* ATCC 19434*,* as well as *Escherichia coli* ATCC 25922 (often used as a standard MIC strain, it is a human clinical isolate from Seattle, WA collected in 1946), *E. coli* BW25113 *ΔbamBΔtolC*, *E. coli* BW25113 *pLacI* NDM1 *ΔbamBΔtolC*, *Candida albicans* ATCC 90028, and *Candida auris* CBS 10913. For bacteria, cation-adjusted Mueller Hinton Broth was used as the growth medium for the screen, and for yeast, RPMI 1640 was used.

Yeast cultures were streaked for single colonies on YPD and allowed to grow at 30 ^{o}C for 48 hours. Bacterial cultures were streaked on cation-adjusted Mueller Hinton agar (caMHA) for single colonies and grown at 37 ^{o}C for 24 hours. From these cultures, a final concentration of 1/200 OD_{625} 0.1 for bacteria and a final concentration of 1/2000 OD_{530} 0.11 for yeast was obtained. The addition of 1 µl of crude/conditioned media/fraction was added to 49 µl of culture. Media and bacteria were added to the plates for screening via the Formulatrix Tempest Liquid handler. High throughput

screening was performed on the Biomek Fxp Integrated Liquid Handler for inoculating the pre-fractionated library into sterile 384 well plates. For bacteria, the 384 well plates were grown for 24 hours at 37 ^{o}C, and for yeast, they were incubated for 48 hours at 30 ^{o}C. Positive controls of 8 µg/mL ciprofloxacin for *S. aureus* and *K. pneumonia,* 8 µg/mL amphotericin B for *C. albicans* and *C. auris,* and 32 µg/mL ciprofloxacin for the *E. coli* strains and the rest of the ESKAPE pathogens were used. The plates were read at OD_{600} on an EnVision, SpectraMax, or Biotek Neo microtiter plate reader. The raw data were exported from the plate reader and analyzed through Microsoft Excel.

2.5 The Vault - Data Management

Screening results were uploaded to the Centre for Microbial Chemical Biology (CMCB) Comp Vault. Hits were tracked directly from the plate map to the WAC# of the bacteria producing the active natural product.

2.6 HEK Cell Screen

Human embryonic kidney toxicity analysis was performed against ATCC CRL-1573 HEK-293 during generation 14. Cells were seeded at 15000 cells/well in Dulbecco's modified eagle medium (4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate) (DMEM) containing 10% fetal bovine serum (produced from the blood drawn from a bovine fetus and used to supplement cell culture medium and increase growth) (FBS), 2 mM L-glutamine and incubated for 18 hours at 37 ^{o}C +5% CO_2 for 24 hours by multichannel pipette or added to assays plates using Formulatrix Tempest. The PFL was screened at 1% concentration as 2% DMSO is toxic to HEK cells. After incubation, the cells were

washed with phosphate-buffered saline (PBS), and 50 µl of CellTiter-Glo 2.0 luciferase was added. Assay plates were shaken on a plate shaker for 2 min and then left stationary for 10 min before reading. The assay plates were read for luminescence on a Biotech Neo. A positive control of 32 µg/ml amphotericin B was used in each assay.

2.7 Data analysis

Hits were defined as 2 standard deviations or more from the interquartile mean using Excel. Data were normalized on a per assay basis by dividing every well value by the interquartile mean.[59] Interquartile mean was calculated using the =TRIMMEAN(start:end,0.5) function.[59] Replica plots were created using the statistical programing language R (**Figure S-2)**.

2.8 Bioassay for reproducibility confirmation

Growth inhibition assays was performed to both confirm reproducibility of active strains from the high-throughput screening process as well as during the bioactivity-guided purification of the WAC11161 active compound. Conditions and controls were identical to screening, except the assays used 100 µL in 96 well U-bottom plates from Fisher Scientific (Cat No. 07000295), instead of 50 µL in 684 well plates. For the yeast follow-up assays, 48 µL of the media, RPMI 1640 was added to each well. Two µL of the sample was added to each assay well in duplicate to produce a 2% concentration. Fifty µL of inoculated media was added to each well. Sterile medium was added to wells as a negative control, and 16 µg/mL amphotericin B was added to wells as a positive control. Plates were incubated at 30 ^{o}C for 48 h. Plates were examined for growth inhibition using a Biotek Synergy H1 microtiter plate reader at OD_{600}.

2.9 WAC11161 active compound purification

Purification of the active compound began with an 8 L fermentation of WAC11161 in YPD at 30 ^{o}C for 6 days. After centrifugation, the 8 pellets were extracted with 400 mL of methanol. The methanol extract was concentrated on a rotary evaporator (Heidolph Rotavap) and resuspended in 50 mL DMSO. Five mL of this sample was then separated on an 86 gram C18 column on the Combiflash. A 30 min run was performed with a rate 66 mL/min with water + 0.1% formic acid and acetonitrile + 0.1% formic acid, and 144 fractions were collected **(Figure 3-6)**. These fractions were evaporated with the Genevac and and resuspended in 100 µL DMSO. Bioactivity testing showed reproducible activity in fractions 96 to 123. Active fractions were combined and separated on a 100 g Sephadex LH-20 column with a 500 mL methanol wash. Testing for activity again, fractions 7-11 from the size exclusion protocol were active. These fractions were combined and purified further on an HPLC (Agilent ELSD 1260 Infinity) with the solvent methanol on a C8 column (Eclipse XDB-C8 5 µm 4.6 x 150mm) **(Figure S-3)**. The purified compound was then confirmed for activity with another bioassay.

Chapter Three – Results and Discussion

For over a decade, the Wright laboratory has maintained a library of over ten thousand natural product extracts. This natural product platform is derived from bacterial strains present in the Wright Actinomycete Collection (WAC). This library has produced positive results as a drug discovery resource, resulting in the discovery of several new antibiotics, antifungals, anticancers, and antimalarial agents. [49,60–65] However, crude natural product extracts can be problematic in modern high-throughput screening technologies. Crude extracts are highly viscous, which leads to errors in robotic tip dispensing and acoustic liquid handling. Moreover, crude extracts may hold multiple compounds of interest where a single positive hit is masking others.

3.1 Natural product library

The WAC consists of over 11,360 isolates and is derived from soil samples, vegetation, insects, caves/etc collected primarily in Canada, but with several samples from elsewhere in the world. Extracts from over 10,600 isolates from the WAC have been prepared – the natural product library (NPL). The NPL is produced from the WAC by creating methanol extracts of microbes grown on solid media **(Figure 3-1)**. These extracts are preserved for long-term storage in dimethyl sulfoxide and 96 well plates at -30 ^{o}C. From these assay plates, many high-throughput screens have been performed in the Wright lab, and this library has been productive in its output of novel bioactive compounds.[49,60–64]

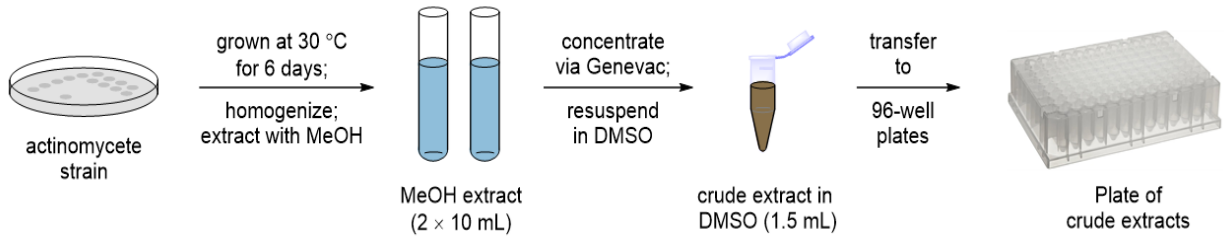


Figure 3-1. Natural Product library production for crude extracts.

3.2 Prefractionated library

Building on the NPL, which is composed entirely of crude extracts, a fraction library was built. Reverse-phase chromatography fractionation through a C18 column can address some issues prevalent in the high-throughput screening of crude extracts. The prefractionation library (PFL) was built with the crude extract, conditioned media, and 8 fractions suspended in DMSO from 381 WAC strains. Fractions can be more highly concentrated than crude extracts, and the separation by polarity prevents one hit from masking other compounds (**Figure 3-2**). A library of 48 96-well plates can be screened in a high throughput manner to advance potential drug discovery **(Figure 3-3)**. The pilot PFL described in this work is derived from 191 bacterial cultures and 190 fungal cultures.

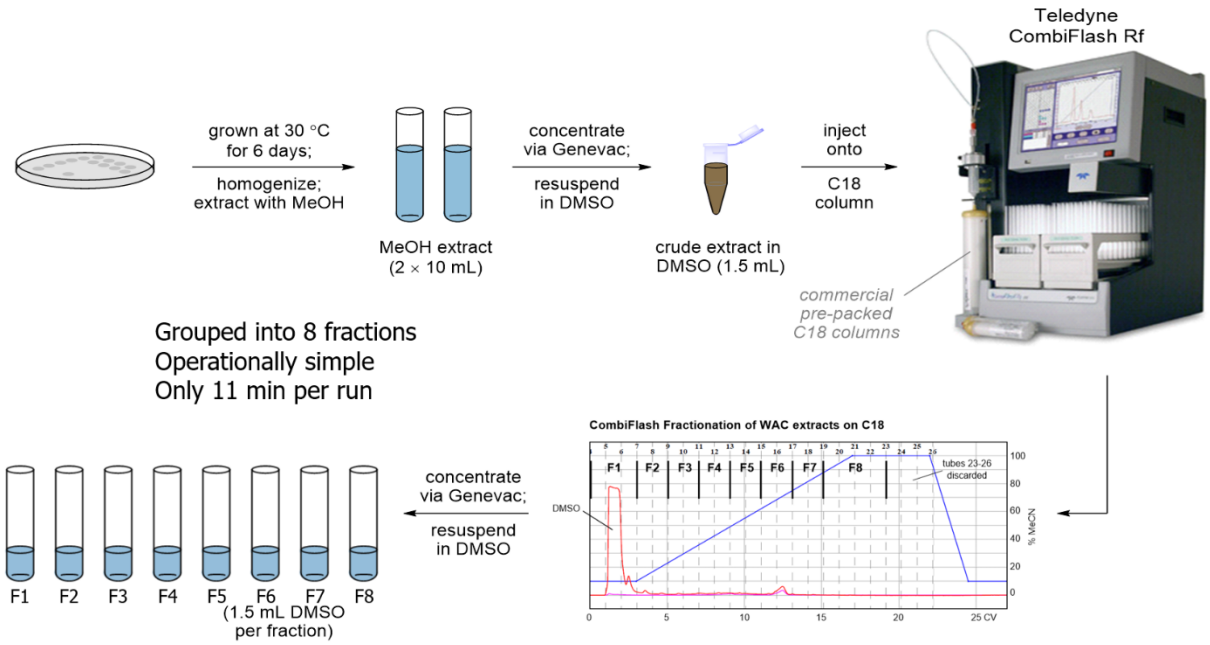


Figure 3-2. Fractionation of crude extracts to form a pre-fractionated library of natural products

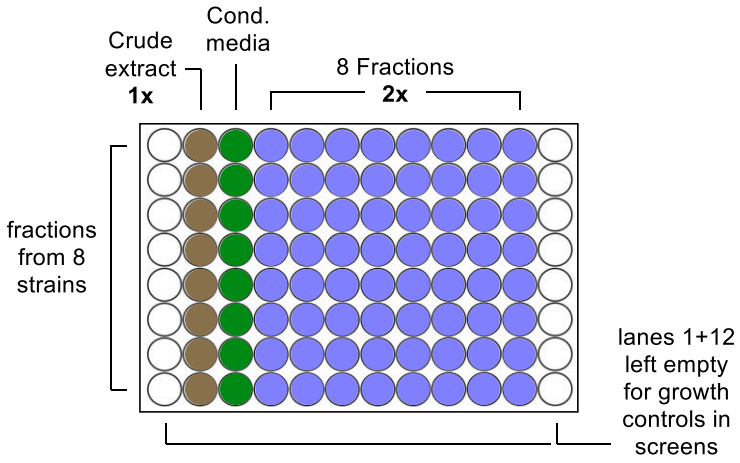


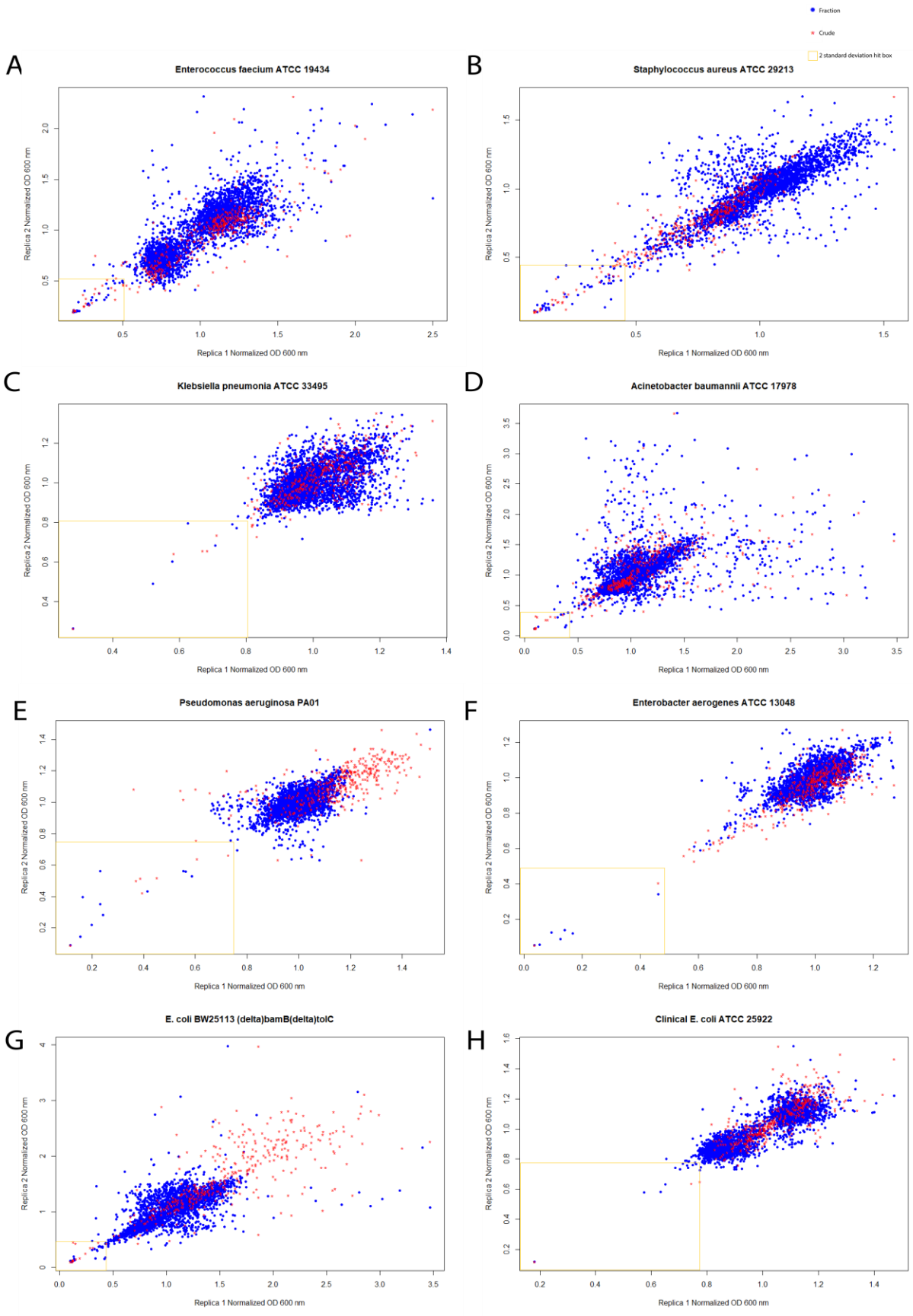
Figure 3-3. PFL plate map displaying the well locations of the crude, conditioned media, and fractions.

A general cell-based screen of the library was conducted to determine the advantages of a fractionated library over a simple crude extract library. Screening for growth inhibition of a panel of ESKAPE pathogens, *C. albicans*, *C. auris*, clinical *E. coli*, *E. coli* BW25113 Δ*bamB*Δ*tolC, E. coli* BW25113 *pLacI* NDM1 Δ*bamB*Δ*tolC,* and HEK-293 cells show that fractionation of crude extracts generates additional hits in susceptibility testing. Generally, screens were performed at a final concentration of 2% PFL sample (1 µL in a 50 µL assay well) except for the HEK cell assay, as prior control runs concluded that 2% DMSO was damaging to the cells *in vitro* **(Table 3-1).** The HEK cell assay was thus performed at 1% DMSO. The *E. coli* BW25113 *pLacI* NDM1 Δ*bamB*Δ*tolC* screen was performed in the presence of 0.125 µg ml^{-1} meropenem, to test for inhibitors of the NDM-1 metallo-β-lactamases (MBL). Unfortunately, issues arose with the conditioned media wells present in the PFL. Transfer from the PFL plates to the 384 well assay plates could not be reliably completely by the robotic screening machines. This was due to a variety of factors, including evaporation of conditioned media wells of the daughter plates due to their high water content. As well as the high viscosity of some of the conditioned media despite being filtered. Due to this, the results from the conditioned media wells have been excluded from this analysis.

The PFL is comprised of both extracts and fractions from bacterial and fungi fermentations. The first 381 strains are close to equal in number. Interestingly, more of the hits arose from the fungi extracts than from the bacterial extracts **(Table 3-1)**. Perhaps fungi are an undermined resource compared to bacteria such as actinomyces, which have traditionally been a large producer for drug discovery and clinical application.

Table 3-1. List of the lab strains and pathogens that were screened against the Pre-fractionated Library (PFL), the screening concentrations and the total number of hits from PFL samples produced by fungi and bacterial WAC strains.

Strain	PFL concentration	Fungi extract hits	Bacterial extract hits
E. faecium ATCC 19434	2%	26	43
S. aureus ATCC 29213	2%	31	30
K. pneumoniae ATCC 33495	2%	11	3
A. baumannii ATCC 17978	2%	43	20
P. aeruginosa PA01	2%	8	8
E. aerogenes ATCC 13048	2%	4	8
C. auris CBS 10913	2%	43	28
C. albicans ATCC 90028	2%	27	17
clinical E. coli ATCC 25922	2%	10	4
E. coli BW25113 Δ*bamB*Δ*tolC*	2%	53	39
E. coli BW25113 *pLacI* NDM1 Δ*bamB*Δ*tolC*	2%	34	26
HEK-293	1%	23	15


Fraction
Crude
2 standard deviation hit box
A
Enterococcus faecium ATCC 19434
B
Staphylococcus aureus ATCC 29213
C
Klebsiella pneumonia ATCC 33495
D
Acinetobacter baumannii ATCC 17978
E
Pseudomonas aeruginosa PA01
F
Enterobacter aerogenes ATCC 13048
G
E. coli BW25113 (delta)bamB(delta)tolC
H
Clinical E. coli ATCC 25922
Replica 1 Normalized OD 600 nm
Replica 2 Normalized OD 600 nm

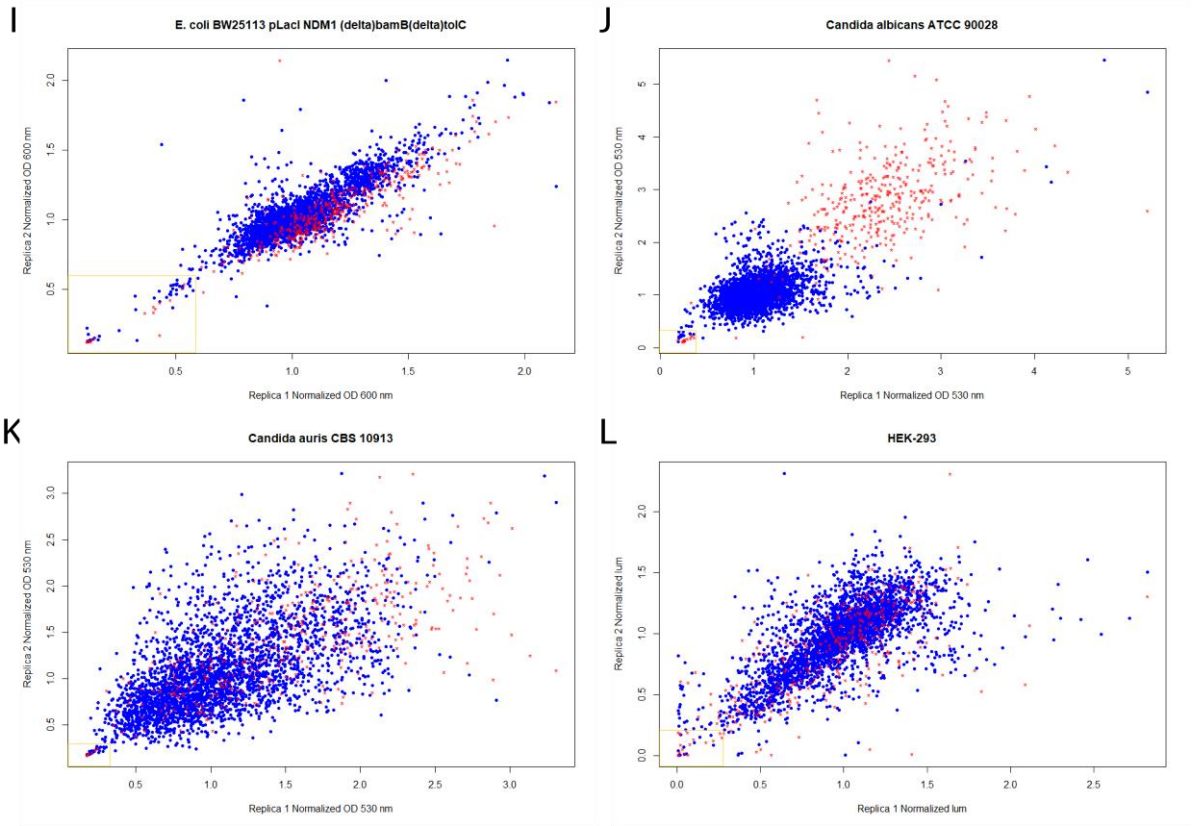


Figure 3-4. Replica plots show the normalized results of a cell growth inhibition assay of 2% extract (1% HEK cell). The blue dots represent fractions and the orange dots represent crude extracts. The orange hitbox shows the hits defined at 2 standard deviations (A) *Enterococcus faecium* ATCC 19434 (B) *Staphylococcus aureus* ATCC 29213 (C) *Klebsiella pneumonia* ATCC 33495 (D) *Acinetobacter baumannii* ATCC 17978 (E) *Pseudomonas aeruginosa* PA01 (F) *Enterobacter aerogenes* ATCC 13048 (G) *E. coli* BW25113 Δ*bamB*Δ*tolC* (H) Clinical *E. coli* ATCC 25922 (I) *E. coli* BW25113 *pLacI* NDM1 Δ*bamB*Δ*tolC* (J) *Candida albicans* ATCC 90028 (K*) Candida auris* CBS 10913 (L) HEK-293

3.3 Analysis of Screens

High throughput screening assays were conducted using all 48 plates of the PFL, resulting in 381 strains, 381 crude extracts, and 3048 fractions being tested in duplicate. The replica plots were normalized using the interquartile mean, and hits were identified as assay wells that showed a decrease in growth by at least 2 standard deviations from the mean (**Figure 3-4)**.[59] The *E. faecium* ATCC 19434 assay produced 42 positive hits from fractions and 27 from crude extracts, a 1.55 fold increase. The screening of *S. aureus* ATCC 29213 showed 34 fraction hits and 22 crude hits, a ratio of 1.54. *K. pneumonia* ATCC 33495 had 9 fraction hits and 5 crude hits, 1.8 times as many fraction hits as crude. The *A. baumannii* ATCC 17978 screen resulted in 43 fraction hits and 20 crude extract hits, meaning a ratio of 2.15 fractions to crude hits, and the *P. aeruginosa* PA01 assay had 13 fraction hits and 3 crude hits, which means there were 4.33 times as many hits from fractions compared to crude. High throughput screening of the PFL against *E. aerogenes* ATCC 13048 produced 10 fraction hits and 2 crude hits, 5 times as many fractions as crude. In total the ESKAPE screen had just over double the number of hits from fraction wells than from the crude extract wells.

A large number of hits were identified using the hyperpermeable efflux deficient mutant *E. coli* BW25113 Δ*bamB*Δ*tolC,* due to its increased susceptibility, 64 hits from fractions and 28 hits from crude extract, a 2.29 times ratio. In contrast, clinical *E. coli* ATCC 25922 showed only 11 hits from fractions, and 3 crude hits (ratio of 3.67). The *E. coli* BW25113 *pLacI* NDM1 Δ*bamB*Δ*tolC* screen, which looked for metallo-β-lactamase inhibitors in the presence of 0.125 μg ml^{-1} meropenem, resulted in 47 hits from fractions and 18 hits from crude (ratio 2.61). In the yeast screens *C. albicans* ATCC 90028 had

28 from fractions and 16 from crude (ratio of 1.75), *C. auris* CBS 10913 had 53 from fractions and 18 crude hits (ratio of 2.94). The HEK-293 toxicity screen had 31 hits from fractions and 7 hits from crude (ratio of 4.42).

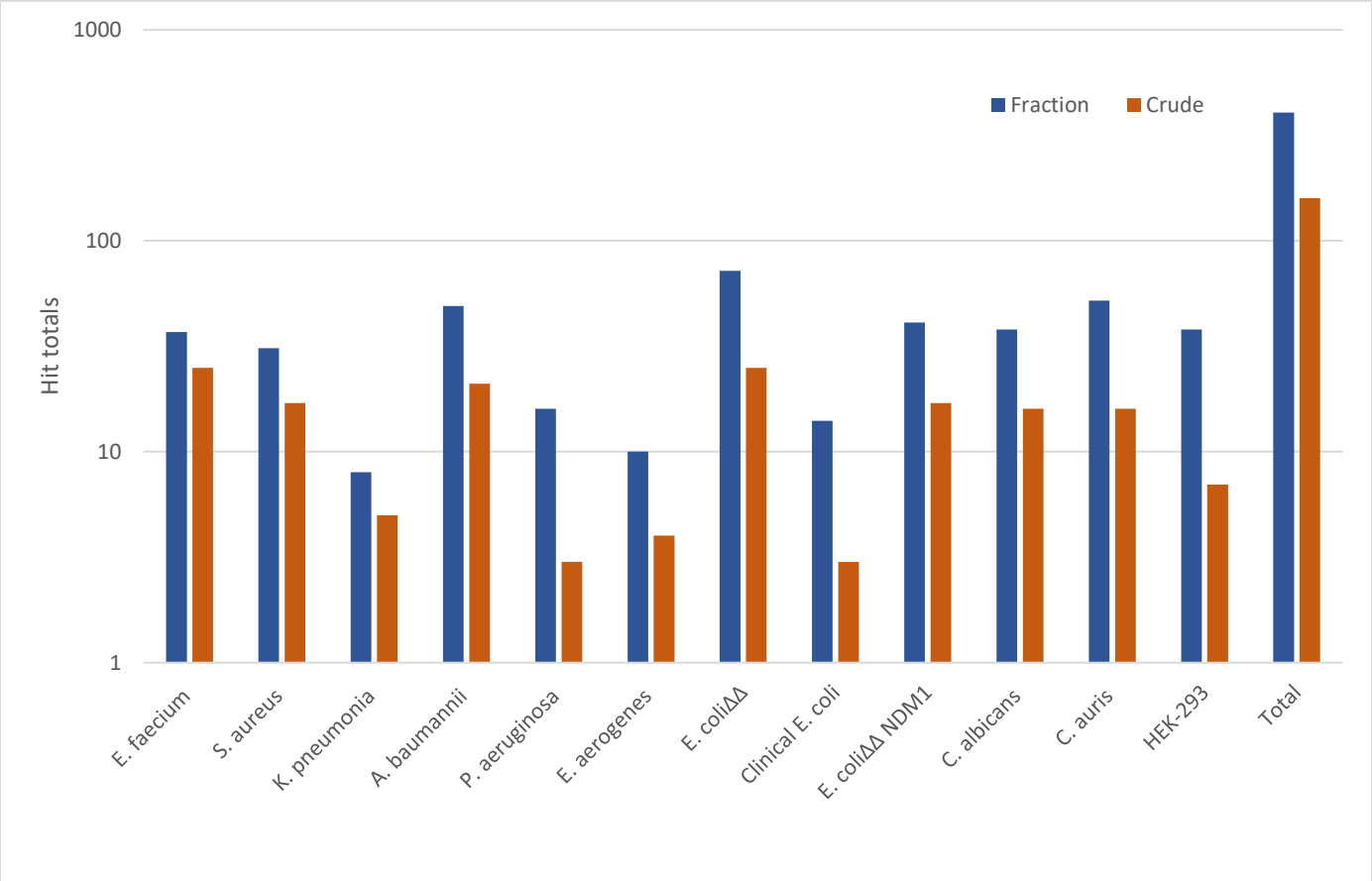


Figure 3-5. Number of fraction hits vs the number of crude extract hits displayed in a log10 bar graph.

Overall, there were 2.55 times more hits from fractions compared to crude extract hits (**Figure 3-5)**. Often, a hit would appear only in the fractionated portion of a strain

row in the PFL, with the crude extra displaying inactivity in the screen. Some of these differences can be attributed to imperfect fractionation and an active compound being spread out across more than one fraction. However, even accounting for that variable, the hits generated from fractions still significantly exceed those generated by crude extract alone.

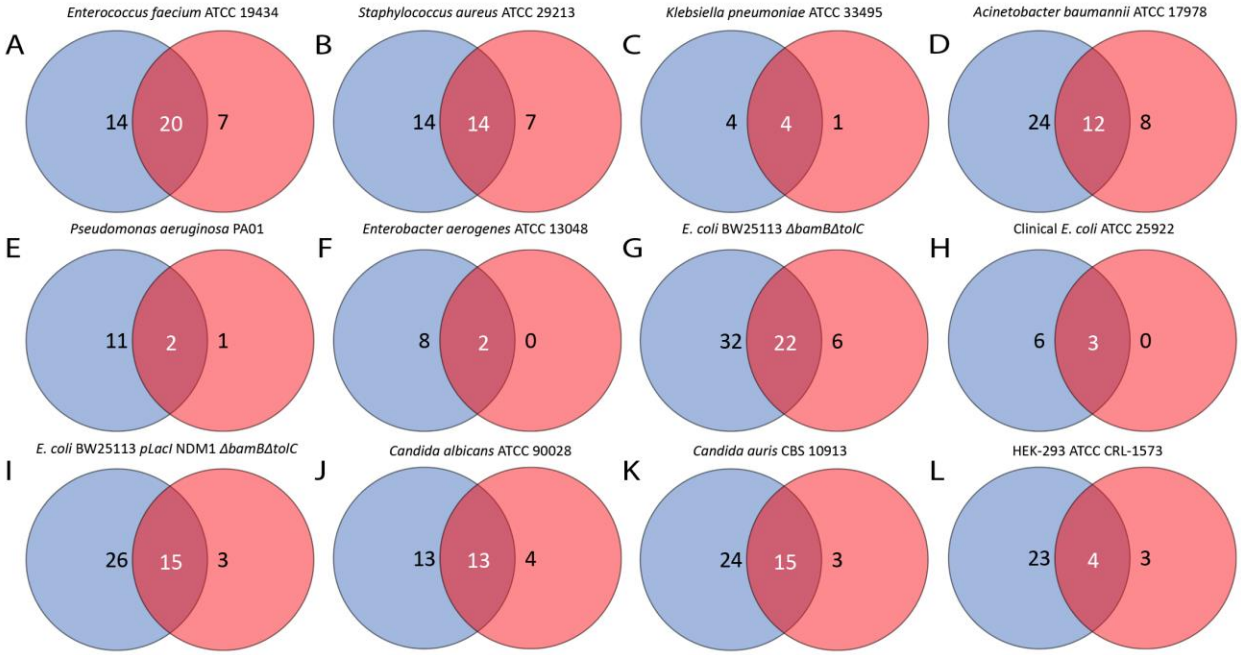


Figure 3-6. Venn-diagrams of the hits from each assay. Fractions (blue), crude extract (red), or in both the fractions and crude of that active producing strain (middle). (A) *E. faecium* ATCC 19434 (B) *S. aureus* ATCC 29213 (C) *K. pneumonia* ATCC 33495 (D) *A. baumannii* ATCC 17978 (E) *P. aeruginosa* PA01 (F) *E. aerogenes* ATCC 13048 (G) *E. coli* BW25113 Δ*bamB*Δ*tolC* (H) Clinical *E. coli* ATCC 25922 (I) *E. coli* BW25113 *pLacI* NDM1 Δ*bamB*Δ*tolC* (J) *C. albicans* ATCC 90028 (K) *C. auris* CBS 10913 (L) HEK-293.

During the 12 screens, the fractions of a particular strain showed up as a hit 199 times when the crude extract displayed no activity, far more than the reverse **(Figure 3-6)**. There were only 43 instances when the crude extract of a strain tested positive and the fractions did not. After the biological screens were conducted and analyzed for hits, active wells must be selected for follow-up work. This involves regrowing the strains to make new extracts and fractions to confirm activity seen in the original screen is reproducible. After a hit is deemed reproducible, the active component must be purified, and various analyses such as HPLC, NMR, tandem MS2 and partial hydrolysis data analysis performed to determine the molecular structure of the bioactive component. Hits of interest were determined by isolating active wells that emerged in related groupings and did not appear in other bioassays as hits **(Table 3-2).** Follow-up work has been done on the MBL inhibitor screen and its hits; however, the reproducibility of these strains was poor, and we were unable to move forward in the protocol of activity-guided purification. There will be continued follow-up work by other members of the lab, but here I will focus on antifungal producing strains.

Table 3-2. Hits of interest for potential follow-up work.

Screen	WAC#	Active Well
ESKAPE Gram +	5858	Crude
	11133	Crude
	11139	F2
	11175	F8
	11201	F8
ESKAPE Gram –	11181	F8
Yeast	11113	F6
	11161	F8
	11243	F8
NDM1	1560	Crude, F4
	2662	F7
	8478	F6
	10964	F8
	10973	F8
	11033	Crude, F6
	11038	F8
Clinical *E. coli*	10988	F1
	11218	F1

3.4 Follow-up; Identification of antifungal compounds in the PFL

After deciding to focus our efforts on follow-up on the antifungal producing hits from the PFL screening. This was done after low reproducibility was shown in the NDM1 follow-up analyses, and we wanted to look for growth inhibitors of *C. neoformans.* We started with the three strains that showed activity only against *C. auris* and *C. albicans.* All 3 of these productive extracts and fractions were created from fungi isolates in the WAC collection. To test the reproducibility of the results seen in the high-throughput screens, we first began with regrowing the strains from the original WAC frozen stocks. Incubation and methanol extraction was performed under the same conditions described in the creation of the PFL. However, after fractionation, the 24 fractions were each resuspended in 100 µL rather than combined. The new fractions and crude samples were assayed for bioactivity in 96 well format in duplicate. The fractions were tested against both *C. auris* and *C. albicans,* as well as *Cryptococcus neoformans* H99. Unfortunately, no activity was found for strains WAC11113 and 11243. However, in fraction 18 of this growth protocol (fraction 8 in the PFL), activity was confirmed in WAC11161 **(Table 3-3)**. This is especially interesting as the crude extract continues to display no antifungal activity.

Table 3-3. Hits active against only *C. auris* and *C. albicans*.

WAC#	18S Identification	Active Fraction	Reproducibility
11113	*Seiridium cardinale*	F6	-
11161	*Sordariomycetes sp. / Hypoxylon submonticulosum*	F8	yes
11243	*Xylaria sp.*	F8	-

Since 2 of 3 original strains chosen due to what appeared to be specific activity against fungi were not reproduced, the list of antifungal producers discovered in the initial high throughput screen was expanded by 11 to a total of 14. These additional strains displayed some activity in other assays, so they are not specific to fungi. However, reproducibility proved much higher (**Table 3-4)**. In summary, we verified 14 candidates and 11 strains had active fractions, giving a 78% reproducibility rate. Interestingly, many strains show little to no activity present in the crude extract suggested they could be missed using older protocols. Only three strains (WAC1545, 1325, and 11175) showed antifungal activity in crude extracts.

Table 3-4. Additional antifungal producing WAC strains regrown and tested for reproducibility.

WAC#	16S/18S Identification	PFL Active Fraction	Reproducibility	Crude Activity
1325	*Streptomyces sp. K35-27P*	F6	yes	yes
1490	*Streptomyces sp.*	F6	yes	-
1545	*Streptomyces sp.*	F8	-	yes
5858	*Streptomyces sp.*	F5	yes	-
10993	*Sarocladium bactrocephalum*	F8	yes	-
10997	*Pseudoallescheria boydii*	F7	yes	-
11024	*Hypocrea rufa*	F6	yes	-
11084	*Myrothecium inundatum*	F6	yes	-
11175	*Fusarium proliferatum*	F8	yes	yes
11213	*Cochliobolus geniculatus*	F1, F7	yes	-
11256	*Trematosphaeria pertusa*	F7	yes	-

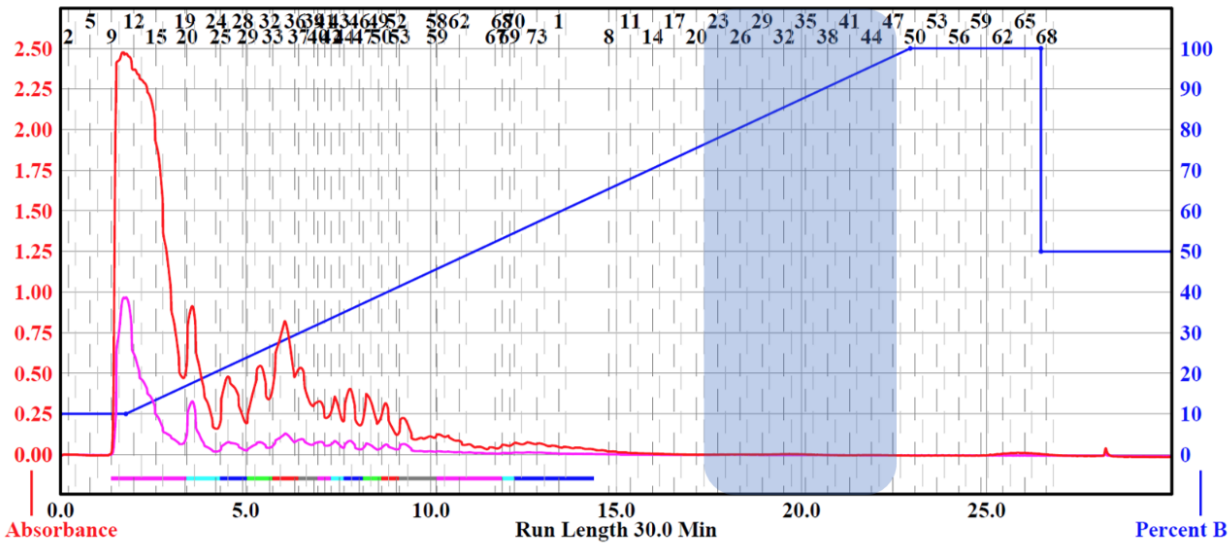


Figure 3-6. Active fractions of WAC11161. Fractions 96 to 123 are highlighted in blue. The blue line is the increase in MeCN%, and absorbance was monitored at 254 nm (red) and 280 nm (purple).

The next step was to identify the compound responsible for the antifungal activity using activity-guided purification. The main active compound of WAC11161 elutes from 75% acetonitrile (MeCN) to 95% MeCN **(Figure 3-6)**. Purification began with a large-scale fermentation. Eight liters of YPD were inoculated with WAC11161 and fermented over 6 days, then the resulting pellets were extracted with methanol. The methanol extract was concentrated by a rotary evaporator and fractionated on the Combiflash with a large 86 g C18 column. The active fractions were then verified by bioassay to purify the compound further, a size-exclusion separation was undertaken. The active fractions were separated on a Sephadex LH-20 column, and the purified compound was separated on HPLC to verify purity after activity was confirmed **(Figure 3-7)**. From this purification protocol, 10 mg of the antifungal compound produced by WAC11161 were recovered.

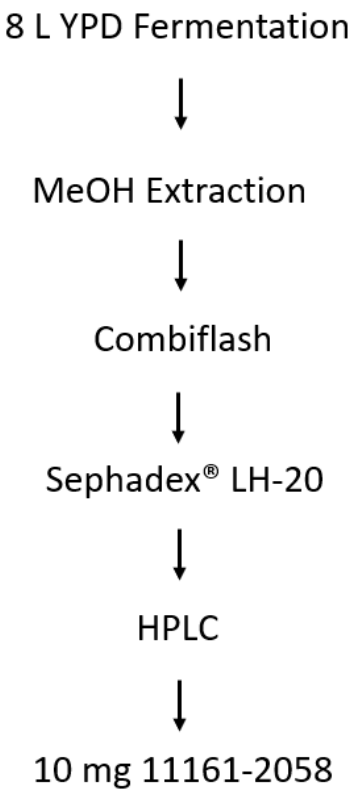


Figure 3-7. Flowchart for purification of WAC11161 compound 2058

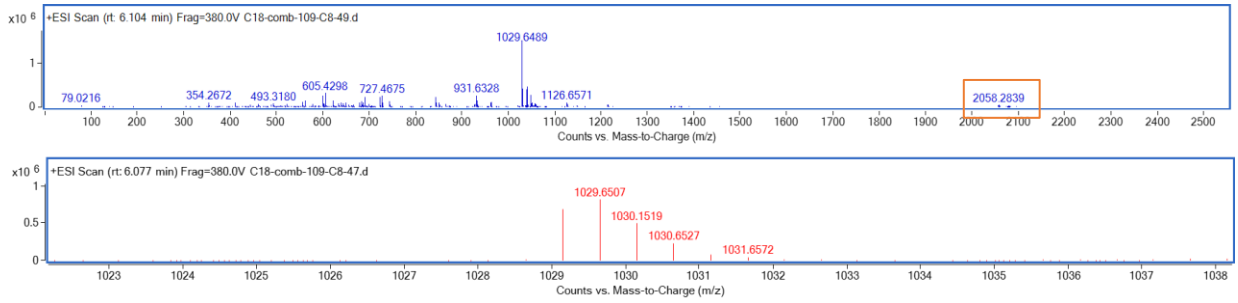


Figure 3-8. High-performance liquid chromatography chromatogram of partially purified active antifungal compound produced by WAC11161.

From the HPLC analysis, an exact mass of 2058.2839 was determined **(Figure 3-8)**. No compounds with this specific mass were listed in the Dictionary of Natural

Products, suggesting a novel molecule.[66] With the compound purified, the next step in identifying the structure was through nuclear magnetic resonance (NMR).

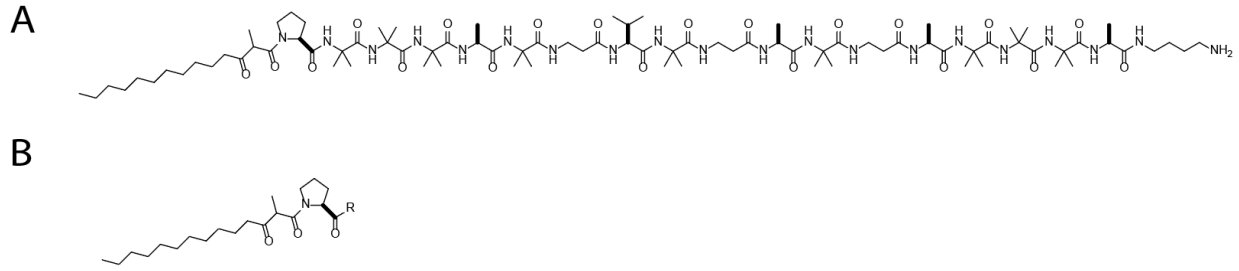


Figure 3-9. (A) Example lipopeptide sphaerostilbellin A[67] (B) Confirmed by NMR beta-keto amide of the active compound present in fractions of Wright Actinomycetes collection strain 11161.

Based on the NMR analysis, the active antifungal compound found in the fractions of WAC11161 is a lipopeptide. The amino acid order is currently unclear due to challenges in solving such a large structure, but progress is being made. The beta-keto amide on the end of the lipopeptide has been confirmed by nuclear magnetic resonance (NMR) and is similar to the lipopeptide sphaerostilbellin A (**Figure 3-9)**. Further work is needed to complete the structure of this potentially novel antifungal natural product.

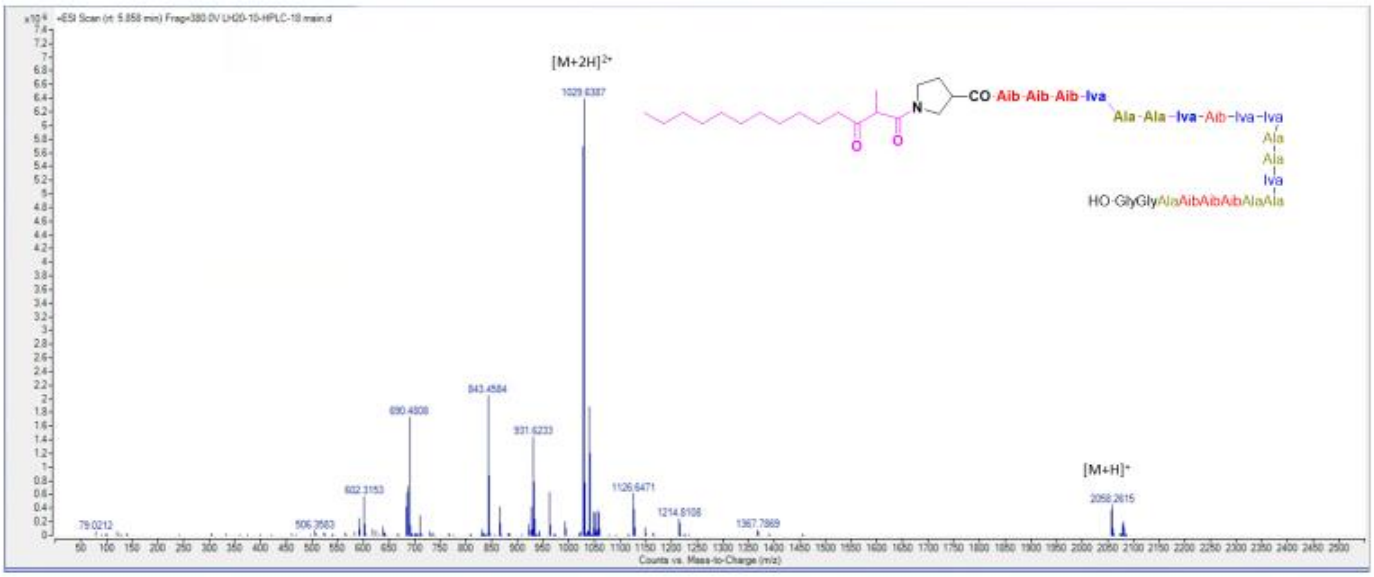


Figure 3-10. HRMS spectra and current proposed structure for the antifungal WAC11161 product.

To further draw closer to the correct sequence of amino acids of the WAC 11161 product, various analyses were performed. These included amino acid analysis, acid hydrolysis for 20h in 6N HCl followed by Marfey's reagent modification **(Figure S-4)**, reference L or DL-Amino acids (over 20 tested). In tandem with MS2, this partial data was used to propose a structure and amino acid sequence. Further analysis is ongoing, and the proposed structure will likely be modified further. A more accurate structure can be suggested when the genome sequence is complete and further NMR analysis is performed. As of now, the closest to a precise structure is a novel lipopeptide antifungal **(Figure 3-10)**.

Chapter 4 – Conclusions and future directions

The world is currently facing an antibiotic resistance crisis, where bacteria such as the ESKAPE pathogens are becoming more difficult to treat effectively and drug resistant fungi such as *C. auris* are emerging as significant threats. The discovery of new compounds must occur to keep up with the race against resistance. Natural products can still be a great source for mining new antimicrobial compounds to combat AMR, but methods for screening for antimicrobials must evolve past the Waksman platform. Fractionation provides a potential option to bring to light compounds that may have been missed in prior screens performed against only crude extracts. Fractionation reduces viscosity, allowing for a higher concentration of potentially active compounds. Further interference between inhibiting compounds in the crude can be reduced in the fractions.

In high throughput screening against a number of bacterial and fungal isolates, the PFL showed an average 2.55 times increased hit rate in the fractions compared to the crude extract control. These results are consistent with data shown in previous literature coming from the Wyeth group, Merlion Pharmaceutical, and NCI. In addition, analysis of the screens resulted in a selection of hits worthy of continued follow-up work.

Focusing on antifungal compounds, WAC11161 produced a potentially novel lipopeptide that exhibits activity against *C. auris, C. albicans,* and *C. neoformans*. The structure is most likely similar to sphaerostilbellin A, sharing a beta-keto amide tail but with a different order of amino acids that have yet to be solved. A novel antifungal compound that displays no activity in the crude, and is only apparent after fractionation,

would be a confirmation of the benefits of screening fraction libraries and the creation of the PFL.

Other than fractionation, there are modern solutions that can be done to expand the IIDR's screening capacity. Some of this is already in the process of being implemented, such as the robotic frozen stock storage solution and the Echo acoustic dispenser is the CMCB. An ultra-high throughput method for screening that could be interesting to investigate further is microencapsulation. Droplets, the volume of a few picoliters, can be used to assay over 100,000 samples in a single day.[68] The IIDR should be receptive to new screening strategies and technologies to stay nimble to fight against a changing dynamic landscape of disease combat.

The prefractionation library can be expanded to encompass the entire WAC, providing a valuable addition to the existing NPL. The PFL has already expanded from the 48 plates (381 strains, 3048 fractions) discussed in this thesis to 80 plates (634 strains, 5072 fractions). Another route that can be pursued to further expand upon these results is continued follow-up on the active compounds from the initial screening results. The structure of the antifungal compound from WAC11161 still needs to be completed, and other antimicrobial hits should be investigated. Overall, the PFL will become a platform that the Wright lab and collaborators can benefit from for years to come.

Chapter 5 – Works Cited

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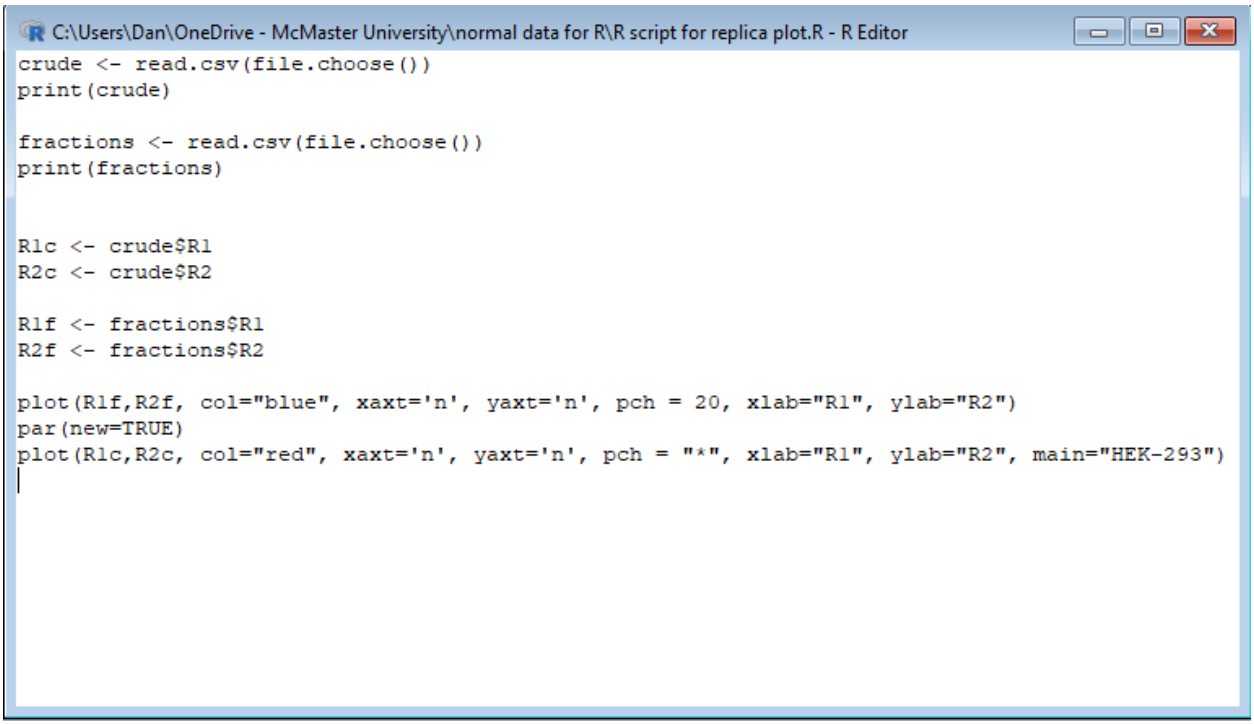
Supplementary Figures

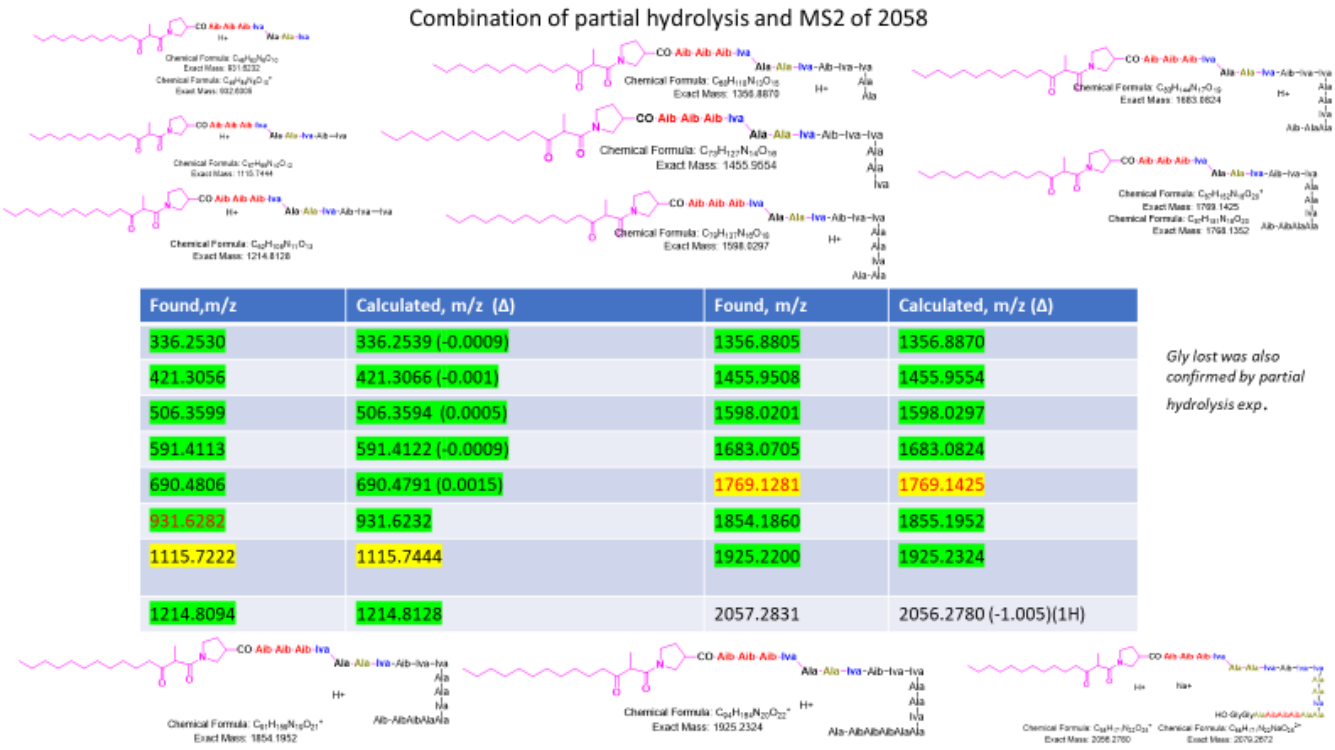
Supplemental Figure S-1. Modified can crusher used squeeze cultured agar into 50 ml falcon tubes.



Supplemental Figure S-2. Script on the statistical R program to produce the replica plots for the bioactivity assays.

Timetable Table (min)	Solution A % (water)	Solution B % (methanol)	Flow (mL/min)	Pressure bar
2.00	70.0	30.0	---	---
3.00	50.0	50.0	---	---
22.00	20.0	80.0	1.000	---
30.00	0.0	100.0	1.000	---
33.00	0.0	100.0	---	---
33.10	70.0	30.0	1.000	---
36.00	70.0	30.0	1.000	---

Supplemental Figure S-3. HPLC protocol for purification of the WAC11161 compound.



Found,m/z	Calculated, m/z (Δ)	Found, m/z	Calculated, m/z (Δ)
336.2530	336.2539 (-0.0009)	1356.8805	1356.8870
421.3056	421.3066 (-0.001)	1455.9508	1455.9554
506.3599	506.3594 (0.0005)	1598.0201	1598.0297
591.4113	591.4122 (-0.0009)	1683.0705	1683.0824
690.4806	690.4791 (0.0015)	1769.1281	1769.1425
931.6282	931.6232	1854.1860	1855.1952
1115.7222	1115.7444	1925.2200	1925.2324
1214.8094	1214.8128	2057.2831	2056.2780 (-1.005)(1H)

Gly lost was also confirmed by partial hydrolysis exp.

Supplemental Figure S-4. Proposed structure work on WAC11161 compound based on tandem MS2 and partial hydrolysis data.