MICROBIAL NATURAL PRODUCT DRUG DISCOVERY THROUGH SYSTEMATIC SAMPLING OF DIVERSE TEXAS SOILS

DISSERTATION

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ABSTRACT

This project aimed to explore a new approach to microbial natural product discovery through collection of soil samples from strategically determined geographic sites in the State of Texas. Morphologically distinctive bacterial and fungal isolates were obtained from the soil samples and a pilot-size, semi-fractionated natural product library was constructed and screened for cytotoxicity against human cancer cell lines. Subsequently, activity-guided purification and structural identification were performed to characterize naturally occurring bioactive small molecules.

Through a pilot fungal-derived natural product study, the library construction methodology was validated and known cytotoxic compounds malformin, palmitic acid, aspergillin PZ, and trichoderone B were isolated and re-identified. Their structural data and previously unknown cytotoxicity against various cancer cell lines were studied and are reported herein.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ACP: Acyl carrier protein

ADC: Antibody-drug conjugate

ALL: Acute lymphoblastic leukemia

AML: Acute myeloid leukemia

AT: Acyltransferase

BGC: Biosynthetic gene cluster

CML: Chronic myelogenous leukemia

CTCL: Cutaneous T-cell lymphoma

DH: Dehydratase

DMEM: Dulbecco's Modified Eagles Medium

ELSD: Evaporative light scattering detection

ER: Enoyl reductase

FBS: Fetal bovine serum

FDA: U.S. Food and Drug Administration

HDAC: Histone deacetylase

HDF: Normal human dermal fibroblast

HPLC: High-performance liquid chromatography

HTS: High throughput screening

IDS: Isoprenyl diphosphate synthase

ISPR: In-situ product recovery

KR: Ketoreductase

KS: Ketoacyl synthase

LB: Liquid broth medium

LogP: Octanol-water partition coefficient

MS/MS: Tandem mass spectrometry

MT: Methyltransferase

NHL: Non-Hodgkin's lymphoma

NRPS: Nonribosomal peptide synthetase

PCP: Peptidyl carrier protein

PD: Pharmacodynamics

PK: Pharmacokinetics

PKS: Polyketide synthase

PTCL: Peripheral T-cell lymphoma

RiPP: Ribosomally synthesized and post-translationally modified peptide

RO5: Lipinski's Rule of Five

TE: Thioesterase domain

TOF-MS: Time-of-flight mass spectrometry

TQMS: Triple quadrupole mass spectrometry

TPS: Terpene synthase

UVD: Ultraviolet detection

CHEMICAL ABBREVIATIONS

ACN: Acetonitrile

$CaCl_2$: Calcium chloride

$CaCO_3$: Calcium carbonate

DCM: Methylene chloride

DMSO : Dimethyl sulfoxide

EtOAc: Ethyl acetate

EtOH: Ethanol

$FeSO_4 \cdot 7H_2O$: Iron (II) sulfate heptahydrate

GSH: Glutathione

H_2O: Water

KH_2PO_4: Monopotassium phosphate

MeOH: Methanol

$MgSO_4 \cdot 7H_2O$: Magnesium sulfate heptahydrate

$MnCl_2 \cdot 2H_2O$: Manganese (II) chloride dihydrate

NaCl: Sodium chloride

Na_2HPO_4: Sodium phosphate dibasic

NaOH: Sodium hydroxide

$(NH_4)_2SO_4$: Ammonium sulfate

PBS: Phosphate buffered saline

TCA: Trichloroacetic acid

$ZnSO_4 \cdot 7H_2O$: Zinc sulfate heptahydrate

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PREFACE

Specific Aims

Aim #1: To construct a pilot library of natural product prefractions derived from microbial isolates systematically obtained from the diverse soils of The State of Texas.

Aim #2: To locate active prefractions that exhibit cytotoxicity to any or all of MIA PaCa-2, SH-SY5Y, or COLO 829 cancer cell lines through cell viability screening.

Aim #3: To purify and characterize bioactive compounds within active prefractions and identify their respective microbial producers.

Significance

This study validated the library construction and screening methodology and thus far yielded compounds which have undergone additional cytotoxic screening at the National Cancer Institute, yielding results which advance our understanding of the aspochalasin sub-group of fungal natural products.

Innovation

An approach of systematic sampling of diverse soils rather than random sampling was employed to obtain microbial isolates for the construction of a new natural product library.

Chapter 1

INTRODUCTION TO NATURAL PRODUCTS, MICROBIAL NATURAL PRODUCT BIOSYNTHESIS AND DISCOVERY

SUMMARY. Natural products are small molecules consisting of carbon, hydrogen, oxygen, sulfur, nitrogen, and infrequent halogens[1] which are produced by living organisms and have long been sought after for their pharmacologic and biologic activities including anticancerous, anti-microbial, and anti-viral characteristics[2]. Among 1,394 small molecule drugs approved by the U.S. Food and Drug Administration (FDA) between 1981 and 2019, 916 (65.7%) were natural products, synthetically modified natural products (semi-synthetic), or synthetic compounds with natural product inspired pharmacophores[3,4]. Approximately 185 small molecule drugs were approved by the FDA in this time frame for treatment of cancerous diseases[4].

Since the discovery of penicillin by Sir Alexander Fleming in 1928, microbial natural products have been deemed a significant source for new valuable lead compounds. However, in the early 1990s there was an paradigm shift[5] away from screening large natural product libraries and instead focusing on vast synthetically derived small-molecule libraries due in-part to the emergence of combinatorial chemistry and high-throughput screening (HTS)[6]. Recently there has been a trend reversal and revitalization or "new golden era"[6,7] of natural product drug discovery. Today synthetic and natural product drug discovery work harmoniously to make up for each other's shortcomings.

1. A BRIEF HISTORY OF NATURAL PRODUCT DRUGS

The modern concept of using naturally produced compounds to treat ailments is by no means a new idea. The earliest documented uses of natural products are found on engravings of Sumerian clay tablets (ca. 2600 BCE) describing, often in great detail, the use of oils from *Cupressus sempervirens* (Mediterranean cypress), *Glycyrrhiza glabra* (licorice), *Papaver somniferum* (opium poppy), and *Commiphora* sp. (myrrh)[8,9].

Discovered by Egyptologist Georg Ebers in 1872, a nearly immaculate papyrus preserved in mummy cloths (ca. 1500 BCE) was translated to reveal an intricate and thorough list of hundreds of natural products and their formulations for use in nearly every disease ranging from dental conditions to treatments of cancerous tumors[8,10-12].

Plant-based natural products were, however, not restricted to Mesopotamia and Northern Africa. The Wushi'er Bingfang (ca. 1100 BCE), Shennong Herbal (ca. ~100 BCE) and the Tang Herbal (ca. 659 CE) all represent the rich history of herbal medicines in Chinese culture[8]. Even Theophrastus, an ancient Greek philosopher and scientist, and Pedanius Dioscorides, a Greek pharmacologist, furthered the scientific understanding of many herbal remedies[9]. To the north a 1,000 year old Anglo-Saxon medical text, Bald's Leechbook, contains an herbal remedy to the common *Staphylococcus aureus* infection among others[13].

Today an estimated 70% - 80% of the global population still uses traditional herbal medicines due to their ease of accessibility and relatively low toxicity[14]. While a majority of history has been spent finding, documenting, and understanding the role of herbal natural products for the treatment of disease, it wasn't until the late 19th century that microbial produced compounds began to be understood as potent cellular growth inhibitors[15].

First reported in 1875, T. H. Huxley and John Tyndall noted that *Penicillium glaucum* inhibited the growth of bacteria within test tubes[16]. Sir Alexander Fleming's serendipitous rediscovery of this phenomenon in 1928[17] led to the development of penicillin by Howard Florey and Sir Ernst Chain and a subsequent 1945 Nobel Prize in Physiology or Medicine for their life-saving WWII-era drug[18].

Driven by the building pressure of antibiotic resistance in the field of infectious diseases[6] and an ongoing cancer threat, understanding the biosynthesis of microbial natural products as well as the efficient screening of libraries containing microbial natural products remains critical to addressing these life-threatening diseases in a timely manner. Since the fortuitous discovery of penicillin, more than 23,000 microbial natural products have been isolated and characterized[19].

The 1950s and 1960s saw a "golden era" of exploring natural products as a primary source for lead compounds in pharmaceutical and academic research[7]. In the 1990s, however, the practice of screening natural product libraries was sidelined and replaced with large combinatorial libraries instead[19-23] due to the lengthy turn-around times associated with natural product discovery, inherent druggability issues of natural products, uncertainty of their structural novelty, and the large fermentation scale-up needed for compounds which are produced at low concentration. From the 1990s to 2000s more compounds were screened through HTS than were screened from the 1930s to 1990 but few lead compounds were identified[24]. Because of this, there was a reemergence of natural product research which still remains a valuable source for lead compounds[25].

While the natural product field has successfully churned out important biologically relevant compounds for centuries, the chemical space still remains largely untapped. For example, compounds which contain only 30 atoms and are made up of only carbon, hydrogen, oxygen,

nitrogen, and sulfur could theoretically be combined in more than 10^{60} unique ways[26]. That is not to say that each arrangement would necessarily be structurally stable or active but serves to illustrate the size of the unknown chemical space, especially when considering that many natural products exceed 30 atoms in size.

Recently, the 2015 Nobel Prize in Physiology or Medicine was awarded to Satoshi Ōmura and William C. Campbell for their discovery of the anti-parasitic avermectin from *Streptomyces avermitilis* which has in-part helped spur this new golden era in microbial drug discovery[7,27]. While a hefty and undoubtedly deserved focus is placed on microbial produced anti-microbial compounds, bacteria and fungi have also made significant contributions to cancer therapy[3].

2. CANCER AND MICROBIAL NATURAL PRODUCTS

Cancer is responsible for 1 in 6 deaths, making it the second leading cause of death behind ischemic heart disease[28]. Globally, by 2030 the annual cancer incidence is projected to reach 21.7 million resulting in 13 million deaths[29]. Despite a steady decline in cancer-related mortality since 1991[30] discovering new anticancer compounds remains an important part of biomedical research.

The first targets for anticancer drug development were leukemias as solid cancer imaging was not possible at the time while blood cancers were visible and easily quantifiable under microscope[31]. The first modern uses of chemotherapeutics in cancer therapy were the utilization of nitrogen-mustard[32] and aminopteroylglutamic acid[33] against leukemias. Since then, both synthetic and naturally produced compounds have been employed as first-line cancer therapy options.

More than 30 approved small molecule drugs have come from or been inspired by microbial natural products despite the high efficacy of plant metabolites and human-derived compounds (**Table 1**). Microbial natural product-sourced cancer therapies are often produced by

actinomycetes and filamentous fungi[34] which are therefore constantly scoured for potential chemotherapeutic metabolites.

Among the microbial-produced natural products, the largest and most prevalent class of metabolites are the anthracyclines. Often regarded as one of the most successful classes of anticancer compounds[35], there are few cancers which are not affected by these streptomycete-produced DNA intercalators.

The topoisomerase inhibiting[36] anthracycline aclarubicin (**1; Figure 1**) was one of 21 analogous compounds isolated from *S. galilaeus*[37]. Daunorubicin (**2**) and its 14-hydroxylated analogue doxorubicin (**3**) were both isolated from *S. peucetius* var. *caesius*[38]. Daunorubicin has also given rise to derivate compounds idarubicin (**4**), epirubicin (**5**) and valrubicin (**6**). Mitoxantrone (**7**), a synthetic compound built upon the anthracycline pharmacophore has also been approved for the treatment of leukemias.

In addition to **1-7**, bleomycin[39], azinomycin B[40,41], chromomycin A3[42], mithramycin (no longer marketed)[43], mitomycin C[44], and streptozotocin[45] are also produced by streptomycetes.

Interestingly, and perhaps indicative of the versatility of microbial-derived natural products, *S. macromomyceticus* produces a large chromoprotein, neocarzinostatin[46], in which a potent yet unstable chromophore (**Figure 2**) is stabilized by a 113 amino acid protein and intercalates DNA which causes free radical damage to deoxyribose, eventually leading to strand breakage or covalent bonding of the drug to DNA[47].

Streptomycetes, of course, do not claim sole possession of the ability to produce small molecules capable of disrupting cancer cell growth. Romidepsin, for example, a bicyclic depsipeptide

inhibitor of histone deacetylase (HDAC) is used to treat T-cell lymphoma and is produced by Gram-negative *Chromobacterium violaceum*[48,49]. Famously from the State of Texas, *Micromonospora echinospora* ssp. *calichensis* within caliche clay was found to produce a potent anti-infective and anticancer metabolite calicheamicin[50] with picomolar potency which was able to elicit *in vitro* transcriptional effects as quickly as 2 minutes post treatment[51]. Due to its remarkable potency, further action was necessary to prevent off-target effects. This effort led to the development of the first FDA approved monocolonal antibody-drug conjugate (ADC) gemtuzumab ozogamicin which targeted the compound to acute myeloid leukemia (AML) with an anti-CD33 IgG4 κ antibody[52]. This ADC strategy has been replicated in inotuzumab ozogamicin which targets calicheamicin to acute lymphoblastic leukemia (ALL) with an anti-CD22 monoclonal antibody[53].

Despite an original belief that the tunicate *Ecteinascidia turbinata* was the source of observed anticancer activity, it was later revealed that a symbiont *Candidatus endoecteinascidia* was responsible for producing trabectedin, a complex cytotoxic molecule which disrupts cancer cell growth by inhibiting nucleotide excision repair[54,55].

Much less extensively studied are the fungal-derived anticancer natural products. Despite the large number of fungal-derived compounds with superb *in vivo* and *in vitro* efficacy, there are currently no approved and marketed fungal compounds for use against cancers[56] notwithstanding compounds such as solamargine from *Aspergillus flavus* and *Solanum nigrum* progressing to clinical trials[57]. This lack of fungal metabolite representation among FDA approved anticancer drugs could be because of low compound titers in fermentation[58]. Because fungal metabolites are often complex molecules, synthetic versions of these compounds are hard to create in a laboratory

setting at quantities sufficient to meet the demand of pre-clinical studies let alone multi-phase clinical studies.

As with many drug production limitations, low production titers can be overcome by carefully studying their biosynthesis or determining effective synthetic schema to produce these complex compounds in a laboratory setting. Despite this lack of FDA approved anticancer drugs, fungal metabolites combine complexity, structural diversity, and drug-like properties to form a group of worthwhile compounds[59]. Given these considerations, there is a clear need for furthering the knowledge of bacterial and fungal-derived natural products for their use as potential anticancer therapies.

Table 1. Chemotherapeutics of Microbial Origin

Compound	Source/Derived Source*	Example Uses
5-ALA	*Alphaproteobacteria* sp.	Barrett's esophagus
aclarubicin	*Streptomyces galilaeus*[36]	AML
amrubicin	*Streptomyces* sp.*[60]	Lung Cancer
asparaginase	*Escherichia coli*[31]	ALL
bleomycin	*Streptomyces verticillus*[61]	Lymphoma/Testicular/Cervical
carzinophilin	*Streptomyces* sp.[40]	Leukemia
chromomycin A3	*Streptomyces griseus*[62]	Various cancers
dactinomycin	*Streptomyces parvulus*[63]	Nephroblastoma/RMS
daunorubicin	*Streptomyces* sp.	AML/ALL/CML/Kaposi's Sarcoma
doxorubicin	*Streptomyces peucetius*	Breast/Bladder/Lymphoma
epirubicin	*Streptomyces* sp.*	Breast/Ovarian/Gastric/Lymphoma
floxoruidine	*Aeromonas salmonicida*[64]	Colorectal Cancer
idarubicin	*Streptomyces* sp.*	AML/ALL/CML
ixabepilone	*Sorangium cellulosum*	Breast
leucovorin	*Leuconostoc citrovorum*[65]	Chemoprotectant
midostaurin	*Streptomyces staurosporeus**[66]	AML/myelodysplastic syndrome
mithramycin	*Streptomyces argillaceus*[43,67]	Testicular/CML
mitomycin C	*Streptomyces caespitosus*	Gastrointestinal/Anal/Breast
mitoxantrone	*Streptomyces* sp.*	AML/ALL
ozogamicin	Antibody-drug conjugate	ALL/AML
padeliporfin	Phototrophic bacteria*[68]	Prostate
peplomycin	*Streptomyces verticillus**[61]	Esophageal/Squamous cell carcinoma
pirarubicin	*Streptomyces* sp.*	Bladder
pixantrone	Natural pharmacophore	NHL
romidepsin	*Chromobacterium violaceum*	CTCL/PTCL
streptozotocin	*Streptomyces achromogenes*	Pancreatic
temsirolimus	*Streptomyces hygroscopicus*[69]	Renal Cell Carcinoma
trabectedin	*Candidatus endoecteinascidia*[55]	Soft-tissue sarcoma / ovarian cancer
valrubicin	Natural derivative	Bladder
zinostatin	*Streptomyces carzinostaticus*	Various cancers

List of approved chemotherapeutics which are directly produced by microbes or are compounds which are inspired by microbial produced compounds. Of this group, many are anthracyclines or are produced by the *Streptomyces* sp.. Despite this, compounds from other genera have been approved for clinical use. Notably, there are no approved fungal-derived small molecules in this list or in clinical use as of July 2021[56].

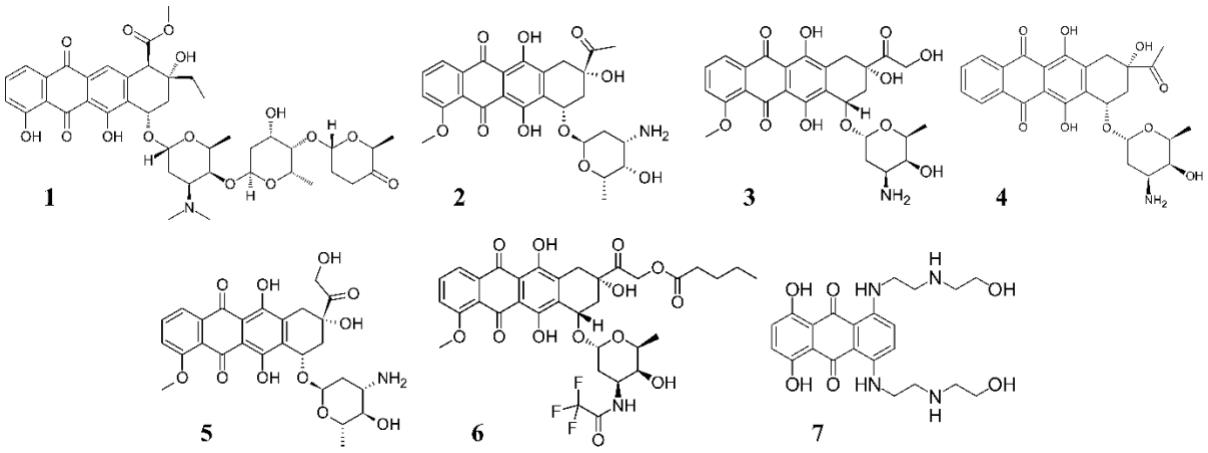


Figure 1. Commonly Used FDA-Approved Anthracycline Chemotherapeutic Compounds

Structures of anthracyclines aclarubicin (**1**), daunorubicin (**2**), doxorubicin (**3**), idarubicin (**4**), epirubicin (**5**), valrubicin (**6**), and mitoxantrone (**7**) which are among the most successful anticancer drugs in use to date. This class of compounds is capable of exerting cytotoxicity against nearly all cancer types through DNA intercalation.

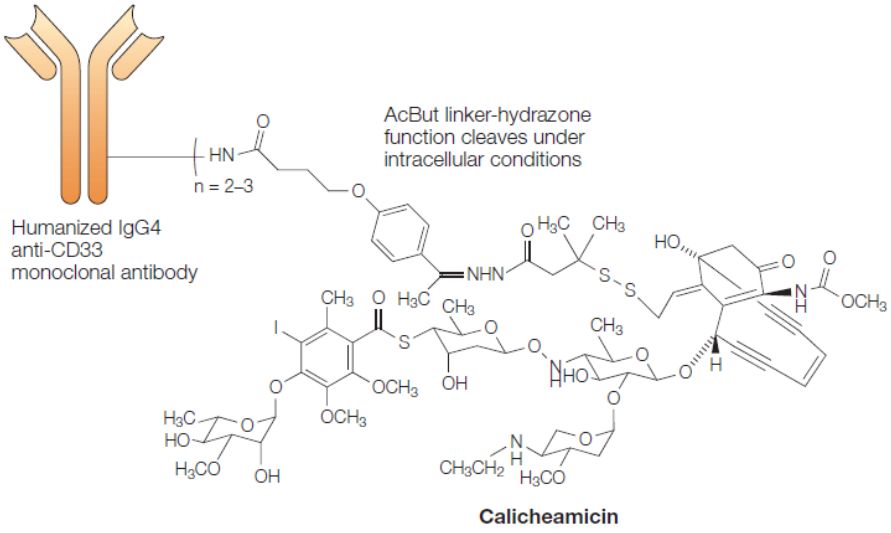


Figure 2. Calicheamicin and its Conjugated Antibody

The structure of highly potent calicheamicin and its attachment to humanized IgG4 anti-CD33 monoclonal antibody to increase specificity for cancer cells due to its picomolar potency causing off-target effects.

Figure adapted from:
Koehn, F. E. and Carter, G. T. The evolving role of natural products in drug discovery. *Nature reviews. Drug discovery* **4**, 206-220, doi:10.1038/nrd1657 (2005).[21]

3. MICROBIAL NATURAL PRODUCT BIOSYNTHESIS

Metabolites which are involved in the growth, development, and reproduction of a microorganism are referred to as primary metabolites[9]. Conversely, those small molecules which are typically produced in the idiophase and do not influence the previously mentioned functions are often used in defense or some survival capacity and are thus classified as secondary metabolites[19,70,71].

For the purposes of microbial natural product drug discovery the term 'secondary metabolites' is widely synonymous with microbial natural products[9] as these are often but not exclusively the compounds of interest for any library screening endeavors.

Within the membranes of fungal and bacterial cells are ever-churning pseudo-assembly-line machineries which linearly and/or iteratively modify growing molecules capable of exerting intracellular or extracellular effects. As the name implies, a biosynthetic gene cluster (BGC) is a group of spatially clustered genes which encode a biosynthetic pathway for the production of small molecules which can in turn be broadly categorized into the two previously mentioned classes; primary and secondary metabolites[72].

A typical bacterial genome contains roughly 30 BGCs while fungal genomes encode even more[23]. For example, the genome of *A. flavus* is predicted to contain 35 polyketide synthases (PKS) and 24 non-ribosomal peptide synthases (NRPS)[73] with each presumably encoding a distinct metabolite. Nearly 10% of *Salinospora tropica*'s genome consists of BGC genes. As of 2021, there were a total of 411,009 identified BGCs in the Integrated Microbial Genomes Atlas of Biosynthetic Gene Clusters[74] database of which only slightly more than 2,500 have had their metabolic product identified[23], a stark illustration of the large disparity between the theoretical number of microbial natural products and those which have been previously identified.

Microbial natural products can be broadly categorized into five major families: polyketides, nonribosomal peptides, RiPPs, terpenes and indole alkaloids[75,76] depending on their source BGCs.

The most abundant class of microbial natural products, polyketides, or molecules with a polyketomethylene group $(-CH_2CO)_n$[77], are produced by type I PKS[75]. The Type I PKS (**Figure 3**) creates carbon chains chiefly by incorporating acetyl-CoA or malonyl-CoA. Three essential domains present in the Type I PKS are: ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP); optional ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains may also be present[75].

A major difference between fungal and bacterial PKSs are the presence of multi-module PKS clusters in bacteria and iterative PKSs in fungi which are capable of repeatedly adding methylmalonyl-CoA eventually terminating as varied polyketides based upon the number of iterative steps, time of cyclization, and post-synthesis modification such as monooxygenation, dehydrogenation, methylation, and esterification[75].

NRPS clusters (**Figure 4**) are responsible for the generation of nonribosomal peptides and NRPSs are broken into modules which are responsible for incorporating each peptide building block into the polypeptide chain[78,79]; serving a purpose similar to transfer RNA and ribosomal RNA in conventional ribosomal systems.

This biosynthetic machinery can be contained within a single module (responsible for incorporating one amino acid) but more frequently NRPS systems exist as multi-module gene clusters (**Figure 5**). Each module requires between 3 and 3.5 kbp which causes NRPS genes to take up a substantial portion of the organism's genome. *Tolypocladium niveum* encodes an 11 module 1.6 MDa NPRS[78] which is responsible for creation of cyclosporine A, an FDA approved

calcineurin inhibitor[80]. Each module contains a substrate recognizer (adenylation-domain; A-domain), substrate holder (peptidyl carrier protein; PCP), and a peptide bond forming domain (condensation domain; C-domain). The first module in multi-module systems lacks a C-domain[78].

The A-domain selects an amino acid building block from a pool of several hundred potential substrates[78] and activates it through adenylation. This process is analogous to the aminoacyl-tRNA synthetases in conventional ribosomal peptide synthesis. After activation, the amino acid is transferred to the PCP. Finally, the C-domain catalyzes peptide bond formation. The major difference between this system and ribosomal peptide synthesis is a lack of proofreading mechanism in the NRPS system[78]. Once the growing peptide is passed to the last PCP in the multi-module system, it encounters a thioesterase (TE) domain similar to the finalization step of the PKS machinery. Importantly at this stage a cyclic or linear peptide can be formed depending on whether H_2O or an internal nucleophile attack the TE-peptide intermediate[78].

Some nuance to this biosynthetic process is the ability for a Cy-domain to be encoded rather than a C-domain. While they both function to incorporate a new amino acid into the growing peptide, the Cy-domain also is capable of heterocyclization[81]. These systems may also include methyltransferase or glycosyltransferase domains[78].

Similarly, secondary metabolites can be produced through a ribosomal system as well. Ribosomally synthesized and post-translationally modified peptides (RiPPs) as their name suggests are peptides which have been post-translationally modified into secondary metabolites following ribosomal peptide synthesis[82]. For production of these compounds, a core peptide is linked to a leader (N-terminal) and/or follower (C-terminal) sequence which help to post-translationally modify the core peptide. After modification, the core peptide is excised from the

leader and/or follower to yield a peptide secondary metabolite[83]. Interestingly for most RiPPs, excision is performed by a peptidase-containing ATP-binding cassette transporter which means that the secondary metabolite is exported from the cell at the instant its synthesis is complete[83]. Thiostrepton is an anticancer RiPP first isolated from *S. azureus* which causes cell cycle arrest through down-regulation of the FOXM1 oncogene[84].

Terpenes, mainly noted for their presence in plants are also produced by microbes and are formed by terpene synthases (TPS) which have been documented in *Gibberella fujikuroi, Fusarium sporotrichioides, A. terreus, S. coelicolor,* and *P. roqueforti* among others[75,85-88]. Further, 262 transcriptionally silent presumptive terpene synthases have been discovered in prokaryotes through bioinformatic analysis[89]. First, isoprenyl diphosphate synthase (IDS) combines isopentenyl diphosphate and dimethylallyl diphosphate to form isoprenyl diphosphate which is then converted by TPSs to terpenes[90]. These newly formed terpenes are then modified by decorating enzymes to form completed terpenes. Though hybrid IDS/TPS enzymes have been discovered in fungal species[91], conventional bacterial and fungal TPS systems are very similar due to a shared ancestry[90].

The final major category of microbial natural products are indole alkaloids, formed by incorporating an indole ring into an alkaloid scaffold. These compounds are commonly seen in endophytic fungi[92]. Indole alkaloids are often formed through NRPS incorporation of L-tryptophan into the nascent molecule or through a dipeptide intramolecular cyclization event in which a free -NH group attacks an aminoacyl thioester. Indole alkaloids have the capability of being extremely diverse due to their electron-rich pyrrole ring[93]. Vinblastine and vincristine are two alkaloids which have been extensively utilized in the clinic. Though originally isolated from *Catharanthus roseus*

(Madagascar periwinkle), they have since been determined to also be produced by endophytes *Alternaria* sp. and *F. oxysporum*[92].

Secondary metabolites may fall under any of these categories but are commonly produced as hybrids of multiple classes of BGCs. For example, both bleomycin and romidepsin are formed through a hybrid PKS/NRPS pathway.

While a microbe faithfully and abundantly producing a secondary metabolite of interest is the best case scenario in the drug discovery process[92], transcriptomics and metabolomics[94] have uncovered a major hurdle to overcome in a microbial natural product focused laboratory. A bacterium or fungus which possesses the necessary BGC to encode a secondary metabolite may have the responsible BGC locus transcriptionally silenced under normal laboratory conditions[88,95] with variances as subtle as filtered tap versus distilled water causing differences in metabolite expression[96]. The common black mold, *A. niger*, expresses less than 30% of its predicted 55 BGCs under a variety of laboratory conditions[97].

Overcoming this obvious impediment to natural product production and thus identification is made easier by 'mining' the genome for regions which encode enzymes involved in the previously described NRPS, PKS, and TPS pathways[23,88]. Indeed, a recently developed genome mining algorithm employed upon 1,154 prokaryotic genomes found more than 10,000 high confidence BGCs including many BGC families without any experimentally characterized members[98] representing a ten-fold disparity between theoretical and experimentally observed BGCs[19,99,100] which could then be targeted to enhance production of the product metabolite.

Most commonly algorithms such as antiSMASH (antibiotics and secondary metabolite analysis shell)[101], PRISM (prediction informatics for secondary metabolomes)[102], BAGEL[103], or SMURF

(secondary metabolite unique regions finder)[104], are employed for this task; though there are dozens of additional tools available for this exact purpose[105,106]. These algorithms are fundamentally based upon the identification of conserved synthase or synthetase genes which are located near genes encoding other compound 'decorating enzymes' which would be indicative of a BGC[107].

The downsides of the algorithmic approach are that it will only work on compounds produced from known BGCs, may be difficult to derive the chemical structure, and cannot predict the druggable potential of the predicted compound without *in vitro* analysis[108]. Alternatively, chemically modifying the metabolism of a given microbe or co-culturing[109] may be sufficient to induce the expression of BGCs which would normally be silent under laboratory conditions[110]. Though overexpression of BGCs through any means does not necessarily imply that their predicted product compounds will be generated or isolatable *in vivo*[111].

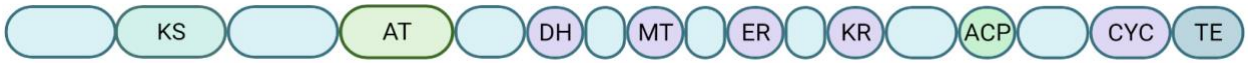


Figure 3. An Example of Polyketide Synthase Domain Structure

An example fungal PKS containing the essential domains: ketoacyl CoA synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). Further modification of a nascent molecule can occur through the dehydratase (DH), methyltransferase (MT), enoyl reductase (ER), ketoreductase (KR), or cyclase (CYC) domains. The growing compound is terminated by the thioesterase (TE) domain.

Figure modified and adapted from:
Keller, N. P., Turner, G. and Bennett, J. W. Fungal secondary metabolism — from biochemistry to genomics. *Nature Reviews Microbiology* **3**, 937-947, doi:10.1038/nrmicro1286 (2005).[75]

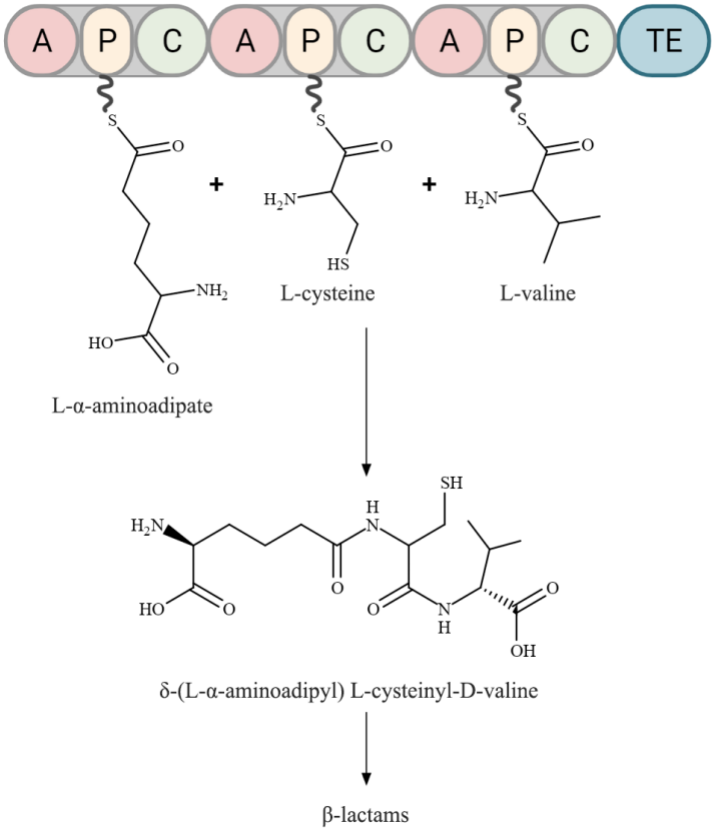


Figure 4. An Example of NRPS Domain Structure

A simple example of a nonribosomal peptide synthetase which produces a precursor molecule in penicillin and cephalosporin biosynthesis. Amino acid recognition is facilitated through the adenylation domain (A), attached as a thioester at the peptidyl carrier domain (P), and covalently bound to the nascent polypeptide at the condensation domain (C). Here, L-α-aminoadipate, L-cysteine, and L-valine are sequentially bonded to form δ-(L-α-aminoadipyl) L-cysteinyl-D-valine which is released by the thioesterase domain (TE).

Figure adapted from:
Keller, N. P., Turner, G. and Bennett, J. W. Fungal secondary metabolism — from biochemistry to genomics. *Nature Reviews Microbiology* **3**, 937-947, doi:10.1038/nrmicro1286 (2005).[75]

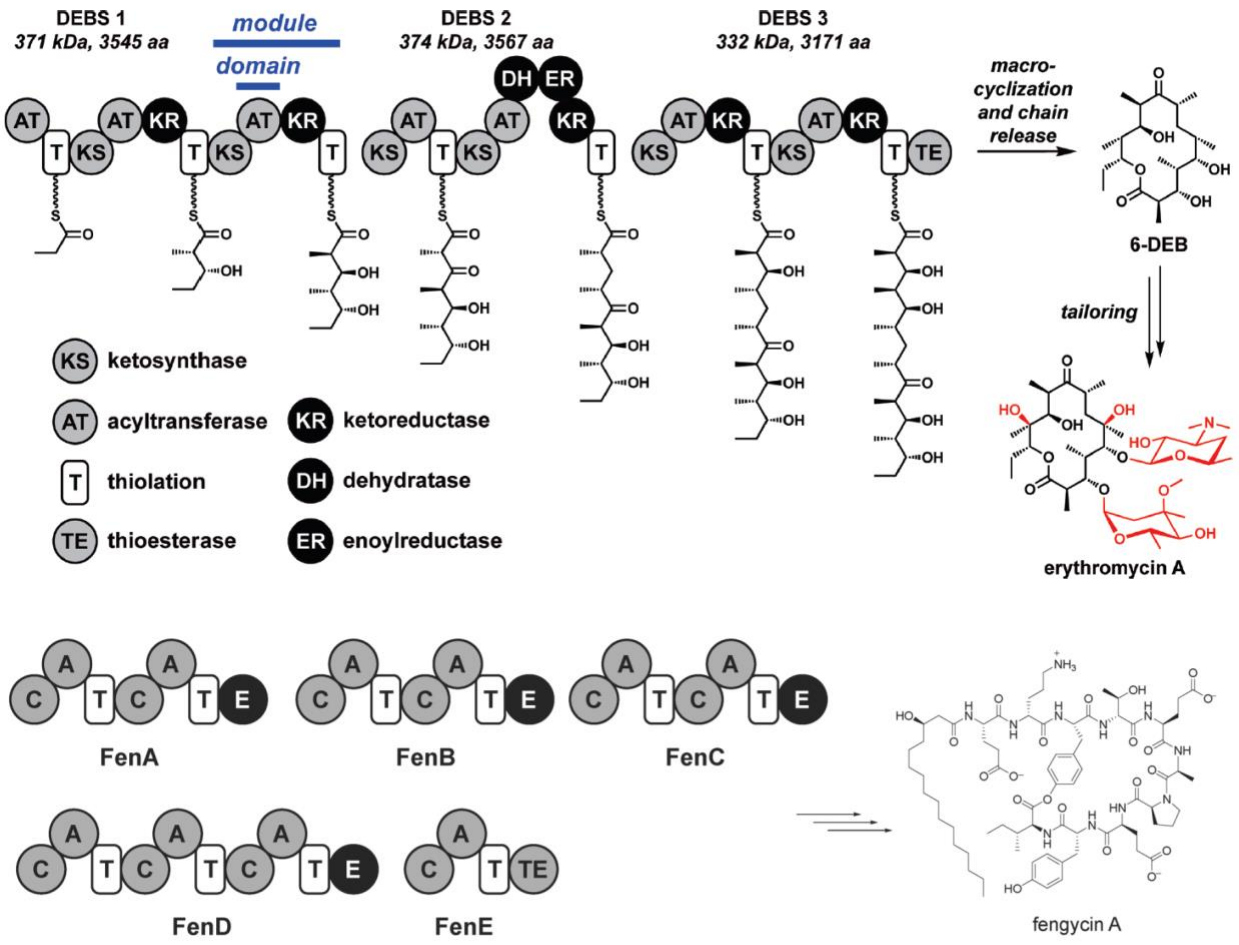


Figure 5. Examples of Biosynthetic Formation of Complex Secondary Metabolites

Legend and example of biosynthetic gene clusters responsible for the production of complex secondary metabolites erythromycin A (PKS-derived) and fengycin A (NRPS-derived).

Exerpted from figures in:
Walsh, C. T. and Fischbach, M. A. Natural Products Version 2.0: Connecting Genes to Molecules. *Journal of the American Chemical Society* **132**, 2469-2493, doi:10.1021/ja909118a (2010).[100]

4. MICROBIAL NATURAL PRODUCT DISCOVERY

Historically microbial drug discovery began as a fairly simple process of co-culturing streptomycetes with an organism of interest to detect antibiotics by measuring inhibition zones, often referred to as the eponymous 'Waksman platform'[112]. Since then, screening has shifted to testing the organism's metabolites rather than the organism itself against the target. This process has been made much more efficient by the development of compound libraries, genome mining, and HTS technology.

There are certain advantages to using natural products in drug development. First, the important understanding that unlike synthetic compounds, almost all natural products have receptor-binding activity[20] which in turn causes them to have elevated hit rates compared to their synthetic counterparts[14]. However, without carefully modifying the natural product to create semisynthetic analogues, natural products often have poor solubility and pharmacokinetics.

Further, despite or perhaps due to their prolific receptor activity, natural products often cause off-target effects and unwanted toxicity[113] making them poor lead compounds without further modification. Analysis of the natural product and medicinal chemistry chemical spaces revealed that there was limited overlap between their biologically relevant compounds indicating that natural products and synthetic compounds have a co-equal role in the drug discovery process[27].

Another advantage of natural products is their relative complexity[19] compared to many *de novo* synthetic compounds. Many libraries of synthetic compounds are constructed under the rigorous constraints of Lipinski's quickly adopted Rule of Five (RO5). Simply, the RO5 laid out four characteristic trends of approved compounds which when followed ought to enhance the solubility and permeability of orally delivered lead compounds: 1) That there be no more than five hydrogen

bond donors. 2) That there be no more than ten hydrogen bond acceptors. 3) That the molecular mass of the compound does not exceed 500 Daltons. 4) That the octanol-water partition coefficient (LogP) does not exceed 5[114]. While this was intended to be a helpful screening tool for complex and diverse libraries, the context and exceptions[115] to this rule were forgotten and the RO5 often becomes dogmatic rather than suggestive. Further amendments such as having no more than ten rotatable bonds, reducing polar surface area rather than logP[116], and limiting the number of atoms to 70[117] have further restricted the desire for pharmaceutical industry to develop or screen complex molecules which would not normally adhere to these rules.

Natural products, which are frequent RO5 rule-breakers, are often, but not exclusively, larger than 500 daltons[22]. In fact, they are explicitly exempt from the RO5 perhaps due to their likeness to endogenous compounds and thus their ability to utilize active transport mechanisms to circumvent LogP constraints[118].

For example, the complex RO5-breaking anthracyclines are typically used for treating infection and have also been used for treatment of various cancers because of their mechanistic intercalation-mediated histone ejection[119]. A strict RO5-following library would have omitted these compounds before they were tested.

It is estimated that there are between 1.5 and 5.5 million fungal species and 40,000 bacterial species globally[120] though only 75,000 to 100,000 species have been cultured and identified[59]. While a gram of soil may contain 10,000 unique microbial species[63] among as many as 10^{10} prokaryotic cells[121,122], only 0.1% to 1% of those species therein may readily grow under normal laboratory conditions[63,123]. Compounded with transcriptionally silent BGCs, it can easily be reasoned that

microbes are a valuable and still relatively untapped source for new pharmaceutical compounds and screening libraries of these compounds is therefore a worthwhile endeavor.

Typically, microbial natural product libraries are constructed with a random approach to sample collection. For example, pharmaceutical companies in the past would encourage their employees to collect soil samples in a random manner during their hometown visits or from vacation sites. Recently, the Citizen Science Soil Collection Program was established by the Natural Products Discovery Group at the University of Oklahoma in 2010. This library originally consisted of fungal isolates taken from the soils of Alaska, Hawaii, and Oklahoma and is now supplemented by "citizen scientists" who are sent soil sampling kits and can collect from far-reaching areas of the United States[124]. Further, the University of Michigan manages a similar library of actinomycetes, fungi, and cyanobacteria which are collected from diverse sites around the world[125]. While these microbial natural product libraries presumably contain more diverse soils due to their wider area of sampling, the current collection methodologies are nonetheless largely random. Because of this and the understanding that soil diversity is necessary for library diversity, there exists a need for a methodically constructed library which is able to incorporate samples from strategically selected diverse soils.

Chapter 2

CONSTRUCTION AND SCREENING OF A PILOT SEMI-FRACTIONATED NATURAL PRODUCT LIBRARY DERIVED FROM TEXAS SOIL-BASED MICROORGANISMS

ABSTRACT. A new pilot-size semi-fractionated natural product library has been constructed from microorganisms isolated from soil samples systematically obtained from distinct geographic regions within the State of Texas. Because of the diverse soil types of these regions, this library presumably contains a wider array of secondary metabolites than would normally be collected through random sampling of soils. This library consists of semi-purified crude extracts in DMSO from fungal and bacterial species which are readily available to be screened for anticancer, anti-microbial, or other endpoints.

1. INTRODUCTION

While establishing a library of natural products is by no means a novel concept, fusing the various methods of collection, fermentation, purification, and screening is a necessary step to tailor a new library so that it is optimized for the instrument capabilities and scale of a small to medium sized academic laboratory rather than a large institutional or industrial library.

Microbial natural product libraries have been an important part of the drug discovery process since the mid-1950s[126] and can range in size from large national libraries such as the National Cancer Institute's 125,000+ prefraction library[127] to small and focused lab-specific libraries.

One major issue with large natural product libraries is the inevitable collection of duplicates of a given microbial isolate or compound. This causes large amounts of waste in man-hours and chemical resources. The process of removing these redundancies is commonly referred to as 'dereplication'. Dereplication can be carried out in various ways, most simply it can involve removing isolates which share a common morphology. Of course, the downside of this could be the removal of distinct species which are morphologically similar. Another method is to perform a mass spectral fingerprinting. For mass spectrometry assisted dereplication, a protein or natural product fingerprint can be obtained with techniques such as MALDI-TOF MS[126] or tandem mass spectrometry (MS/MS). For the latter, efforts have been undertaken to create a community curated database of natural product mass spectrometry data to share discoveries more efficiently within the scientific community[128].

Ultimately, a preventative attempt at dereplicating before library construction is underway is also possible. To do this, the library should be constructed in a manner which is not random by design. By systematically sampling soils, a library is more likely to be constructed from soils which are

different from one another. This soil diversity leads to a diversity in the microbial constituents of the sample but also the secondary metabolites which are being produced. To do this, the soils should be taken from sites with great geographic variety such as those seen in the State of Texas.

The State of Texas offers a unique opportunity for piloting an idea of systematic sampling of soil and microorganisms from its vast and diverse geographic landscape for building a microbial natural product library. Texas is host to 1,300 different soil types located within 21 major land resource areas[129]. Since soil microenvironments help dictate the microorganisms living (and therefore compounds produced) therein[130], systematically sampling from around the state should allow for the construction of a library containing compounds of widely varying structure.

For the purposes of this library, isolates from three major microbial categories (fungal, Gram-positive, and Gram-negative) were collected and fermented in type-specific media. This categorization was done morphologically prior to determining the microbial species; however, the isolates of interest have their phylogeny determined simultaneous to late-stage compound identification.

MATERIALS AND METHODS

2. MATERIALS

2.1 Chemicals

Bracketed annotation denotes which microbial categories (F: Fungal, G-: Gram-negative, or G+: Gram-positive) use each material component.

Solvents: dimethyl sulfoxide (DMSO) [F | G- | G+], ethanol (EtOH) [G-], ethyl acetate (EtOAc) [F | G- | G+], methanol (MeOH) [F | G- | G+].

Media: agar [F | G- | G+], ammonium sulfate ($(NH_4)_2SO_4$) [G+], beef extract [G-], calcium carbonate ($CaCO_3$) [F | G- | G+], calcium chloride ($CaCl_2$) [G+], cycloheximide [G- | G+], glucose [F | G- | G+], iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) [G+], kanamycin [F], magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) [G+], malt extract broth [F], manganese(II) chloride dihydrate ($MnCl_2 \cdot 2H_2O$) [G+], monopotassium phosphate (KH_2PO_4) [F | G- | G+], penicillin [G-], peptone [G-], polymycin B [G+], sodium butyrate [F | G- | G+], sodium chloride (NaCl) [G- | G+], sodium hydroxide (NaOH) [F], sodium phosphate dibasic (Na_2HPO_4) [F | G- | G+], soluble starch [G+], yeast extract [F | G- | G+], zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) [G+].

Adsorbent Resins: HP-20 [G-], silica gel [F | G- | G+], XAD-7 [G-].

2.2 Instruments

Flash chromatography was performed on an AI-580S flash chromatography system (Yamazen Science, Burlingame, CA, USA) with compound elution monitored by ultraviolet detection (UVD) at 230 nm as well as on an evaporative light scattering detector (ELSD). N_2 pressure = 50 PSI, ELSD T = 50°C. Elution occurred on a reversed phase ODS-C18 loaded column (size 2L) with a MeOH:H_2O mobile phase. Large volume (>100 mL) rapid solvent evaporation occurred in a Heidolph HEi-VAP Advantage rotatory evaporator with attached Brinkmann B-169 vacuum aspirator. Medium volume (<100 mL) rapid solvent evaporation occurred in a Labconco Centrivap centrifugal vacuum concentrator with attached Labconco Centrivap cold trap and solvent filter system. Small volume (< 20 mL) H_2O removal was carried out through cryodesiccation in a Labconco lyophilizer at condenser temperature -10°C and 0.010 mBar pressure.

3. MEDIA COMPONENT RATIONALE

In developing a pilot natural product library, certain care must be taken to select methods and materials which ensure cost-effectiveness, titer optimization, and reusability. One often discussed point is the utilization of General Mills Cheerios™ breakfast cereal as the solid fungal medium. Adapted from a previous natural product library protocol[131], using this cereal base for fermentation is not only cost effective but allows the fungi "[to grow] in all the crevices, leaving behind 'fuzzy doughnut shells where the Cheerio used to be" and further provides a standardization of media components which could otherwise affect metabolite production from batch to batch[132]. Using this breakfast cereal as a fungal medium is becoming commonplace in other libraries[133,134] including a large library containing nearly ten thousand fungal isolates currently focusing on antimalarial, anti-leukemic, and antibiotic compounds[135].

Breakfast cereal is not the only example of using a non-traditional medium for fungal growth. Rice has also been used for fungal fermentation and has proven to effectively activate otherwise silent BGCs causing the production of previously undescribed natural products[136,137].

As previously noted, a large issue in natural product drug discovery is the so-called 'cryptic' or transcriptionally silent BGC. Activation of silent gene clusters can be elicited through co-culturing, ribosome engineering, media manipulation, or chromatin remodeling[138].

As most fungal gene clusters are located on highly heterochromatic regions, BGC activity can be increased by co-culturing with HDAC inhibitors[97,139]. To maximize metabolite production, this project has utilized a well-documented HDAC inhibitor, sodium butyrate[140], to promote chromatin remodeling as it is a simple procedural change which can, in theory, greatly amplify our ability to produce and collect otherwise absent small molecules.

Further, many BGCs are regulated by negative feedback pathways to allow the source organism to control the amount of metabolite being produced. If a microorganism has a negative feedback controlled BGC, the concentration of its corresponding metabolite may be reduced during the fermentation stage. To circumvent this regulatory mechanism, the product secondary metabolite must be removed from culture to cause the organism to continue manufacturing the compound. This removal is a hard task to accomplish on solid media but is achievable in liquid fermentation broth such as that used in the Gram-negative growth protocol.

To do this, for Gram-negative fermentation, a HP-20/XAD-16 50% EtOH resin slurry mixture is added to each 2-liter flask partway through fermentation while the flasks are being constantly agitated on a shaker table. This allows for in-situ product recovery (ISPR), a common solid-phase adsorption tactic used to sequester small molecules from their producers so they are stabilized, effectively removed from culture, and negative feedback of their metabolic pathways is prevented[141]. This slurry mixture contains EtOH which may be toxic to the microorganisms. After addition to the fermentation flasks, the final concentration of EtOH is 0.68%. Because of this low concentration and the high concentration of bacteria in the fermentation media, we do not expect this to have a significant effect on the overall production of secondary metabolites[142].

4. MEDIA PREPARATION AND FERMENTATION

4.1 Fungal Isolates

For initial fermentation of fungal isolates, two autoclaved and UV irradiated covered 1-liter flasks with 8 g of Cheerios™ breakfast cereal were again autoclaved for 15 minutes followed by further exposure to ultraviolet radiation for 30 minutes. 48 mL of 30% w/v vacuum-filtered sucrose solution was then added to each flask and the flasks were stored statically at room temperature for

72 hours to ensure no contamination was present which could compromise the purity of the fermentation.

After 72 hours with no sign of fungal or bacterial contaminant growth, 1 ml of sodium butyrate solution was added to each flask. Following the addition of sodium butyrate, a roughly 1 x 1 cm plug of agar from a fully confluent and pure fungal culture was cut and transferred from its petri dish using a sterile wooden toothpick. Each flask was then gently swirled and covered to ensure the fermentation was started and flasks were incubated statically on a flat surface for 10-15 days at room temperature until early signs of fungal colony collapse were observed.

4.2 Gram-negative Isolates

On day one, a seed culture was started by transferring a roughly 1 x 1 cm plug of agar from a fully confluent and pure Gram-negative culture to a 250 mL flask containing 50 mL of sterile 0.2 x liquid broth (LB) medium and the 250 mL flask was then held at room temperature and continuously agitated at 45 rpm for 72 hours. Simultaneously, one 2-liter flask containing 600 mL of sterile M8 medium and one 2-liter flask containing 600 mL of sterile 2S4G medium (**Table 1**) were prepared for each Gram-negative culture and stored for 72 hours to ensure no contamination was present.

After 72 hours, 20 mL of seed culture LB was added to both media containing flasks for each Gram-negative isolate. Flasks were then covered and agitated at 100 rpm for 48 hours at room temperature on a shaker table. After 48 hours, 16.6 mL of HP-20/XAD-16 (6 g / 6 g) 50% EtOH resin slurry mixture was aseptically transferred to each 2-liter flask to begin ISPR. Flasks were once again covered and continuously agitated on a shaker table at 100 rpm for 96 additional hours at room temperature.

4.3 Gram-positive Isolates

For each Gram-positive isolate, 8 x 150 mm glass petri dish plates containing 62 mL of ISP2/ISP4 (50%/50%) agar were prepared (**Table 1**). After the plates cooled, a sterile plastic spreader was used to transfer and thoroughly apply bacterial isolate from the seed culture plate. Plates were then statically fermented for 14 days while monitoring daily for any observed fungal or bacterial contamination. Any plate which contained contamination during the 14-day fermentation period had the contaminate growth excised or the plate was discarded to ensure fermentation purity.

Table 1. Per Liter Fermentation Media Components for Bacterial Isolates

M8 Medium	2S4G Medium	ISP2	ISP4
5.0 g glucose	40.0 mL glycerol	4.0 g yeast extract	10.0 g soluble starch
5.0 g peptone	20.0 g HySoy peptone	10.0 g malt extract	1.0 g K_2HPO_4
3.0 g NaCl	2.0 g $(NH_4)_2SO_4$	4.0 g dextrose	1.0 g $MgSO_4 \cdot 7H_2O$
1.2 g Na_2HPO_4	0.1 g $MgSO_4 \cdot 7H_2O$	20.0 g agar	1.0 g NaCl
0.5 g KH_2PO_4	2.0 g $CaCO_3$		2.0 g $(NH_4)_2SO_4$
			2.0 g $CaCO_3$
			1.0 mg $FeSO_4 \cdot 7H_2O$
			1.0 mg $MnCl_2 \cdot 2H_2O$
			1.0 mg $ZnSO_4 \cdot 7H_2O$
			20.0 g Agar
pH 7	Natural pH	pH 7.2	pH 7.2

5. METABOLITE EXTRACTION

5.1 Fungal Isolates

After first signs of colony collapse were observed, the dried fungal mass was removed from each 2-liter flask by addition of EtOAc and vigorous agitation. The dislodged mass was transferred to a flat bottom glass tray and extracted twice with 200 mL EtOAc for 4 hours with rigorous agitation. The EtOAc extract was then filtered into a 500 mL bottle and the remaining fungal mass was air-dried completely in a ventilated chemical hood to remove any residual EtOAc. The dried fungal mass was then extracted twice with MeOH under the same conditions. The MeOH extracts were

filtered into a separate 500 mL bottle. Both the EtOAc and the MeOH chemical extracts were then concentrated separately with rotary evaporation and resuspended in up to 20 mL of respective solvent and transferred to separate pre-weighed 20 mL glass vials.

5.2 Gram-negative Isolates

Media from both the M8 and 2S4G flasks were poured through a fine metal sifter to separate the resins from the fermentation broth. Resins were air-dried on the sifter and then poured or scraped into a 250 mL flask for extraction. Resins containing the secondary metabolites were extracted twice with 200 mL EtOAc for 4 hours with vigorous agitation and filtered into a new 500 mL bottle. The resins were then air-dried and extracted twice more with 200 mL MeOH under the same conditions. Both the EtOAc and MeOH chemical extracts were concentrated separately with rotary evaporation and resuspended in up to 20 mL of respective crude extract solvent and transferred to separate pre-weighed 20 mL glass vials.

5.3 Gram-positive Isolates

After the large glass petri dishes were fully confluent (approximately 14 days), the agar was gently lifted from the plate surface and sliced into a grid of small pieces. The Petri dish lids were left slightly open to allow the water within the agar to evaporate off yielding dried agar. Once dried, the agar pieces for each isolate were consolidated into a single 500 mL flask and extracted twice with 200 mL EtOAc for 4 hours with vigorous agitation prior to being filtered into a 500 mL bottle. The remaining agar pieces were then air-dried once again and extracted twice with 200 mL MeOH under the same conditions. Both the EtOAc and MeOH chemical extracts were then concentrated separately with rotary evaporation and resuspended in up to 20 mL of respective solvent and transferred to separate pre-weighed 20 mL glass vials.

6. PREFRACTIONATION

6.1 Rationale

While it is possible and theoretically appropriate to screen the library at this crude extract stage, often there are issues which arise from screening crude extract mixtures with varying polarities, solubilities, and stabilities. One common issue is the effect of counteracting small molecules[127]. Whereby one molecule may induce cell death while another promotes proliferation or survival thus masking a potential hit or understating its potency.

Secondly, biologically active metabolites may simply have their activity 'drowned out' by more abundant small molecules – this effect could cause secondary metabolites produced at low concentrations to not be present in the reaction wells at a sufficient concentration to outcompete the rapid division of cancerous cells or to elicit their cytotoxicity. Further, the general complexity of the mixture may exert undue stress upon the cultured cells; potentially leading to false positive results. By performing an initial round of flash chromatography before adding the compound mixtures to the natural product library, the overall mixture complexity can be reduced thereby minimizing these unwanted effects[143].

Of course, there are downsides to screening a library based only on preliminary fractionation, namely a compound may not exert its full effect if not in the presence of another co-produced small molecule – referred to as a 'synergistic' effect. By separating the crude extract and therefore the two synergistic compounds, this may result in biologically active compounds going undetected. Conversely, semi-purified library screening may not go far enough; the complexity of a semi-purified extract does not have known concentrations of each compound, therefore concentrations of trace components may not be sufficient for detection[21].

As with most aspects of scaling a library, taking all these points into consideration is a delicate balancing act requiring time management, resource savings, and working with often small amounts of crude extract material. Given these concerns, it is important to take this extra flash chromatography step to reduce the complexity of the crude extract. However, it is also equally important to assay the crude extract to determine if there are any synergistic bioactivities as these activities are rare and could be important discoveries. As a compromise to these considerations, for this project the crude extract and prefractions for each isolate were both collected and tested.

6.2 Crude Extract Preparation

For each isolate, its EtOAc and MeOH crude extracts were air dried until a dry solid or oily mixture remained. The crude extract mass was then recorded, and the crude extract was resuspended to a final concentration of 50 mg/mL in its respective solvent. 1.0 mL of each crude extract was sequestered, and its solvent was replaced with DMSO for addition to the final 96-well natural product library plate. 5.0 mL of EtOAc crude extract suspension and 5.0 mL of MeOH crude extract suspension were then combined into a 20 mL vial containing 3 g of silica gel. The crude extract/silica gel suspension was air-dried in a chemical hood until it was completely dry yielding a fine silica gel powder.

6.3 Flash Chromatography

An empty injection column was mounted above the separation column and the separation column was equilibrated with 20% MeOH for 15 minutes at 10 mL/min. Following equilibration, the injection column was dry loaded by pouring the dried crude extract/silica gel powder mixture. The column system was then subjected to 20% MeOH for 1 min, followed by a linear gradient from 20% to 100% MeOH over 30 minutes, and finally by 100% MeOH for 30 min with a flow rate of 10 mL/min (**Figure 1**).

The collected eluates were then separated into 10 prefractions with approximately equal volume of eluate per prefraction. The 10 prefractions were concentrated using a centrifugal evaporator SpeedVac system and transferred into a pre-weighed 20 mL glass vial. Finally, the residual H_2O from each concentrated prefractions was removed through cryodesiccation until a dried powder, solid, or oily substance remained.

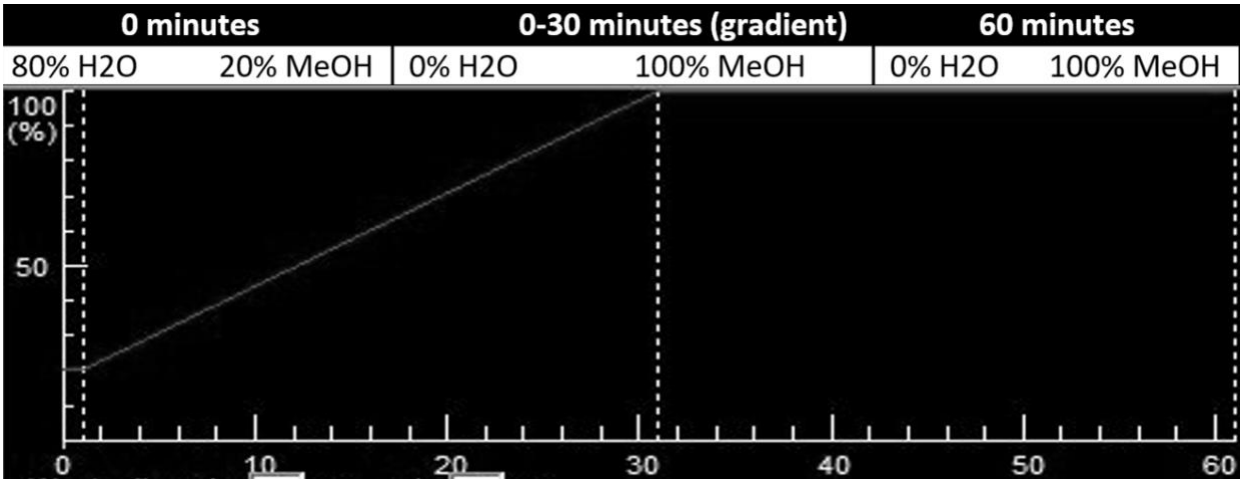


Figure 1. Flash Chromatography Method for Prefractionation

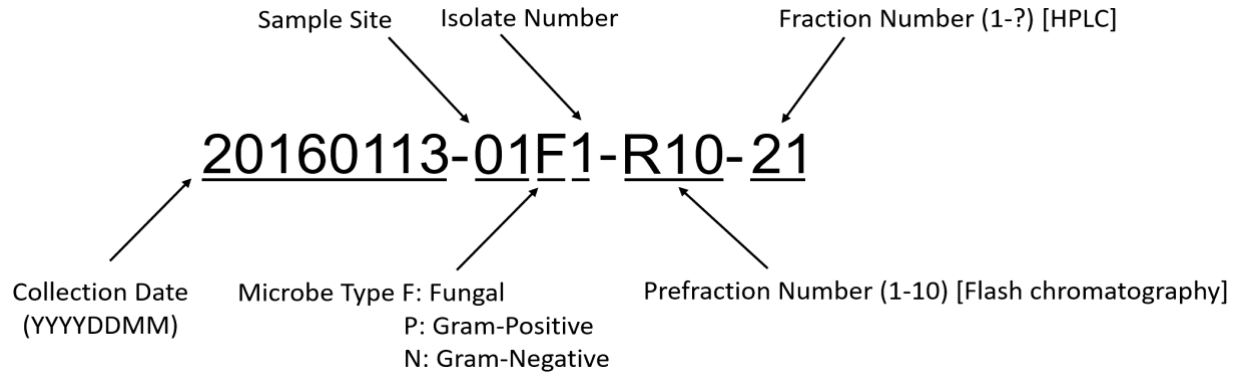
Solvent gradient for crude extract purification through flash chromatography. A 30-minute linear gradient from 20% MeOH to 100% MeOH followed by 30 minutes of isocratic 100% MeOH elution yields 10 prefractions which are then added to the natural product library.

7. LIBRARY CONSTRUCTION

After cryodesiccation, each vial was once again weighed so that the accurate total prefraction mass could be determined. Finally, the prefractions were prepared for final addition to the microbial natural product library. To ensure accurate and standardized concentrations as well as easy interpretation of results when the library is screened, each dried prefraction was resuspended to a concentration not exceeding 20 mg/mL in DMSO. If less than 20 mg was present in a prefraction, it was resuspended in 1 mL of DMSO, and its adjusted concentration was documented. 1 mL of

each resuspended prefraction was transferred to the library plate and the remaining prefraction was transferred and stored in extra volume storage.

In day-to-day lab work and throughout this paper a naming system has been adopted to track isolates, prefractions, fractions, and compounds which have yet to be identified.



8. SCREENING OF PILOT NATURAL PRODUCT LIBRARY

8.1 Abstract

Following isolation, fermentation, extraction, and prefractionation, a pilot-size natural product library containing 800 prefractions from 80 Texas soil-obtained fungal isolates (**Figure 2)** was screened for cancer cell cytotoxicity as measured by a luciferase luminescence assay against human cancer cell lines (MIA PaCa-2, SH-SY5Y, COLO 829); and a normal human dermal fibroblast (HDF) line. This study represents a validation of the collection and purification methods and also produced a short-list of interesting prefractions for follow-up through activity-guided purification to identify the responsible bioactive compounds.

MATERIALS AND METHODS

8.2 Materials

Chemicals: HyClone Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1640, fetal bovine serum (FBS), penicillin-streptomycin (Pen-Strep), trypsin, phosphate buffered saline (PBS), DMSO.

Instruments: Synergy H1 plate reader with Gen5 software (BioTek, Winooski, VT, USA), Light Microscope.

Kits: CellTiter-Glo® luciferase luminescence assay (Promega, Madison, WI, USA).

Cell Lines: MIA PaCa-2 (ATCC® CRL-1420™) human epithelial pancreatic carcinoma , COLO 829 (ATCC® CRL-1974™) human fibroblast melanoma, SH-SY5Y (ATCC® CRL-2266™) human neuroblastoma.

8.3. Experimental Design

At T+0 hours, cells were plated into 96-well half-area flat bottom plates with 25 µL of cell suspension at a concentration of 2,000 cells/25 µL. MIA PaCa-2 cells were cultured in DMEM + 5% FBS + 1% Pen-Strep. COLO 829 and SH-SY5Y cells were cultured in RPMI 1640 + 10% FBS + 1% Pen-Strep. At T+24 hours, the stock natural product library plates were diluted tenfold in DMSO yielding a final concentration of 1-2 mg/mL depending on initial stock library concentration and 2 µL of the DMSO diluted library was transferred to 100 µL of appropriate media for each cell type. Each mixing plate's well A1 was left as a negative control blank (sacrificing the EtOAc crude extract in A1). 25 µL of the diluted library was transferred from the

mixing plates to each well of the assay plates (**Figure 3**) in triplicate. Throughout this experiment plates were stored statically in an incubator at 5% CO_2 and 37 °C.

At T+72 hours, cell viability was determined by measuring luciferase luminescence in each reaction well. 50 µL of CellTiter-Glo® media was transferred to each well of the assay plates. Plates were gently agitated at 20 rpm for 5 minutes at room temperature and then rested for 5 minutes at room temperature before measuring luminescence on a cell plate reader.

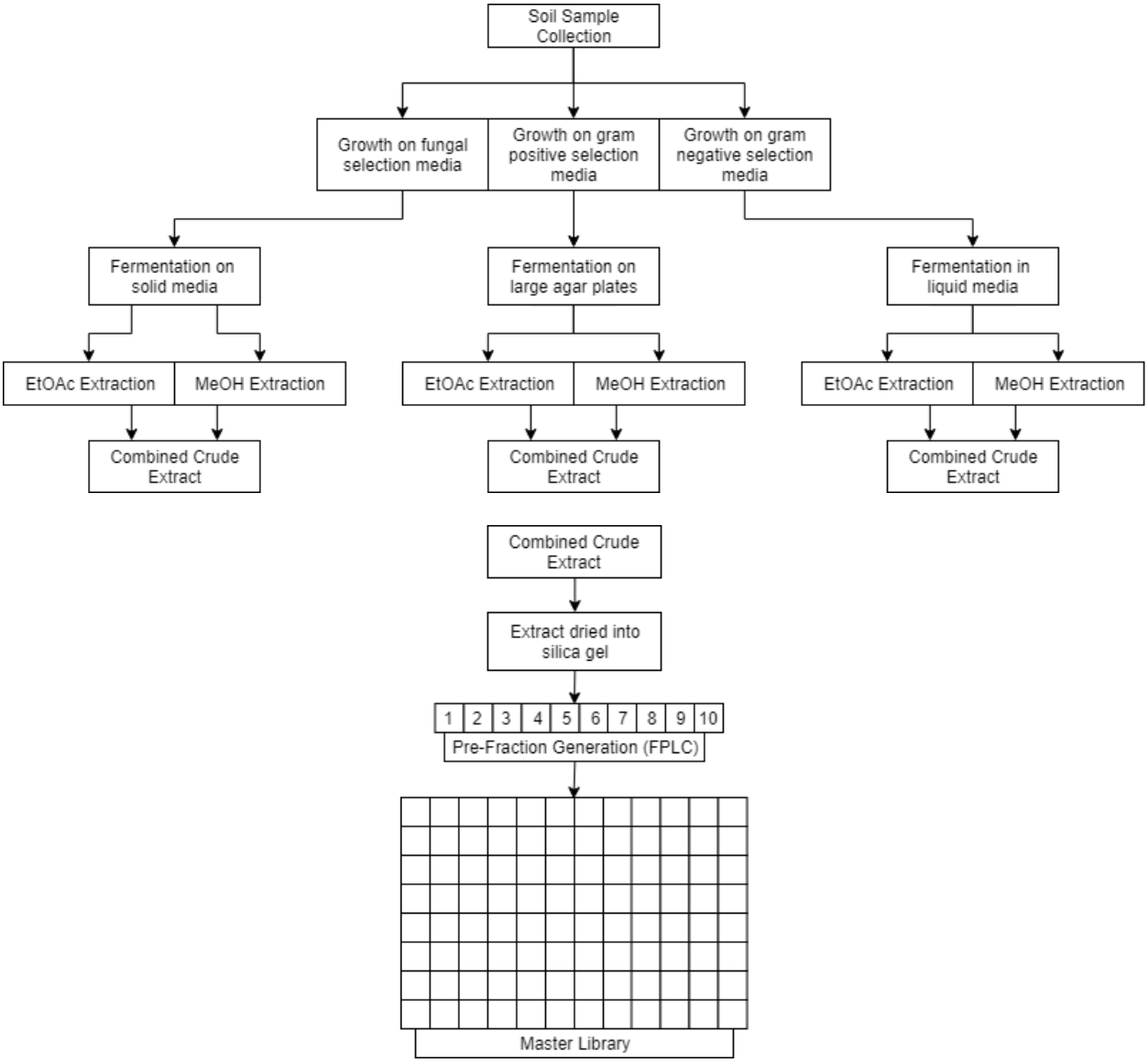


Figure 2. Fermentation and Prefractionation Schema

Schema for microbial growth and prefractionation. All collected isolates are grown on respective type-specific media (Fungal, Gram-positive, Gram-negative) followed by EtOAc and MeOH extraction and then flash chromatography to generate 10 roughly equal prefractions which are added to the natural product library at concentrations not exceeding 20 mg/mL and eventually screened for cytotoxicity. After 10 master library plates were completed (80 fungal isolates; 800 prefractions), the pilot-size natural product library was screened for cytotoxicity against MIA PaCa-2, SH-SY5Y, and COLO 829 cancer cell lines.

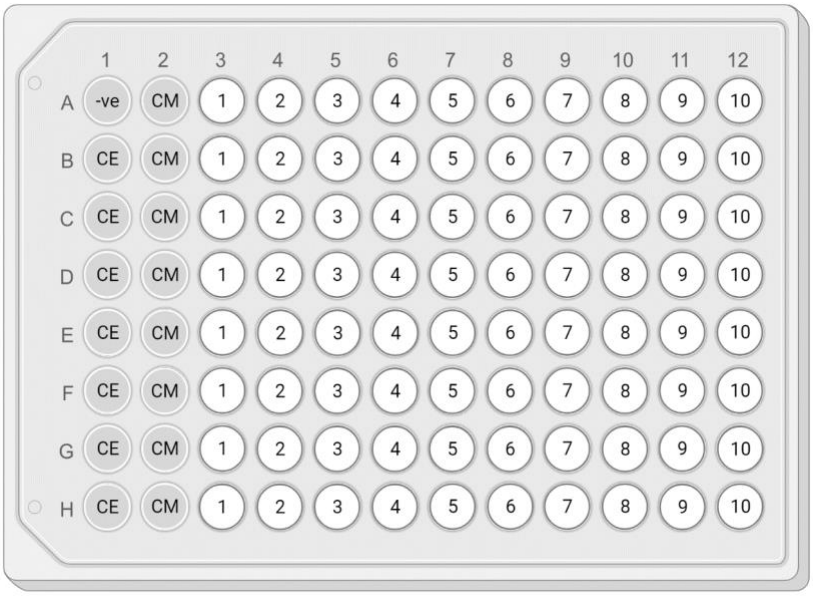


Figure 3. Layout of 96 Well Half-Area Plates Used for Cell Viability Assay.

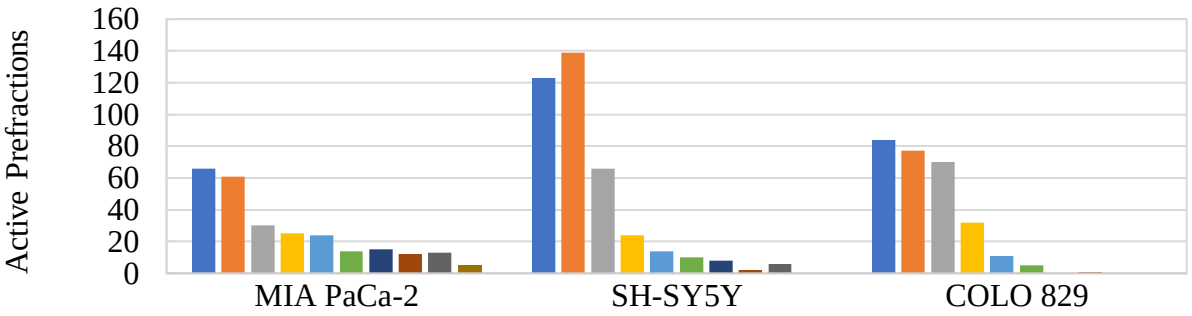
-ve: Negative control (cells + media), CE: EtOAc crude extract, CM: MeOH Crude extract. Well A1 on each plate is sacrificed for inclusion of a negative control. Each row corresponds to one bacterial or fungal isolate. Columns 1 and 2 are reserved for the EtOAc and MeOH crude extracts for each isolate. Columns 3-12 correspond to prefractions 1-10 for each isolate. Plates are run in triplicate for cell viability analysis.

9. RESULTS

Of the 800 prefractions screened against MIA PaCa-2, 265 (33.1%) had lower levels of ATP compared to control with 59 (7.4%) exhibiting a 50% or greater loss in cell viability. For SH-SY5Y, 392 (49.0%) had lower cell viability and 26 (3.3%) exhibited a 50% or greater loss in cell viability. The COLO 829 screening group had 280 (35.0%) prefractions causing lower levels of cell viability but only 6 (0.75%) prefractions caused a 50% or greater loss in cell viability **(Figure 4)**. Given the large difference in number of prefractions causing greater than 50% reduction in cell viability, it is apparent that this pilot-size natural product library already contains compounds which are capable of eliciting cancer cell line specific cytotoxicity.

To further investigate these active compounds, additional purification must be done on the active library prefractions. From this pilot library screening group, those prefractions exhibiting a 30% or greater loss in cell viability were advanced to a confirmative screening round to verify the observed bioactivity and to screen against the HDF line. Following confirmative screening, a short-list of interesting (characterized by high activity, high specificity, or broad activity/low normal cell line activity) was compiled and advanced to the purification and identification stage.

Attention was given to adjacent prefractions as it is important to remember that the prefractionation process does not necessarily yield high peak resolution and therefore there is likely overlap in the metabolite contents of adjacent prefractions. For those prefractions which contained compounds with interesting activities, any which did not have at least 20 mg of prefraction were refermented and prefractionated before proceeding in activity-guided purification as a 1-10 mg of purified compound is typically needed to efficiently identify an unknown natural product.



	MIA PaCa-2	SH-SY5Y	COLO 829
0-9%	66	123	84
10-19%	61	139	77
20-29%	30	66	70
30-39%	25	24	32
40-49%	24	14	11
50-59%	14	10	5
60-69%	15	8	0
70-79%	12	2	1
80-89%	13	6	0
90-100%	5	0	0

Figure 4. Active Prefractions from Pilot Study of 800 Fungal Prefractions

Among 800 fungal prefractions studied, MIA PaCa-2 had the most prefractions which caused a greater than 50% reduction in cell viability. COLO 829 had the least with only 6. This disparity in number of active prefractions suggests the presence of active compounds within this pilot-sized library which demonstrate cell-line specific cytotoxicity.

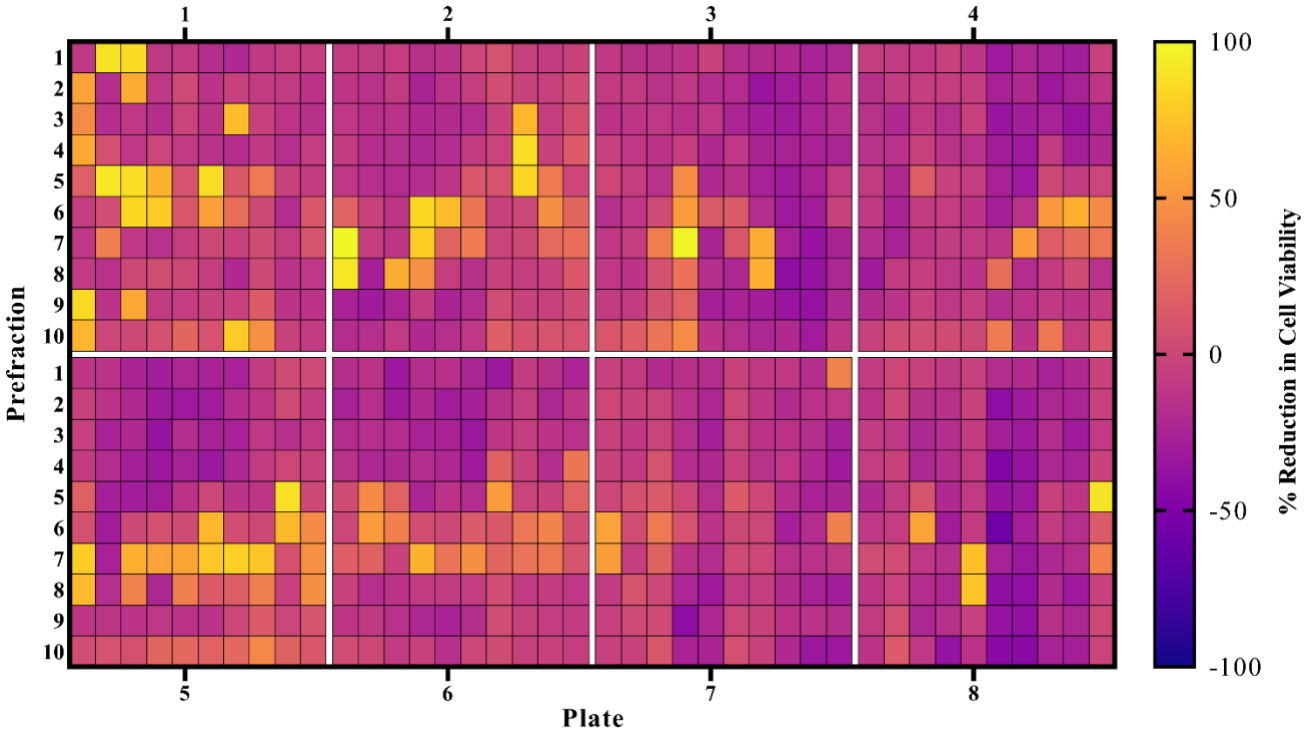


Figure 5. Heat Map of Fungal Prefraction Activity Against MIA PaCa-2

From the pilot library screening of secondary metabolites against three cancer cell lines, MIA PaCa-2 had the fewest active prefractions. Despite this, there were 18 prefractions which exerted an 80%+ reduction in cell viability. Special consideration should be given to the prefractions which have activity in adjacent wells as this could be indicative of poor compound separation or a class of similarly active compounds.

10. CONCLUSIONS

Screening of the new, pilot-size semi-fractionated natural product library represented an important progression in the overall project. This stage of library construction allowed verification of the previously employed methods (fermentation, extraction, and flash chromatography) by demonstrating the ability to take microbial isolates from soils and assay their metabolites for bioactivity. The results obtained demonstrate that the library already contains a promising set of secondary metabolites which in certain cases exerted cytotoxic effects with great potency or cancer cell line specificity.

While including a normal human dermal fibroblast cell line in the confirmative round helped to narrow the short-list of prefractions which would undergo additional purification, further study into the effects of these compounds on tissue specific non-cancerous cell lines would be more indicative of potential off-target effects as the nuance for compound specificity ideally should be more selective than simply cancerous versus non-cancerous. For this reason, the HDF results are not presented but were nonetheless important in determining which compounds to purify and attempt to identify.

Chapter 3

PURIFICATION AND IDENTIFICATION OF CYTOTOXIC NATURAL PRODUCTS

ABSTRACT. After determining active prefractions through cell viability assaying of the pilot natural product library, a short-list of active prefractions was prioritized based upon criteria such as selectivity, HDF cytotoxicity, mass of available prefraction, and potency.

From this selected group of prefractions, activity-guided purification was carried out through continued cell viability assaying coupled with HPLC yielding purified compounds aspergillin PZ, trichoderone B, palmitic acid, and malformin all of which display cytotoxic activity against MIA PaCa-2, SH-SY5Y, or COLO 829.

Subsequent to compound identification, species identification was performed through fungal genetic barcoding of the 18S-ITS-28S rRNA gene region, providing positive identification of *Aspergillus flavipes, Aspergillus niger,* and *Cladosporium* sp..

1. INTRODUCTION

Once a flash chromatography generated prefraction has been determined to have activity against the cell line of interest, additional 'activity-guided' purification must be undertaken to isolate a single compound from a mixture of dozens of compounds. The major tool in this purification step is high-performance liquid chromatography (HPLC).

A multistep method must be developed to ensure the adequate purification of the active compound prior to proceeding to compound identification. While it is possible to perform basic mass spectrometry on the mixture of compounds, it is not until at least one round of HPLC purification has been performed that it can be determined which compound is exhibiting activity due to the inherent complexity of the library's prefractions.

For this stage, the purification protocol is broken into two rounds, a semi-preparative round, and an analytical round. Most of the compound separation legwork is undertaken in the semi-preparative round. This allows large multi-milligram quantities of prefraction to be purified based upon elution characteristics. The major drawback of this stage and reason for an additional round of analytical HPLC, is the lower peak resolution offered by semi-preparative HPLC compared to analytical HPLC in exchange for supporting a higher loading volume.

MATERIALS AND METHODS

2. MATERIALS

Solvents: Acetonitrile (ACN), methanol (MeOH), filtered H_2O, formic acid.

Instruments: Agilent 1260 Infinity LC System (HPLC), Agilent 6230 TOF-LC/MS, Agilent 6460 QQQ-LC/MS, NMR, Small volume (<10 mL) rapid solvent evaporation occurred in a Labconco

Centrivap centrifugal vacuum concentrator with attached Labconco Centrivap Cold Trap and solvent filter system. Residual H_2O removal occurred through cryodesiccation in a Labconco lyophilizer at condenser temperature -10°C and 0.010 mBar pressure.

Column Information: HPLC was carried out on an Agilent Technologies 1260 Infinity LC system with two Agilent Technologies PrepStar solvent delivery modules. Analytical HPLC was performed on reversed-phase Zorbax StableBond-Phenyl column [4.6 x 250 mm; 5-micron] (880975-912) or reversed-phase Zorbax StableBond-Aq column [4.6 x 250 mm; 5-micron] (880975-914). Semi-preparative HPLC is performed on a reversed phase Zorbax StableBond-Phenyl column [9.4 x 250 mm; 5-micron] (880975-212).

3. ACTIVITY-GUIDED PURIFICATION

A simple yet powerful tool for purifying bioactive metabolites often employed by drug discovery processes is activity-guided purification; simply using the activity of a given metabolite to inform the purification process. For the purpose of this project, activity-guided purification consists of repetitive cell viability assaying throughout the HPLC process to determine and follow the location of the metabolite of interest in the HPLC generated fractions (**Figure 1**).

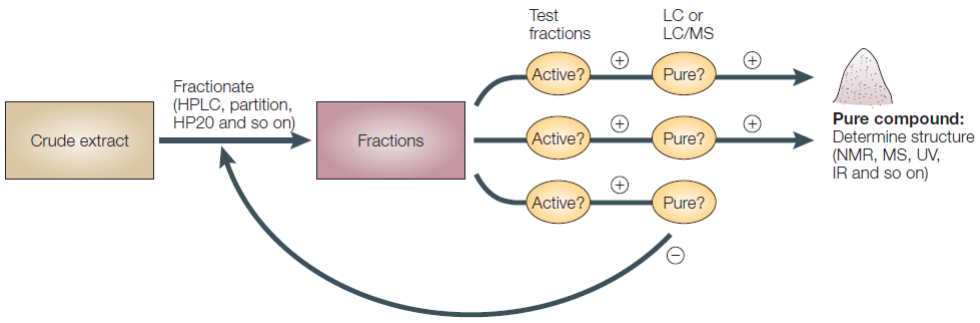


Figure 1. An Example of Activity-Guided Purification

Figure adapted from: Koehn, F. E. and Carter, G. T. The evolving role of natural products in drug discovery. *Nature reviews. Drug discovery* **4**, 206-220, doi:10.1038/nrd1657 (2005).[21]

4. MALFORMIN

20160113-02F1-R7, a fungal prefraction within the pilot microbial natural product library exhibiting cytotoxicity against MIA PaCa-2 and SH-SY5Y with limited viability reduction of COLO 829 (**Figure 2**) was the first prefraction selected for follow-up activity-guided purification from the pool of 800 fungal prefractions within the pilot library.

Initially, 914.3 milligrams of crude extract from 20160113-02F1, a then unidentified fungal species, collected from clay soil under tall grass in Tarrant County, TX was removed from crude extract cold storage and re-purified through flash chromatography using a 30 minute 20% to 100% linear MeOH:H_2O gradient followed by a 30 minute 100% MeOH isocratic elution identical to the flash chromatography methodology described in the prefractionation step of the library construction procedures.

Following flash chromatography, the dried and weighed prefraction was resuspended in 10 mL of 20% ACN and 200 µL per-run was manually injected into a reversed-phase HPLC system with a Zorbax StableBond-Phenyl column. Eluate from each run was monitored through UV detection at 210 nm and 230 nm. Each detected compound peak was collected separately and later pooled with identical chromatogram peaks from 30 repeated runs; for the purpose of this project, these pooled peaks are now classified as fractions.

Aliquots from each detected fraction (**Figure 3**) were plated in a 96-well plate and underwent cell viability testing against MIA PaCa-2 through the previously described cell viability methods with the addition of a positive control well which contained cells, media, DMSO, and the parent prefraction (20160113-02F1-R7).

Luminescence results were exported to Microsoft Excel and cell viability for each well was compared to the negative control group. Fraction 27 (20160113-02F1-R7-27; **Figure 4**) caused an average 99.05% reduction in cell viability when compared to negative control.

Purity of the concentrated semi-preparative fraction was assessed through time-of-flight mass spectrometry (TOF-MS). As the peak resolution and TOF-MS indicated that the sample was not sufficiently purified, a subsequent round of reversed-phase HPLC purification on a Zorbax StableBond-Aq analytical column was performed. For this, the dried fraction was resuspended in 1 mL 20% MeOH and manually injected 20 μL/run into the HPLC system for a total of 50 identical runs.

TOF-MS was utilized on the purified compound to begin preliminary compound characterization. Through TOF-MS, the compound's accurate molecular mass as well as the isotope distribution were gathered to make informed conclusions about the compound's molecular formula. 20160113-02F1-R7-27 was determined to have a molecular weight of approximately 529.24. This information was passed through Scripps Research's METLIN Metabolite and Chemical Entity Database[144], a tool used to efficiently screen the known chemical space for any deposited compounds with similar mass/charge and compound formulae.

A bicyclic pentapeptide, malformin[145-147], with a formula of $C_{23}H_{39}N_5O_5S_2$ (**Figure 5**) and with similar molecular weight to the observed compound was noted and efforts to determine similarity were undertaken. First, a literature search identified the source organism as *Aspergillus niger* which had close morphologic similarities to the then unidentified fungal isolate 20160113-02F1. Second, through MassHunter Qualitative Analysis software the possible chemical formulae were produced based upon similarities between the theoretical and observed molecular weight and

isotope abundance distributions. The observed isotope abundances of 20160113-02F1-R7-27 demonstrated remarkable similarity to $C_{23}H_{39}N_5O_5S_2$ (**Table 1**).

Table 1. Malformin Natural Isotope Distribution

[M+H] (Observed)	[M+H] (Calculated)	20160113-02F1-R7-27	C23 H39 N5 O5 S2
530.24 m/z	530.2465 m/z	69.6%	68.4%
531.24 m/z	531.2493 m/z	19.9%	19.8%
532.24 m/z	532.2458 m/z	8.7%	9.6%
533.24 m/z	533.2471 m/z	1.8%	2.2%

Due to malformin's signature disulfide bond between its cysteine residues which forms its notable bicyclic structure, a chemical reduction could be performed to attempt to break the exposed disulfide linkage[148] which would then be represented and identifiable by a 2 m/z shift on TOF-MS. Indeed, following addition of glutathione (GSH), the mass of GSH reacted 20160113-02F1-R7-27 was measured on TOF-MS and showed a 2 m/z shift indicative of an addition of 2 hydrogens and therefore a likely reduction of the disulfide bond (**Figure 5**).

An unsuccessful attempt was made to crystallize this unknown compound. To further determine whether 20160113-02F1-R7-27 was in fact malformin, ^{1}H-NMR and ^{13}C-NMR were performed on the unknown compound which showed agreement with the historical NMR data for malformin.

Malformin has previously shown to be an active metabolite produced by *A. niger* with *in vivo* anticancer activity against Colon 38 (IC_{50}: 0.27 ± 0.07 µM) and HCT 116 (IC_{50}: 0.18 ± 0.023 µM) in xenografted BDF1 mice[149]. This compound elicits its cytotoxicity by arresting cell cycling at the G2 checkpoint[149,150] and causes an up-regulation of H2A.X, p53, cleaved CASPASE 3, and LC3 causing necrosis, apoptosis, and autophagy[149]. Further, it has been shown to have an antibiotic effect against Gram-positive organisms *Arthrobacter tumescens, Bacillus cereus, B. subtilis, Mycobacterium phlei, M. smegmatis, Nocardia corallina, N. rubra, Streptomyces griseus,* and *S.*

venezuelae as well as Gram-negative organisms *Achromobacter liquefaciens, Aerobacter aerogenes, Agrobacterium radiobacter, E. coli, Pseudomonas andropogonis,* and *Xanthomonas stewarti*[151]. It is well documented in its synthesis[152,153].

While there is potential to modify malformin to cause its activity to be more selective, it has a small and concerning therapeutic index[149] which makes it of little value without synthetic modifications to decrease its off-target toxicity.

To finalize characterization of this compound now known as malformin, fungal barcoding was performed to determine whether this compound was isolated from the typical source of *A. niger* or from another fungal species.

For identification of isolate 20160113-02F1, DNA barcoding was carried out by sequencing the unknown isolate's 18S SSU, ITS1, 5.8S, ITS2, and 28S LSU rDNA regions. 9 primers, NS1, NS2, NS3, NS4, LROR, LR3, LR3R, LR6, and ITS1 (Eurofins) were used to cover all domains of the 18S-ITS-28S rRNA gene (**Figure 6**).

Isolate 20160113-02F1 was grown to 90%+ confluence on Fungal Selection Media[154] in five petri dishes. Mycelia were gently scraped from the agar surface using a sterilized wooden toothpick with great care to ensure no agar was transferred in the process. The collected mycelia from the five replicate plates were combined into a single 25 mL centrifuge tube and washed/resuspended with 10 mL TE Buffer to inactivate DNase enzymes. The tube containing collected mycelia was frozen and lyophilized to yield freeze-dried mycelia.

Genomic DNA from 20 mg of the freeze-dried mycelia were extracted with a Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek) and success of DNA isolation was confirmed on 0.7% agarose gel electrophoresis following two elution steps.

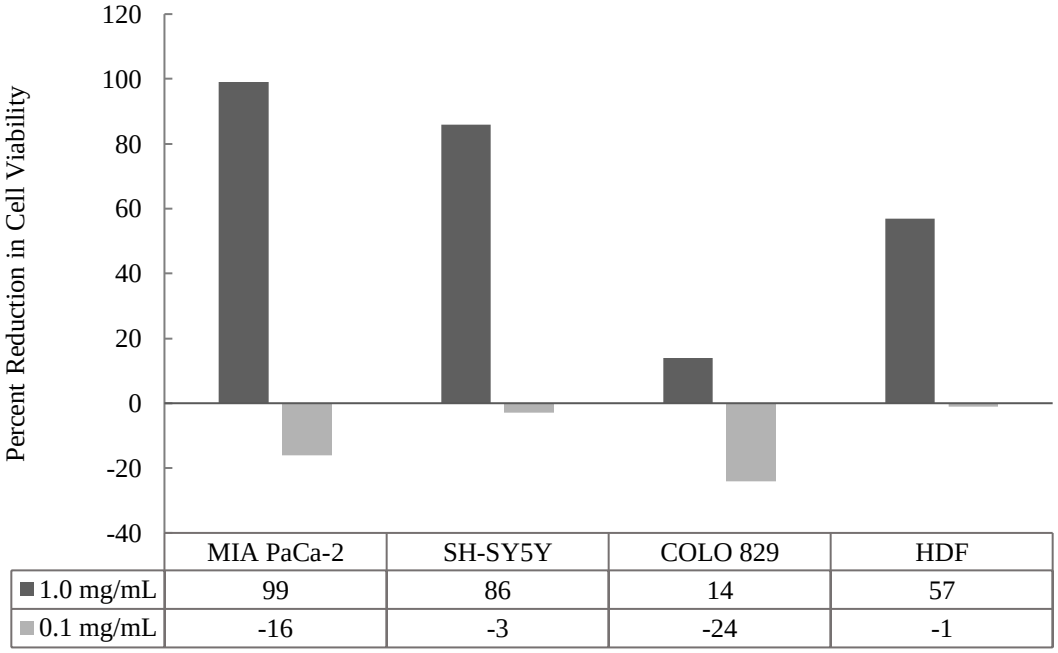
The 18S SSU, ITS, and 28S LSU domains were amplified using 3 primer pairs; NS1:NS4 (~1200 bp), ITS1:LR3 (~1100-1150 bp), and LROR:LR6 (~1200 bp). Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare) containing Taq polymerase, BSA, dATP, dCTP, dGTP, and dTTP were used for this amplification step.

For each reaction tube 1 µL x 25 pmol forward primer, 1 µL x 25 pmol reverse primer, 2 µL template DNA, and 21 µL ddH_2O were added. A Touchdown PCR protocol was used to optimize reaction specificity. Initial denaturation occurred at 95 °C for 5 minutes followed by 10 cycles of 94 °C for 30 seconds, a 58 °C → 50 °C 30 second touchdown step (decreasing 1 °C every cycle), and 72 °C for 1.5 minutes. Subsequently, 30 cycles of 94 °C for 30 seconds, 50 °C for seconds, and 72 °C for 1.5 minutes were carried out followed by a final extension step of 72 °C for 8 minutes.

Size and purity of DNA amplicons were confirmed on 1% agarose gel with a 100 bp ladder. DNA amplicons were then recovered from the gel using a QIAEX II® Gel Extraction Kit (Qiagen). DNA concentrations and A260/A280 ratios were measured on a Take3 microdroplet plate using Gen5 plate reader software (BioTek).

50 ng of PCR amplicon in pH 8.5 10 mM Tris, 10 pmol primer, and ddH2O were combined into each PCR strip tube for Sanger sequencing. From these results, two contigs (**Appendix A**) were generated and processed using GeneStudio Pro for 20160113-02F1's 18S SSU-ITS and 28S LSU region (**Figure 6**) and run through an NCI-BLASTn search. Both the 18S SSU-ITS and 28S LSU

for 20160113-02F1 showed high identity with deposited genetic sequences of *Aspergillus niger* (99.30% and 98.76%, respectively).



	MIA PaCa-2	SH-SY5Y	COLO 829	HDF
1.0 mg/mL	99	86	14	57
0.1 mg/mL	-16	-3	-24	-1

Figure 2. 20160113-02F1-R7 Preliminary Screening Results

20160113-02F1-R7 was screened against MIA PaCa-2, SH-SY5Y, COLO 829, and a normal cell line (Human Dermal Fibroblast) at two concentrations, 1.0 mg/mL and 0.1 mg/mL in triplicate. A reduction in cell viability was seen in all four cell lines at the 1.0 mg/mL concentration and no reduction in cell viability was seen at the 10x diluted 0.1 mg/mL concentration.

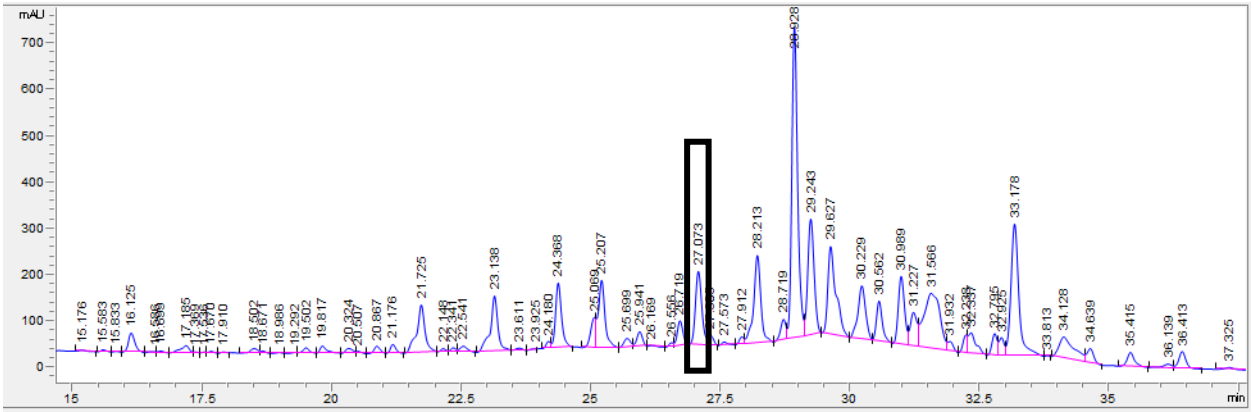


Figure 3. 20160113-02F1-R7 Semi-Preparative HPLC

A 210 nm UV chromatogram from HPLC of 20160113-02F1-R7 on a semi-preparative column with an ACN:H_2O mobile phase. Each peak was collected separately and screened against MIA PaCa-2 for effects on cell viability. The boxed peak at 27.073 minutes was most active with 99.05% reduction in cell viability. The compound at peak 27 was later identified as malformin.

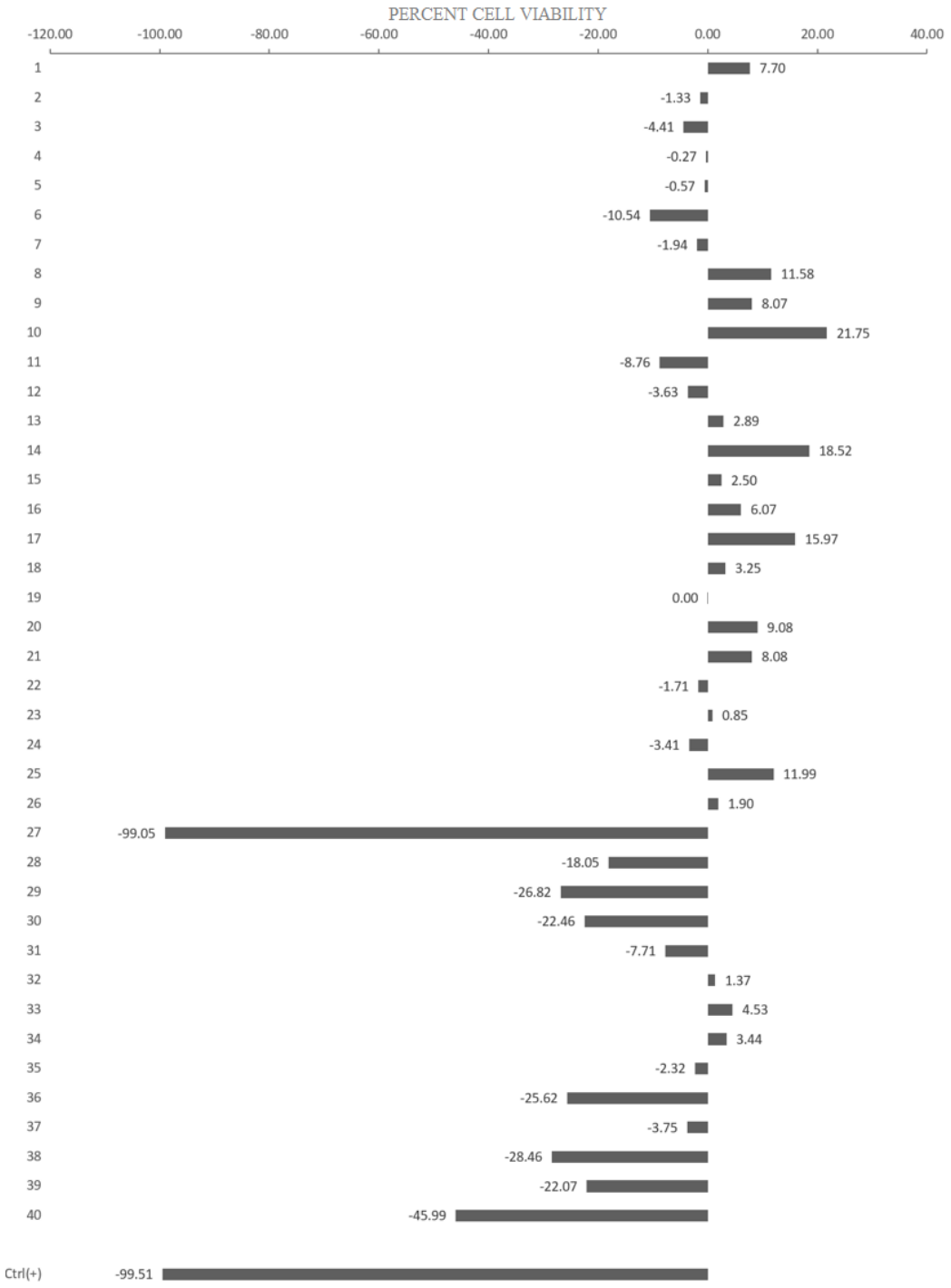


Figure 4. Percent Cell Viability of 20160113-0F1-R7 Fractions

To track the active fraction during semi-preparative HPLC purification, each peak from the HPLC chromatogram is collected separately and tested in triplicate against MIA PaCa-2. Values to the left of 0.00 indicate a reduction in cell viability and values to the right of 0.00 indicate an increase in cell viability (more cells) compared to control. Positive control (Ctrl(+)) was the active prefraction at 1.0 mg/mL. Cell viability from fractions was tested in triplicate.

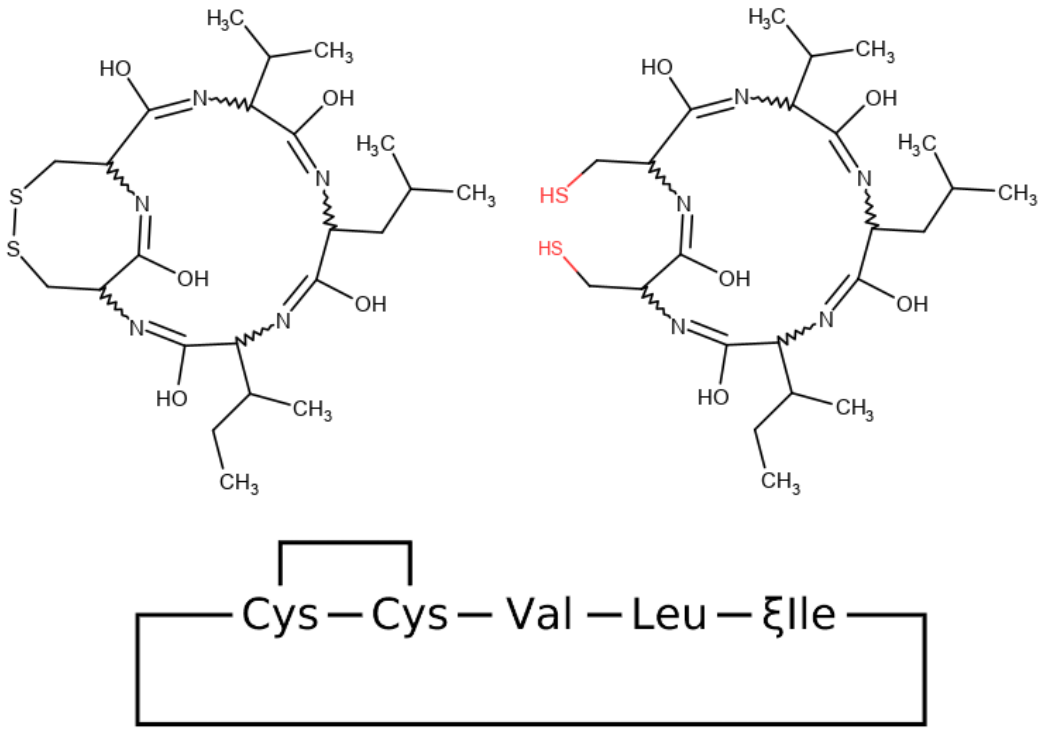


Figure 5. Structure of Malformin and Reduced Malformin

Malformin (**1**) and malformin with a reduction on the disulfide bridge (**2**) linking its constituent cysteine residues. Amino acid connectivity of this bicyclic pentapeptide is shown at bottom of figure.

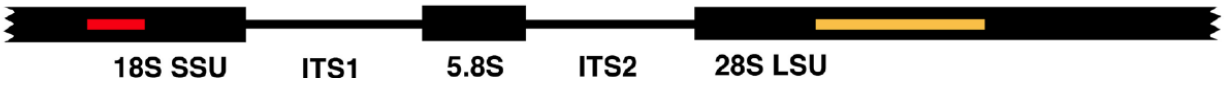


Figure 6. Fungal 18S-ITS-28S rDNA Region

The 18S/28S rRNA gene is an important tool for fungal species "barcoding". The two subunits, 18S SSU (small) and 28S LSU (large) as well as the Internal Transcribed Spacers (ITS) can be amplified and sequenced through the use of primers ITS1, LR3, LROR, LR6, and LR3R. Once sequenced, contigs for this region can be assembled and run through a BLASTn search to determine the identity of an unknown fungal isolate.

Figure excerpted from: Raja, H. A., Miller, A. N., Pearce, C. J. and Oberlies, N. H. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *Journal of natural products* **80**, 756-770, doi:10.1021/acs.jnatprod.6b01085 (2017).

5. PALMITIC ACID

20160113-01F1-R8, a prefraction from a then unidentified fungal source exhibited an 85% reduction in viability of MIA PaCa-2 at 8.3 mg/mL concentration on initial screening and no activity against SH-SY5Y (-11%) or COLO 829 (3%) compared to control. Furthermore, the 20160113-01F1-R8 prefraction did not appear to elicit cytotoxicity to the HDF cell line (**Figure 7**).

Because the initial round of fermentation only produced 8.3 mg of prefraction 8, a second batch fermentation of 20160113-01F1 was undertaken to yield enough crude extract for the purification process. Following fermentation of 20160113-01F1 in twenty 1-liter flasks, 1,792.9 mg of crude extract was recovered and purified through the previously described flash chromatography methods yielding 40.8 mg of prefraction 8 which was confirmed to contain the active compound through cell viability screening.

Activity-guided purification was then carried out through semi-preparative and analytical HPLC. Due to the poor water solubility of this compound, an adjusted protocol was developed and used for reversed-phase purification. The dried prefraction was resuspended in 10 mL 80% ACN and 200 μL per run was manually injected into the HPLC system and passed through a Zorbax StableBond-Phenyl semi-preparative column under a 10 minute 80% ACN isocratic elution method. Similar to the malformin procedure, the eluate was monitored on UV detection at both 210 and 230 nm and eluate peaks were collected and pooled separately.

Though activity was confirmed through cell viability assaying, compound purity was again of concern due to a broad right-hand shoulder on the 210 nm chromatogram (**Figure 8**). This broad shoulder was likely from a compound with similar structure, similar polarity or from increased column retention of this compound. To improve compound purity, an additional round of HPLC

was carried out on a Zorbax StableBond-Aq analytical column with a change in mobile phase from ACN:H_2O to MeOH:H_2O. 20160113-01F1-R8 was passed through the analytical column under a gradual 11 minute 85% MeOH to 100% MeOH gradient. A single large peak was present on the 210 nm chromatogram (**Figure 8**) and the collected fraction was observed to be largely hydrophobic with a white cloudy appearance which turned into a yellow oil when MeOH was evaporated off of the MeOH:H_2O solution.

Subsequent to confirmation of activity in the newly purified fraction, identification of this unknown compound was carried out by MS/MS analysis on a triple quadrupole mass spectrometer (TQMS). This fragmentation chromatogram revealed a likely long hydrocarbon chain (**Figure 9**) which aligned closely with the observed solubility and physical characteristics. To further elucidate the structure, an attempt at crystal growth was performed. Using KOH to aid in crystal growth, a crystal was successfully produced, and the structure was determined to be that of palmitic acid following x-ray crystallography (**Tables 2-4**; **Appendix B**) of a single 0.10 x 0.04 x 0.02 mm^3 colorless plate-shaped crystal (**Figure 10**).

While this is not a new compound, it is notable to observe such a common metabolite eliciting selective cytotoxicity against cancer cell lines. The precise mechanism of action of this observed activity is unknown. However, palmitic acid derived compounds[29] such as brefeldin A[155] (from *Penicillium brefeldianum*) have shown cytotoxicity against colon cancer and induction of ER stress-mediated apoptosis (EC_{50}: 0.016 µg/mL) in the MDA-MB-231 human breast cancer cell line[156].

Similar to the previously isolated and identified *A. niger*, the 18S-ITS-28S rDNA of 20160113-01F1 was sequenced to identify the producing species. Two contigs were generated from Sanger

sequencing of the 18S SSU, ITS1, 5.8S, ITS2, and 28S LSU regions. The 18S-ITS contig showed 99.31% identity to *Cladosporium* sp. and the 28S contig showed 98.53% identity to *Cladosporium cladosporioides* (**Appendix A**). Because the Cladosporium genus has a highly conserved ITS region[157] there was a high degree of sequence similarity between 20160113-01F1 and other species. Therefore, this fungal isolate has been tentatively identified as *Cladosporium* sp..

Table 2. 20160113-01F1-R8-6 Bond Lengths

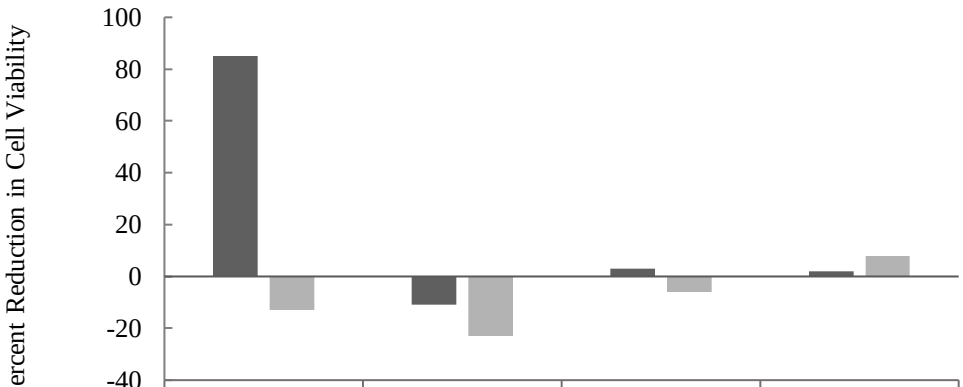
Atom	Atom	Length/Å
O1	C1	1.246
O2	C1	1.284
C1	C2	1.494
C2	C3	1.513
C3	C4	1.504
C4	C5	1.522
C5	C6	1.517
C6	C7	1.514
C7	C8	1.524
C8	C9	1.521
C9	C10	1.526
C10	C11	1.521
C11	C12	1.530
C12	C13	1.519
C13	C14	1.527
C14	C15	1.516
C15	C16	1.528

Table 3. 20160113-01F1-R8-6 Bond Angles

Atom	Atom	Atom	Angle°
O1	C1	O2	123.2
O1	C1	C2	121.4
O2	C1	C2	115.3
C1	C2	C3	116.5
C4	C3	C2	111.4
C3	C4	C5	116.0
C6	C5	C4	112.9
C7	C6	C5	114.9
C6	C7	C8	113.1
C9	C8	C7	114.1
C8	C9	C10	113.7
C11	C10	C9	113.9
C10	C11	C12	113.8
C13	C12	C11	113.3
C12	C13	C14	113.9
C15	C14	C13	113.8
C14	C15	C16	113.8

Table 4. 20160113-01F1-R8-6 Torsion Angles

Atom	Atom	Atom	Atom	Angle/°
O1	C1	C2	C3	-3.6
O2	C1	C2	C3	175.6
C1	C2	C3	C4	178.4
C2	C3	C4	C5	175.7
C3	C4	C5	C6	-179.0
C4	C5	C6	C7	177.4
C5	C6	C7	C8	-178.6
C6	C7	C8	C9	179.9
C7	C8	C9	C10	-179.4
C8	C9	C10	C11	-179.7
C9	C10	C11	C12	179.9
C10	C11	C12	C13	179.4
C11	C12	C13	C14	179.3
C12	C13	C14	C15	179.5



	MIA PaCa-2	SH-SY5Y	COLO 829	HDF
1.0 mg/mL	85	-11	3	2
0.1 mg/mL	-13	-23	-6	8

Figure 7. 20160113-01F1-R8 Preliminary Screening Results

20160113-01F1-R8 was screened against MIA PaCa-2, SH-SY5Y, COLO 829, and a normal cell line (Human Dermal Fibroblast) at two concentrations, 1.0 mg/mL and 0.1 mg/mL in triplicate. A reduction in cell viability was seen only in the 1.0 mg/mL concentration against MIA PaCa-2 and no reduction in cell viability was seen at the 10x diluted 0.1 mg/mL concentration.

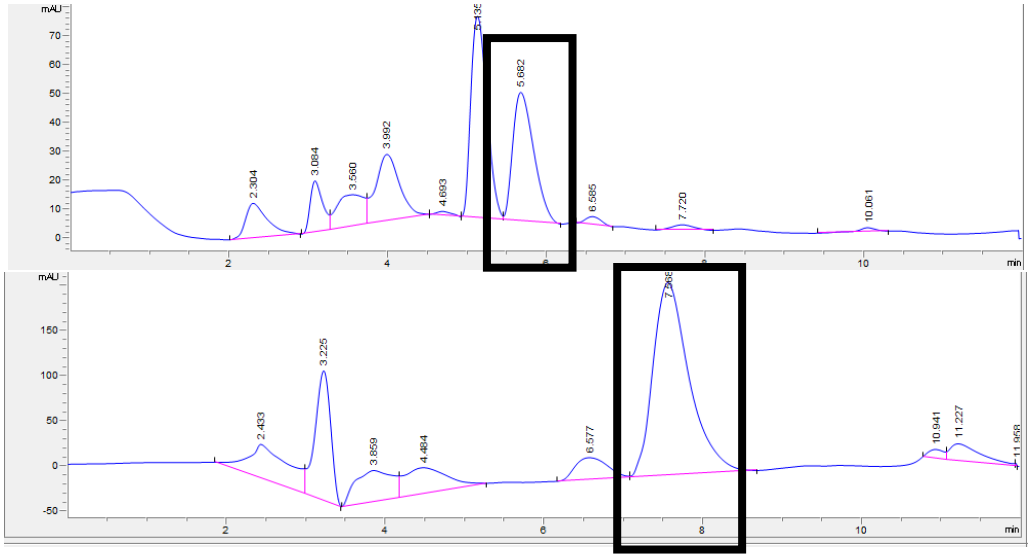


Figure 8. HPLC Chromatograms for 20160113-01F1-R8-6

Semi-preparative (top) and analytical (bottom) 210 nm UV chromatograms for 20160113-01F1-R8-6. While peak resolution is acceptable between the compound of interest and the peak to its immediate left (top), the broad right-hand shoulder on the semi-preparative chromatogram causes concern for compound purity. Therefore, an additional round of analytical HPLC was performed.

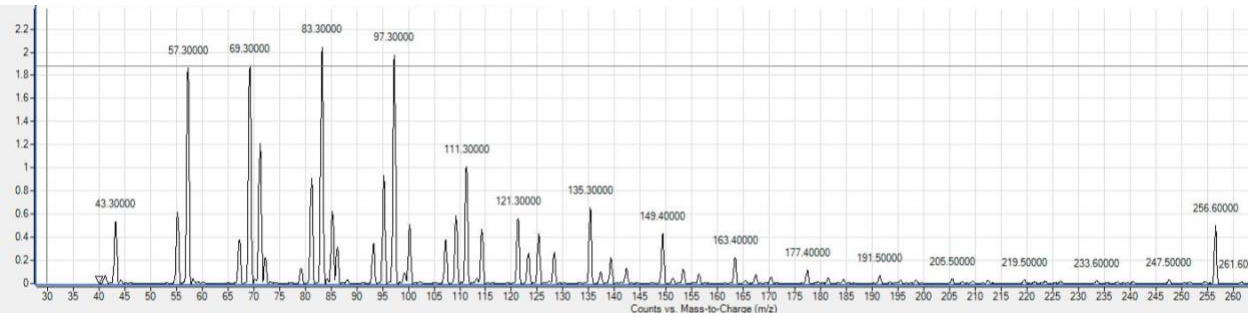


Figure 9. Palmitic Acid MS/MS

MS/MS of unknown compound 20160113-01F1-R8-6 later identified to be palmitic acid. Spectrum indicates the presence of a long hydrocarbon chain with stepwise loss of 12-14 m/z from the parent compound consistent with loss of C or CH_2. This assessment is supported by the physical characteristics of the unknown compound.

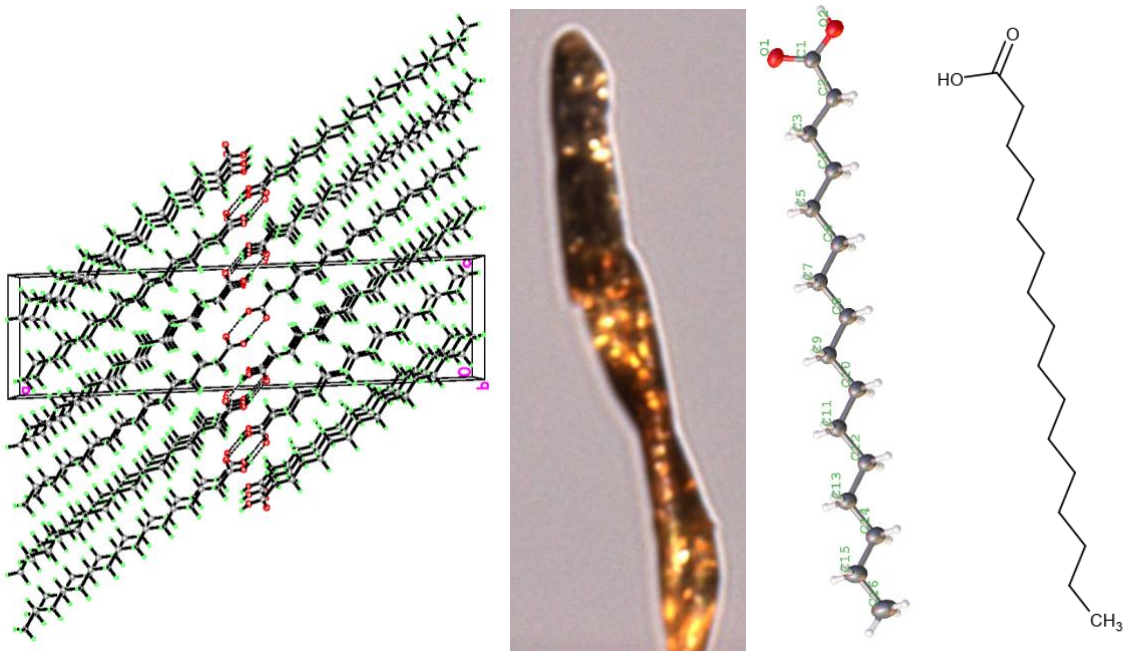


Figure 10. Palmitic Acid Crystal Structure

(Left) 3-dimensional structure of palmitic acid crystal generated from x-ray crystallography of a KOH grown crystal of fraction 20160113-01F1-R8-6. (Center-left) Plate shaped crystal grown in KOH. (Center-right) Structure of palmitic acid generated from x-ray crystallography atom coordinates. (Right) Chemical structure of palmitic acid.

6. CONCLUSIONS

Though at this stage of the project the library has yet to provide a new compound from the pilot study, the methods of library development and screening have been fully tested and verified to produce compounds which are purifiable and verifiably active against MIA PaCa-2, SH-SY5Y, and/or COLO 829.

Further, purification of malformin and palmitic acid have provided confirmation that many methods of compound identification can be employed on this library to positively identify compounds of interest. Between these two compounds, TQMS, TOF-MS, ^{1}H-NMR, ^{13}C-NMR, MS/MS, and x-ray crystallography were all used to identify these active compounds.

Chapter 4

ASPOCHALASINS, A STRUCTURALLY DIVERSE FUNGAL DERIVED BIOACTIVE SUB-GROUP OF CYTOCHALASANS AND THEIR ACTIVITIES

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Modified from:

Aspochalasins, A Structurally Diverse Fungal Derived Bioactive Sub-group of Cytochalasans and Their Activities

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ABSTRACT. Aspochalasins are a structurally diverse subgroup of cytochalasans produced by fungal secondary metabolism. While much is still unknown about their precise mechanisms of action, they appear to elicit cytostatic and cytotoxic effects similarly as their parental group. Those effects include antibacterial, anti-tumoral, phytotoxic, anti-proliferative, and anti-viral activities. Their potencies are often as varying as their structures with minor modifications on their frequently substituted macrocyclic scaffold causing complete loss or significant enhancement of activity. Due to their various biomedically relevant effects and a large number of macrocyclic carbons with tolerance for diverse functional group substitutions, aspochalasins appear to be good candidates for structural optimization which may produce compounds with potent and targeted effects. This chapter aims to categorize the underlying features of this subgroup of naturally produced compounds.

1. INTRODUCTION

Cytochalasans are a large class of fungal secondary metabolites with varying anti-tumoral, anti-microbial, and other biological activities[158]. Since their first discovery in 1967, the size of this class has steadily grown and is now made up of at least 300 compounds[159], which are characterized by a highly substituted isoindole ring fused to a larger often 11- or 12-membered macrocyclic ring (**Figure 1**).

Cytochalasans with a 2-methylpropyl substitution at C-3 of the isoindole unit are sub-grouped as aspochalasins (**Figure 1**). By 2004 only 12 such compounds containing this substitution had been identified[160]. As of 2021, this number has swelled to greater than 100. A majority of aspochalasins have been isolated as white or pale-yellow amorphous powder from *Aspergillus* species; however, they have also been found to be produced by other fungal genera such as *Phoma*[161-163], *Periconia*[164-167], *Spicaria*[6,168,169], *Trichoderma*[170-173] or *Westerdykella*[162,174]. This small but rapidly expanding group of fungal natural products have demonstrated proclivity for vast structural diversity and varying biological activities. This chapter aims to categorize the underlying features of aspochalasins.

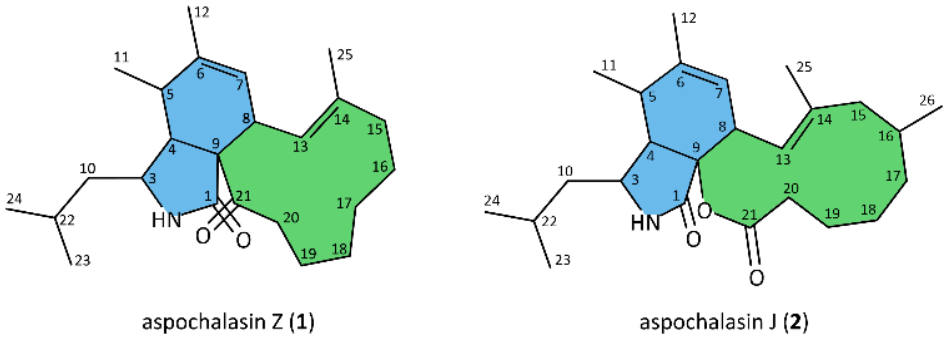

Figure 1. Planar Structures of Aspochalasin Z and Aspochalasin J

Representative planar structures of aspochalasin Z (**1**) with a 11-membered macrocyclic ring and aspochalasin J (**2**) with a 12-membered macrocyclic ring. Isoindole unit is drawn in blue and macrocycle in green.

2. STRUCTURAL DIVERSITY AND CATEGORIZATION

While aspochalasins are easily characterized and recognizable by their C-3 2-methylpropyl group, the macrocycle affords the most opportunity for structural novelty via addition of functional groups or attachment of large complex molecules on the C-17, -18, -19, and/or -20 positions.

Commonly, aspochalasins are described based upon their overall ring structure. The most common ring arrangement is the tricyclic 5/6/11 organization as seen in aspochalasin Z (**1**; **Figure 1**); however, other ring arrangements such as 5/6/12 as seen in aspochalasin J (**2**; **Figure 1**), or 5/6/6/7, 5/6/9, and 5/6/7 have also been identified.

In addition to the previously mentioned ring structural categorization, aspochalasins can also be classified as ketone aspochalasins for having a key C-21 ketone or ester aspochalasins for having a C-21 ester on the macrocycle fused to C-9 of the isoindole ring (**Figure 1**), or as open-ring aspochalasins for lack of a macrocyclic ring. The exceptions to this rule are a hydroxyl group seen on C-21 of aspochalazine A attributable to its iconic azabicyclo moiety[175], a replacement of the ester ketone with NH in flavichalasine O[176], and periconiasins D-F due to their severely truncated macrocycles[166].

2.1. Ketone Aspochalasins

Roughly 2 of every 3 (80/117) aspochalasins identified to date contain what can be considered the basic form of the macrocycle with a C-21 ketone moiety (**Figure 2**). Compound **1** is thought to be the "basic version" of all aspochalasins with the emblematic C-5, -6, and -14 methyl groups but having no further modifications to the macrocycle or the isoindole ring[177].

Though this group contains the most common and basic 5/6/11 ring structure, it nonetheless encompasses interesting compounds such as iizukine C (**3**) with a unique 1,2,4-triazole group attached to C-19[178], in addition to the more familiar compounds such as aspochalasins C-E (**4**-**6**) with hydroxyl groups at the frequently substituted C-17, -18, and/or -19 positions (**Figure 3**)[179]. Spicarins A-B (**7**-**8**) have four acetyloxy groups attached to C-17 and their macrocycle-fused isobenzofuran ring[168]. Tricochalasin A (**9**) markedly contains a heavily modified 5/6/6 tricyclic ring attached to C-18, -19, and -20[136].

Despite the presumed availability of C-13 and C-15 on the macrocycle for functional group substitutions, there is no example of substitution on those atoms other than internal ring closure as seen in flavichalasines C-E (**10**-**12**)[180], trichalasin H (**13**)[172], aspergillin PZ (**14**)[181], 16-hydroxymethylaspergillin PZ (**15**)[174], and trichoderone B (**16**)[173]. Ring closure often generates an epoxy moiety fused to the macrocyclic ring.

Apart from C-13 and C-15, the next most uncommonly substituted atom in the macrocycle is C-16 (**Figure 3**). Phomacin C (**17**)[161,163] and several naturally occurring analogs of **17** have been isolated and found to have an atypical hydroxymethyl attachment at C-16 including bioactive **17** diastereomer 19,20-dihydrophomacin C (**18**)[162]. Among these analogs, several were also determined to have an equally rare methoxy group. While methoxy has not yet been observed at C-17, it is seen at C-18 in aspochalasin K (**19**)[160]. Methoxy has also been seen at C-19 of trichalasin G (**22**)[172], which is an analog of aspochalasin I (**20**)[160] and flavichalasine F (**21**)[180]. Finally, a methoxy group is present at C-20 in flavichalasine L (**23**)[180].

Complex ring systems expand far past the relatively simple tricyclic, tetracyclic, and pentacyclic structures yielding compounds such as the cage-like hendecacyclic epicochalasines A-B, which

are thought to be generated from the fusion of an epicoccine dimer to **5**[182] similar to asperflavipines A-B, which are characterized by their tetradecacylic and nonacyclic structures, respectively[159]. Epicoccine is likewise integrated into the structure of asperchalasines A-H which theoretically arise from aspochalasins B and P (**24**-**25)**[183-185] and aspergilasines A-D from **5**[186].

Multifarious ring structures are not the only characteristics which give aspochalasins their rich diversity; many identified aspochalasins have interesting groups attached to their macrocycle rather than complex interconnections. Aspochalamins A-D are notable for a tripeptide sequence attached to the macrocycle of **1** or **5** and were only found in stationary culture[177,187].

Within the ketone aspochalasin group are other oddities such as bisaspochalasin A wherein a **5** monomer is homodimerically linked at C-17, -18, and -19 to the cleaved macrocycle of another[188]. In the same way, bisaspochalasins B and C are homodimerized **24** but are instead linked by a peculiar thioether bridge and differentiated by bisaspochalasin C's equally odd C-7 peroxy seen only one other time in trichalasin E[172,188]. While sulfur is rarer than carbon, nitrogen and oxygen in microbial natural products methylthio-containing aspochalasins V and W[189] as well as modified cysteine-containing cyschalasins A and B[190] have been described. Periconiasin H rounds out the sulfur-containing aspochalasins; however, its sulfoxide group is not its most striking structural feature. The periconiasins are much smaller than typical aspochalasins with ring structures as small as 5/6/7 seen in periconiasin G[165]. Periconiasins D-F are technically not ester nor ketone aspochalasins due to a C-15/18 ring closure on their truncated macrocycle[164,166]. Regardless, the periconiasins still retain the other key aspochalasin characteristics such as the C-3 2-methylpropyl group and isoindole subunit[164-166].

The last common modification is addition of a hydroxyl group to C-7 of the isoindole ring, as seen in **16,** aspochalasins L, U and W[191,192], and trichalasin C[171]. This group of compounds represent the lone non-macrocyclic aspochalasin modifications thus far documented (**Figure 3**).

2.2. Ester Aspochalasins

Ester aspochalasins have their macrocycle connected to the isoindole unit by what is now known to be a Baeyer-Villiger generated C-21 ester[193,194] and are largely 5/6/12 structured compounds, though unsurprisingly this is not the only possible ring structure (**Figure 4**). Aspochalasin F (**26**) was the first documented occurrence of a C-21 ester aspochalasin reported in 1997[195]. This group is no less diverse than the ketone aspochalasins and often contains compounds which share structural similarity with analogous ketone counterparts. For example, the uncommon 17,18-epoxy group found in C-21-ketone aspochalasin G is also seen in the ester-containing **26**[195]. Likewise, many ester aspochalasins are also present with hydroxyl or carbonyl groups attached to C-17, -18, and/or -19 (**Figure 3**)[160,178,180,193,196] with internal ring structures such as those seen in amiaspochalasins B and C (**27**-**28**)[193].

Exceedingly unique amiaspochalasin A (**29**) has a methyl group affixed to C-16 rather than the largely ubiquitous 14-methyl placement seen in nearly all of its aspochalasin congeners (**Figure 3**)[193]. Similar absence of 14-methyl is only seen in periconiasin F and flavichalasine A[180] which both contain 14-methylene making **29** the only recognized C-14 unsubstituted aspochalasin. Similar C-16 methylation is seen in 16α-methylaspochalasin J (**30**)[174] and phomacin A[161] which both, however, retain 14-methyl; interestingly, none of these 16-methyl bearing compounds were isolated from *Aspergillus* sp.

2.3. Open-ring Aspochalasins

Opening of the macrocycle rather than a ring closure is the source of the last major category of aspochalasins (**Figure 5**)[197]. The products of those ring breakages have been from both ketone and ester aspochalasins and can occur at many locations on the macrocycle. For example, amiaspochalasin F and G (**31**-**32**)[193] are thought to be generated by cleavage of the C-21 ester group from their immediate precursors. Similar ring opening is seen in amiaspochalasin I[197], secochalasins A and B (**33**-**34**)[190], and a monomer of the strangely cross-linked bisaspochalasin A[188]. Overall, the open-ring aspochalasins are a small and rare subset of aspochalasins (**Figure 3**). Whether those are true terminal secondary metabolites, shunt intermediates during biosynthesis or simply breakdown products from the isolation process is unknown as they have not been reported to have meaningful bioactivities.

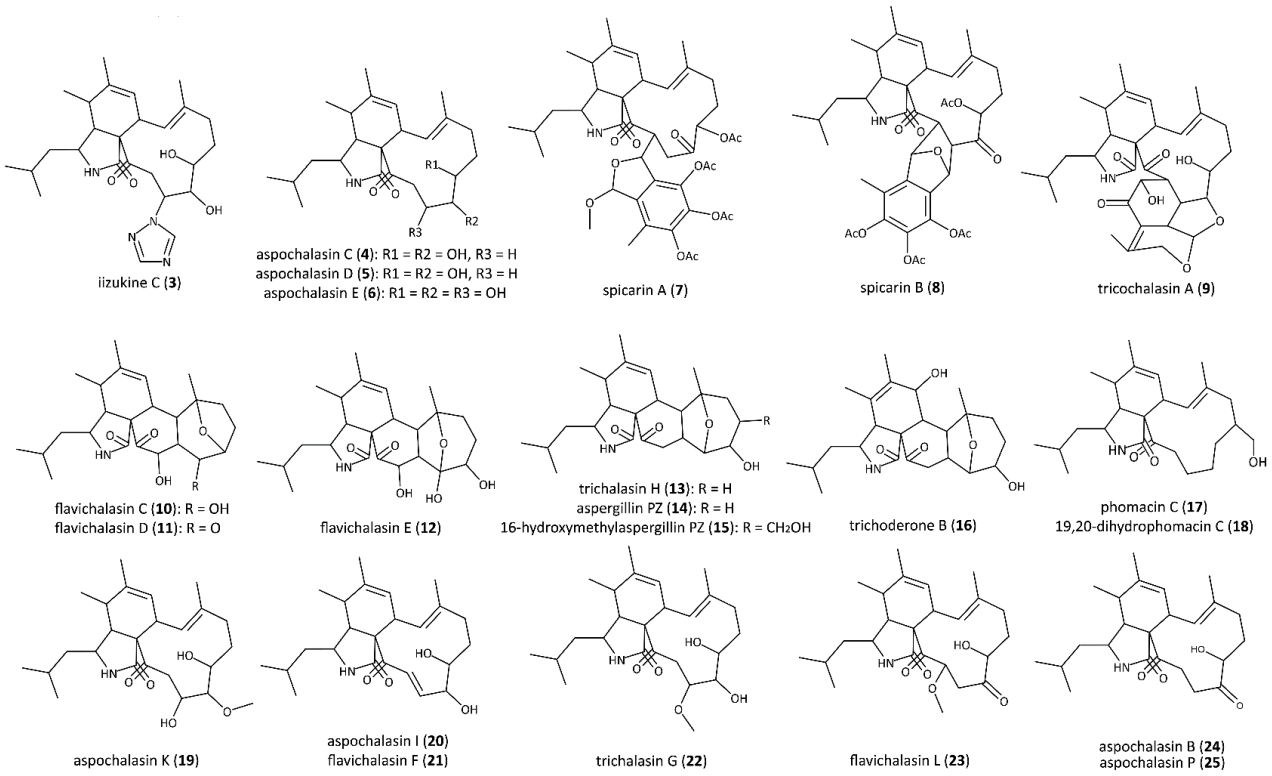

Figure 2: Representative Planar Structures of the Ketone Subgroup of Aspochalasins

Representative planar structures of compounds within the ketone subgroup of aspochalasins. These compounds are notable for a ketone attachment of the macrocycle to the isoindole ring. Many of these compounds have analogous compounds with an ester rather than ketone functional group.

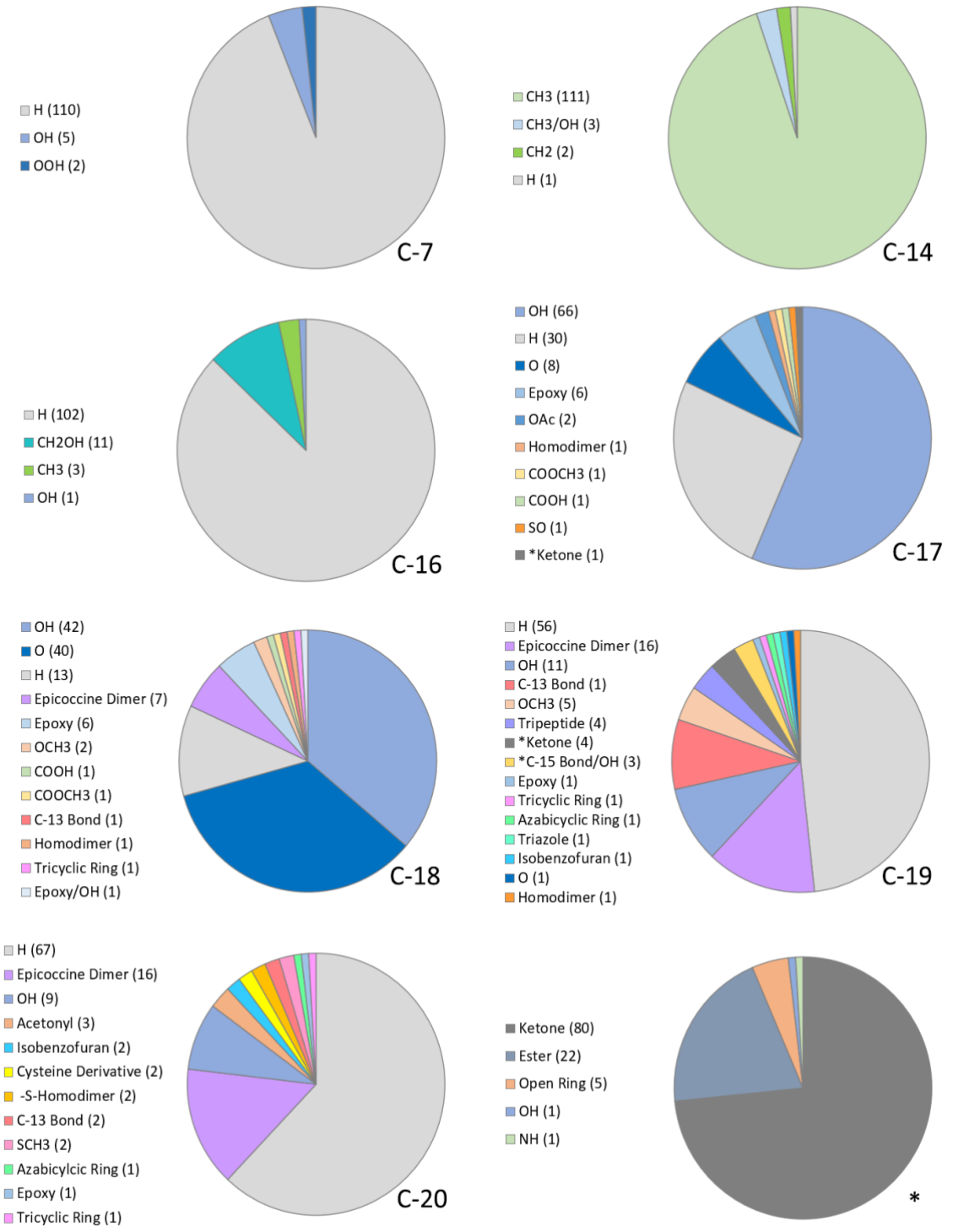

Figure 3. Occurrences of Functional Attachment to C-7, -14, -16, -17, -18, -19, and -20

With few exceptions, aspochalasins typically have a methyl group attached at C-14 and an unsubstituted C-16. For C-17, -18, -19, and -20, substitutions are often oxygenated functional groups or larger high MW modifications.

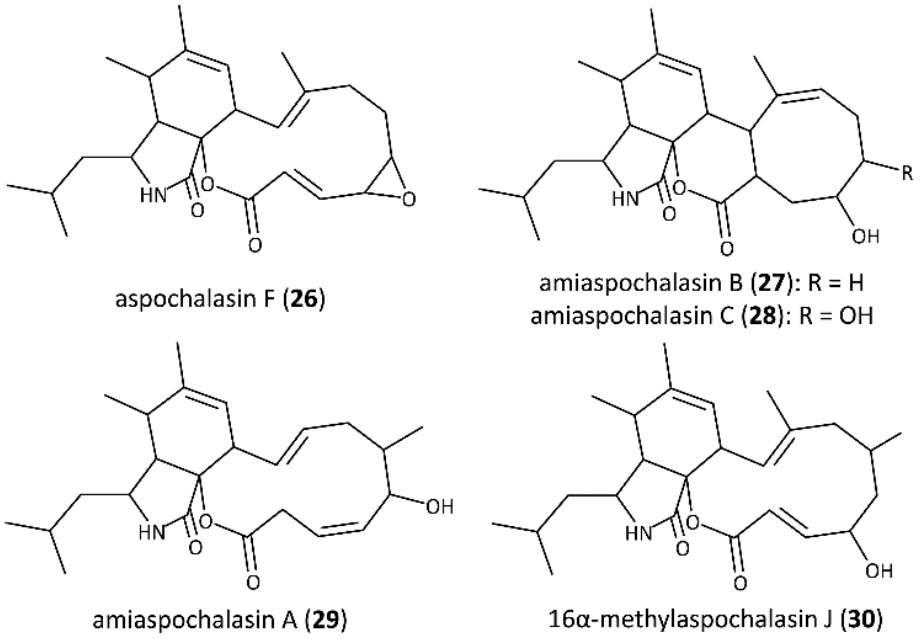

Figure 4. Representative Planar Structures of the Ester Subgroup of Aspochalasins

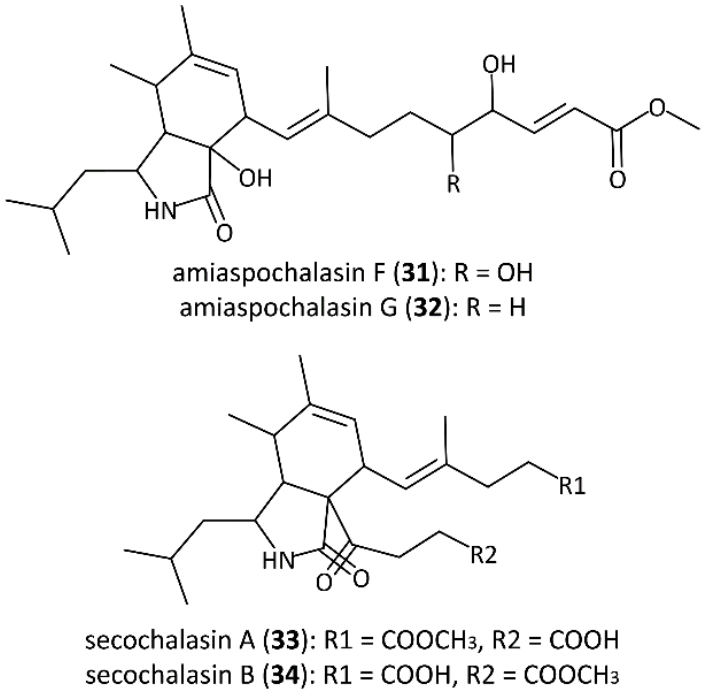

Figure 5. Representative Planar Structures of the Open-ring Subgroup of Aspochalasins

3. BIOSYNTHESIS OF ASPOCHALASINS

While the biosynthesis of aspochalasins is not as thoroughly documented as cytochalasans, limited studies demonstrate that their formation is similar to the parental cytochalasan class which arises from hybrid PKS/NRPS biosynthetic pathways[198,199]. As aspochalasins are solely distinguished from cytochalasans by their characteristic C-3 2-methylpropyl group, it was postulated that their origins are due to incorporation of leucine in the nascent structure rather than tryptophan, phenylalanine, tyrosine, valine, or alanine[136,198] which gives rise to other well-known cytochalasan subgroups such as the chaetoglobosins, pyrichalasins, and alachalasins[190,198].

Specifically, a 36,705 bp biosynthetic gene cluster, *ffs*, was identified in marine-derived *A. flavipes* CNL-338 that yields many aspochalasins including **4, 6**, **14**, **21**, aspochalasin M, TMC-169, and flavichalasine G[200]. The *ffsA* PKS module iteratively incorporates malonyl-CoA building blocks followed by addition of *L*-leucine by an NRPS module[200].

Similarly, a PKS/NRPS gene cluster *phm* was upregulated by overexpression of transcriptional regulatory gene *phmR* in *Parastagonospora nodorum*, which resulted in the production of a previously characterized phomacin derivative[162] that was renamed phomacin D (**35**) and phomacins E-F (**36**-**37**; **Figure 6**)[201]. Further, when *phmA* and *phmE* were expressed in *A. nidulans*, prephomacin (**38**) was produced. While **38** is not a conventional aspochalasin, it was proposed that this compound is the precursor to **35**, which can logically be derived following enolization and a Diels-Alder addition[201].

37 notably has a C-20 acetonyl group[201], which is similarly seen in aspochalasins N and O[6]. Whether this functional group is naturally occurring is unclear; though they appear to be an artifact

of using acetone during extraction processes[6,201]. Experiments to verify this theory have emphasized the reactivity of C-20[201].

Synthetically generating these often-complex molecules from their biologic precursors complements the ability to understand their biosynthetic origin. This has proven to be an achievable endeavour even in the intricate asperchalasine structures[183,184] to aid in understanding their ability to selectively inhibit cell cycling[202].

The proposed biosynthetic pathway for the unique sulfur-containing aspochalasins involves a 1,4-addition of cysteine to intermediate **24** followed by either a deamination-methylation or acylation-decarboxylation step to arrive at the structure of cyschalasins A and B, respectively[190]. A similar biosynthetic strategy is proposed for the sulfur-containing bisaspochalasins B and C wherein cysteine is also postulated to conduct a nucleophilic addition to the unsaturated C-20 of **24**[188].

The complexly linked epicochalasines A and B are suggested to be formed through a Diels-Alder reaction of **5** and two epicoccine molecules followed by [3 + 2] cycloaddition and a subsequent nucleophilic addition step to yield epicochalasines A or B depending on the original orientation of epicoccine at the time of cycloaddition[182]. A similar biogenetic pathway is proposed for the creation of aspergilasine A which undergoes an intramolecular [3 + 2] cycloaddition to arrive at its final structure[186].

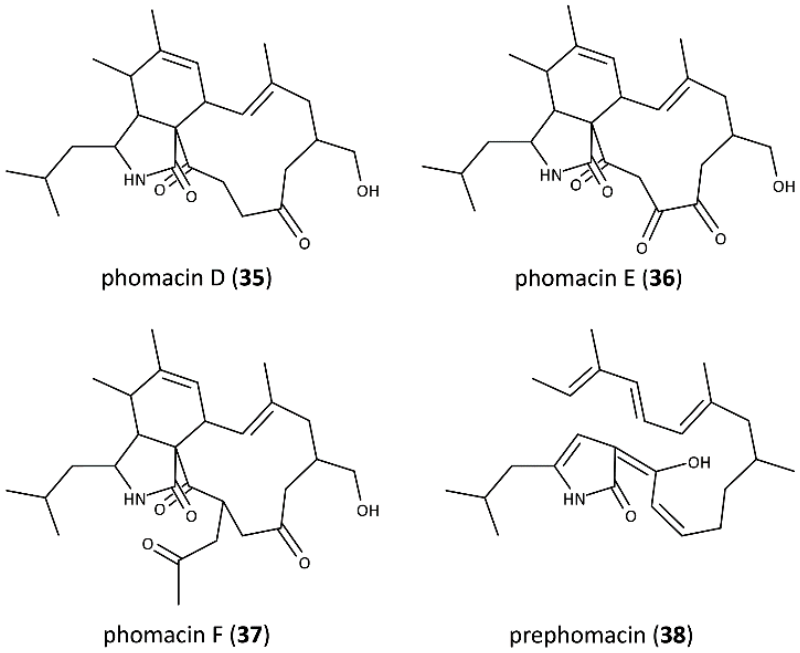

Figure 6. Planar Structures of Phomacins D-F and Prephomacin

4. BIOLOGICAL ACTIVITIES

As indicated by the Greek root meaning 'cell relaxation'[203], cytochalasans are known to elicit anti-proliferative activity through targeting and capping the actin cytoskeleton[204]. This actin capping affects normal cell processes such as cell adhesion, motility, signalling, and cytokinesis[158,190]. Though the biological activity of cytochalasans is well documented, aspochalasins have sporadically reported data (**Table 1**) which obfuscates our ability to determine their importance, structure-activity relationships, and any specific mechanisms of action which vary from current understanding of cytochalasan activity. For example, cytochalasans have been shown to elicit cytotoxic as well as cytostatic effects, which would presumably allow for the use of the cytostatic compounds in cancer therapy despite their lack of cytotoxicity[198]; whether aspochalasins operate similarly is not extensively studied. Importantly, there is a need for some levels of modification on the macrocycle to cause cytotoxicity as the aspochalasin 'basic version', **1**, has no documented

bioactivities[177]. Compounds **35**-**36** inhibited actin polymerization and **37** did not. These findings suggest that aspochalasins block actin polymerization similar to cytochalasans, but these activities are not present in the erroneously generated **37**. Further supporting the suggestion of actin inhibition as a mechanism of action, **35**-**36** both exerted anti-germinative phytotoxicity with **35** selectively preventing germination of only monocot *Avena sativum* and not dicots *Arabidopsis thaliana* or *Lepidium sativum*[201].

To date, the most potent aspochalasin reported is TMC-169, a rather simple C-18-hydroxyl analog to **1**. Despite or possibly due to its simplicity, it has shown remarkable cytotoxicity against U937, Jurkat, HL-60, WiDr, and HCT-116 (IC_{50}: 0.81, 0.2, 0.68, 0.83, 0.78 µg/ml, respectively)[205]. Another minimally substituted compound, **5**, caused cell death in dexamethasone inducible *ras*-dependent Ba/F3-V12 cells (IC_{50}: 1.9 µg/ml), PC3 cells (IC_{50}: 11.14 µg/ml), and exhibits moderate antimicrobial effects against Gram-positive microorganisms[206].

That is not to say that simplicity is a requirement for activity in aspochalasins. Complex compounds such as the tripeptide-linked aspochalamins elicited moderate growth inhibition zones against Gram-positive microorganisms at 1 mg/mL and aspochalamins A-C had GI_{50} less than 10 µg/mL against HM02, MCF7, HepG2, and Huh7 cancer cells[177]. Though it was cytotoxic to NCI-H460, MCF-7, and SF-268[160], **20** interestingly demonstrated no cytotoxic effect on Mel-Ab cells at <100 µM; however, **20** inhibited the ability of Mel-Ab to carry out melanogenesis (IC_{50}: 22.4 µM) by blocking tyrosinase activity[207].

Table 1: Reported Bioactivities of Aspochalasins Against Cell Lines or Microorganisms.

Compound	Notable Inhibitory Bioactivities	Refs.
aspochalasin C (**4**)	B16-F10, HCT-116, NCI-H460, MCF-7, SF-268	160,179
aspochalasin D (**5**)	*A. globiformis*, *A. aurescens*, *A. oxydans*, *A. pascens*, *B. subtilis*, *R. erythropolis*, *S. aureus*, *S. epidermidis*, Ba/F3, NCI-H460, MCF-7, SF-268, HeLa, PC3	136,160,171,177,196,206
aspochalasin E (**6**)	B16-F10, HCT-116, NCI-H460, MCF-7, PC3, Jurkat, HL60, NB4, HEP3B, RKO, Caspase activation, PARP degradation	160,175,179,180
aspochalasin F (**26**)	HL60, MH60	195
aspochalasin G	*B. subtilis*, *S. aureus*, *M. luteus*, *A. laidlawii*, *P. oryzae* HL60, MH60	195
aspochalasin I (**20**)	*S. epidermidis*, *S. aureus*, NCI-H460, MCF-7, SF-268, Melanogenesis inhibition in Mel-Ab cells (IC_{50}: 22.4 μM)	160,196,207
aspochalasin J (**2**)	MCF-7, SF-268	160
aspochalasin K (**19**)	NCI-H460, MCF-7, SF-268	160
aspochalasin L	Anti-HIV integrase (IC_{50}: 71.7 μM)	192
aspochalasin U	TNF-α inhibition	191
aspochalasin V	PC3, HCT-116	189
amiaspochalasin D	HL60, A549, SW480	193
amiaspochalasin E	HL60, A549, Hep3B, U87, SW480	193
asperchalasine A	cyclin A, CDK2, CDK6, F-actin disruption	202
asperflavipine A	MDA-MB-231, RKO, Hep3B, HCT116, Jurkat, NB4, HL60	159
aspochalamin A	*A. pascens*, *B. brevis*, *R. erythropolis*, HM02, MCF7	177
aspochalamin B	*R. erythropolis*, HM02, MCF7, HepG2, Huh7	177
aspochalamin C	*A. globiformis*, *R. erythropolis*, *HM02*, *MCF7*, *HepG2*, *Huh7*	177
aspochalamin D	*R. erythropolis*	177
aspergillin PZ (**14**)	*S. epidermidis*, *K. pneumoniae*, *P. aeruginosa*, HL-60, A2780, PC3, LNCaP, DU145, A2058	196,208
bisaspochalasin A	Anti-T-cell proliferation (IC_{50}: 15.8 μM)	188
cyschalasin A	*S. aureus*, MRSA, *C. albicans*, HL60, Hep3B, MCF-7, SW480	190
cyschalasin B	MRSA, HL60, A549, Hep3B, MCF-7, SW480	190
epicochalasine A	Caspase activation, PARP degradation	182
epicochalasine B	HL60, NB4, Caspase activation, PARP degradation	182
flavichalasine F (**21**)	Jurkat, HL60, NB4, Hep3B, HCT-116, RKO, Caspase activation, PARP degradation	180
iizukine C (**3**)	HL-60, A549	178
periconiasin A	HCT-8, BGC-823	164
periconiasin B	HCT-8, BGC-823, Bel-7402	164
phomacin A	HT-29	163
phomacin B (**17**)	MCF-7, HT-29	162,163
phomacin C (**18**)	HT-29	163
TMC-169	U937, Jurkat, HL-60, WiDr, HCT-116	205
trichoderone B (**16**)	HeLa	173

Common to many aspochalasins is having a C-17 hydroxyl or C-18 hydroxyl moiety (**Figure 3**). There is evidence to support the claim that hydroxyl at C-18 on the macrocycle can be necessary for antibacterial activity. The C-18 hydroxyl of aspochalamin C[177,187] was necessary for its activity against *Arthrobacter globiformis* when compared to the hydroxyl-lacking aspochalamin D. Similar activity against *A. globiformis* is seen in **5** which also bears a C-18 hydroxyl. Interestingly, **5** exerted antibiotic activity against many Gram-positive species which were not inhibited by aspochalamins A-D, indicating that their C-19 tripeptide linkage had a negative effect on their antibiotic potencies[177].

When compared for antibiotic activity, **5**, **14**, and **20** all caused growth inhibition against *S. epidermidis* and *S. aureus;* however, this inhibition is greatly diminished in **14** and **20** (MIC: 20 µM vs. 10 µM)[177,196]. Neither **14** nor **20** are inhibitory to *E. coli* or *B. cereus* whereas **5** (MIC: 10 µM) is inhibitory. Compound **24** was not inhibitory to any of these organisms (MIC: >20 µM)[196]. Given their structural similarities, it is clear that antibacterial potency among simple 17,18-diol compounds is greater in the C-21 ketone variant and this activity is not enhanced by changing C-17 hydroxyl to C-17 carbonyl or C-17 epoxy. Likewise, the C-17,18-diol-19,20-epoxy bearing aspochalasin H, demonstrated no antibiotic activity against these targets[196]. Aspochalasin H also did not prompt cytotoxicity against Ba/F3 cells whereas **5** had high potency (IC_{50}: 0.49 µg/ml) against the same target[209], indicating that this observed C-17 hydroxyl preference may carry over to anticancer effects as well.

While there was little difference in the anticancer activity between **26** and aspochalasin G against HL-60 and MH-60, there was a substantial difference in inhibition zones when 30 µg of either was added to cultures of Gram-positive and Gram-negative species. Compound **26** was inactive whereas aspochalasin G was inhibitory to *B. subtilis, S. aureus, M. luteus, A. laidlawii, P. oryzae,*

and L-form bacteria[195]. Given this, it appears that there is a role for C-21 ketone in antibiotic activity.

As for the effects of internal epoxides, **13-15** have shown anticancer and antimicrobial effects[173,174,181]. Other uncommon compounds such as the 1,2,4-triazole-carrying **3** and the sulfur-containing cyschalasins also have documented cytotoxicity against cancer cells[178,190].

On the isoindole ring, aspochalasins with C-7 hydroxyl also exhibit important biological activities. Aspochalasin U has shown moderate dose-dependent anti-TNFα activity in L929 cells which led to increased cell survivability by blocking necrotic cell death[191]. Aspochalasin L, which similarly contains C-7 hydroxyl, has shown to inhibit HIV-1 integrase activity but not HIV replication in HuT78 T-cells (IC_{50}: 71.7 μM)[192].

Compound **22** is a C-7-H structural analog to aspochalasin L. Whether **22** has similar anti-HIV integrase activity is unknown. Further study of the comparative effects of these two compounds would aid in determining the importance or lack thereof for the uncommon yet often active C-7 hydroxyl aspochalasins. No activity was observed in C-7 hydroxyl, C-20 methylthio-containing aspochalasin V whereas its C-7 unsubstituted analog aspochalasin W was active against PC3 (IC_{50}: 30.4 μM) and HCT-116 (IC_{50}: 39.2 μM)[189]. Further SAR is possible here due to the number of identified aspochalasins with hydroxyl or methoxy groups (**Figure 3**).

The number of hydroxyl groups on the macrocycle is not necessarily indicative of the degree to which a compound will be cytotoxic. For example, amiaspochalasins D-E both have C-18 hydroxyl but amiaspochalasin D also has a C-17 hydroxyl. Despite being more substituted, amiaspochalasin D was significantly less cytotoxic to HL60, A549, Hep3B, U87, and SW480 than its structural analog[193].

Investigation of the role of C-16 methyl moiety is possible through comparison of **2** and **30**. Though they have not been tested against the same targets, both compounds have shown moderate cytotoxicity[160,174]. Amiaspochalasin A lacked activity against a panel of cancer cell lines[193]. This deficiency in activity may have more to do with the absence of the extremely pervasive C-14 methyl rather than the presence of C-16 methyl.

Further, phomacin A demonstrated potent toxicity against HT-29 cells (IC_{50}: 0.6 µg/mL) but the toxicity is reduced in C-16 methoxy carrying phomacin B (IC_{50}: 1.4 µg/mL) or its C-21 ketone analogue **17** (IC_{50}: 7.4 µg/mL)[163]. Of a group of six **17** analogs which have been described, none demonstrated cytotoxicity (IC_{50}: >50µM) against HT-29 except for **18** (IC_{50}: 49.09 µM)[162].

Interestingly, the size of aspochalasins does not appear to be determinative of their activities. Compounds as small as a MW of 359.5 and as large as a MW of 976.2 have been found to be biologically active[164,188]. The smallest active aspochalasins, periconiasins A-B, both showed sub-micromolar potency against HCT-8 (IC_{50}: A: 0.9, B: 0.8 µM). Though they differed only by the orientation of their C-17 hydroxyl moiety, they had dissimilar potency against BGC-823 (IC_{50}: A: 2.1, B: 5.1 µM) and periconiasin B was the only compound of the two to be cytotoxic to Bel-7402 (IC_{50}: 9.4 µM)[164]. The largest aspochalasin discovered to date, asperchalasine A, which consists of two **24** monomers attached to a central epicoccine has exhibited ability to selectively arrest the cell cycle at G1 through inhibition of cyclin A, CDK2, and CDK6 in cancerous cells[202]. Other epicoccine-carrying aspochalasins, epicochalasins A-B and asperflavipine A, have shown cancer cell cytotoxicity through the activation of caspase-3 and degradation of PARP[159,182], a process similarly noted in flavichalasine F[180].

5. CONCLUSIONS

Much is now known about the vastness of aspochalasin chemical space, though little is currently known about their structure-activity relationships which dictate their prolific anticancer, antimicrobial, phytotoxic and anti-viral profiles. As the size of the aspochalasin subgroup has now eclipsed 100 compounds, there is a necessity as well as an opportunity to delve deeper into their structure-activity relationships.

Investigation of the responsible biosynthetic gene clusters may be helpful to discover more cryptic aspochalasins or further understand their biogenesis. Though their structures are often complex, many of these secondary metabolites have analogous compounds which differ by only one functional moiety. Because of this and considering their documented potential to elicit a wide array of biomedically relevant effects, the next sensible step is to methodically analyse the structure-activity relationships against common targets or to attempt synthetic structural optimization particularly on the macrocycle which has shown to permit attachment of substitutions that could potentially increase potency or targeting specificity. Given their well-documented *de novo* syntheses and reactive macrocyclic ring, aspochalasins may be appropriate scaffolds for further drug development as it is clear that size does not necessarily preclude aspochalasins from eliciting meaningful activity, though there is some bias for smaller molecules.

Among the potential modifications to the macrocycle, C-14 methyl as well as C-17 and/or C-18 oxygenated functional groups appear to be preferred for bioactivity. **4, 5**, and **24** appear to be functional 'starting blocks' for building a large portion of this set of compounds (**Figure 7**) and would thus be ideal candidates for any optimization attempts as their syntheses have been well documented.

Most importantly, the precise mechanism of action of those compounds is still largely unknown. While their ability to cause cytotoxic or cytostatic activity is not disputed and there are suggestions that aspochalasins operate through actin capping[201], determining whether there are any other mechanistic activities which cause this wide range of intracellular effects is crucial to creating strategies to optimize their structures.

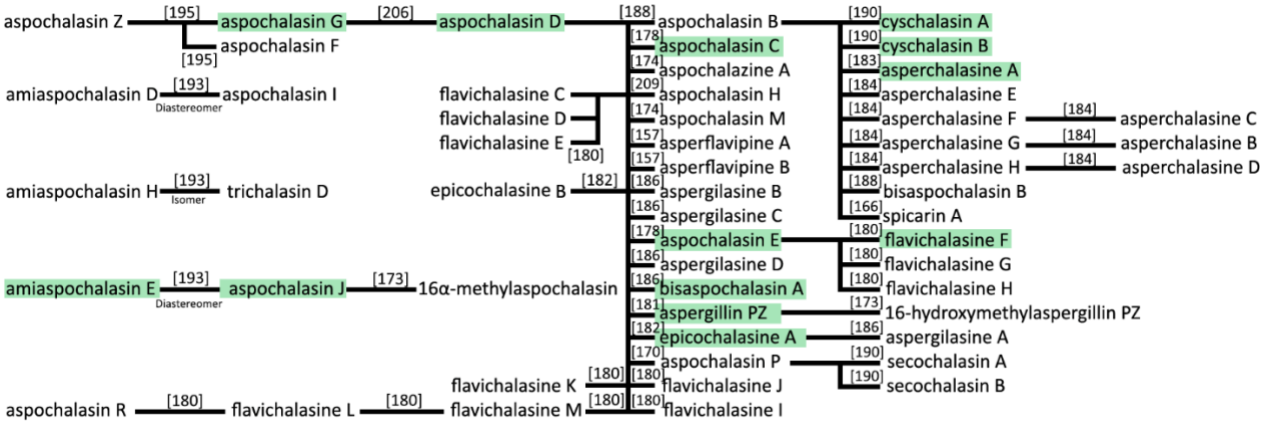

Figure 7: Proposed Plausible Relationships and Biosynthetic Derivations of Various Aspochalasins

From the ‘basic’ aspochalasin Z to more complexly substituted molecules, there exists an interrelation between many ketone aspochalasins. Lines represent related compounds. Compounds highlighted in green have documented biological activity; those not highlighted have no documented biological activity though are not necessarily inactive. Numbers in brackets are reference numbers.

Chapter 5

REDISCOVERY AND CYTOTOXIC ACTIVITY OF ASPERGILLIN PZ AND TRICHODERONE B

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Modified from:

Cytotoxic Activities of Aspergillin PZ and Trichoderone B from an Isolate of *Aspergillus flavipes* sp. Against NCI-60 Human Tumor Cell Lines

Records of Natural Products (2021)

ABSTRACT. In the process of piloting a new approach to natural product discovery from microorganisms isolated from systematically determined geographic sites in the State of Texas, USA, two previously identified and structurally similar bioactive pentacyclic aspochalasins, aspergillin PZ (**1**) and trichoderone B (**2**), were re-discovered from a single isolate of *Aspergillus flavipes* sp.. While the antimicrobial activity and cytotoxic activity of **1** had been documented in several publications, nothing was known about the bioactivities of compound **2**. After a large laboratory-scale fermentation and activity-guided purification, both compounds were enrolled in the NCI-60 Human Tumor Cell Lines Screen program. A preliminary single-dose assay of two natural products shows a similar cytotoxic profile and a low potency against human tumor cell lines with few exceptions at the 10 μM dosage level.

1. INTRODUCTION

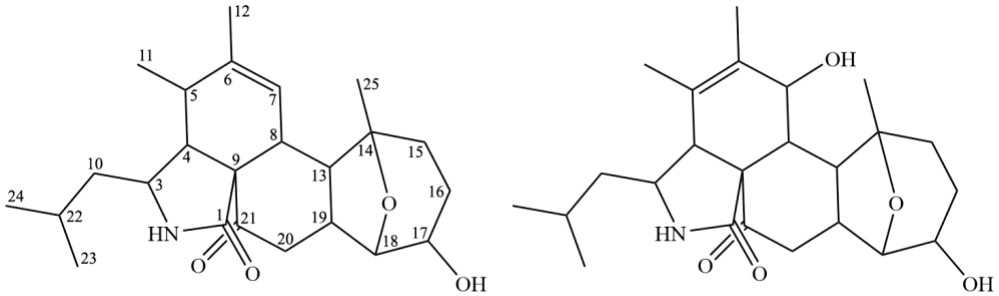
Aspochalasins, a sub-group of cytochalasans, are a class of secondary metabolites commonly produced by many *Aspergillus* species. Cytochalasans are prolific inhibitors of actin polymerization[210] and are recognizable by an isoindole unit fused to one macrocyclic ring and a C-3 2-methyl-propyl group[136,211]. *A. flavipes* produces many cytotoxic aspochalasins including TMC-169 and aspochalasins C, D, E, I, J, and K[205,211]. TMC-169, one of the most potent aspochalasins recorded to date, exhibited antiproliferative activity against U937 human histiocytic lymphoma cells (IC_{50}: 0.81 μM), Jurkat human acute T cell leukemia cells (IC_{50}: 0.21 μM), HL-60 human acute promyelocytic leukemia cells (IC_{50}: 0.68 μM), WiDr human colorectal adenocarcinoma cells (IC_{50}: 0.83 μM), HCT-116 human colorectal carcinoma cells (IC_{50}: 0.78 μM), NCI-H460 human large cell lung carcinoma cells (IC_{50}: 3.5 μM), MCF-7 human breast adenocarcinoma cells (IC_{50}: 5.0 μM), and SF-268 human astrocytoma cells (IC_{50}: 9.5 μM)[205,211]. Demonstrating a proclivity for potential potency among this group of compounds.

Aspochalasins C, D, E, I, J, and K exhibited similar activity against NCI-H460 cells (IC_{50}: in a range of 3.4–55.2 μM), MCF-7 cells (IC_{50}: in a range of 5.3–33.4 μM), SF-268 cells (IC_{50}: in a range of μM)[211], and HeLa human cervical adenocarcinoma cells (IC_{50}: in a range of 5.72–27.4 μM)[173].

Two members of the aspochalasin family, aspergillin PZ (**1**) and trichoderone B (**2**) (**Figure 1**), first isolated from *A. awamori*[212] and *Trichoderma gamsii*[173], respectively, were re-discovered during our pilot exploration of a new approach to natural product discovery from microorganisms isolated from systematically determined geographic sites in the State of Texas, USA. Compounds **1** and **2** have structural similarity to aspochalasins C and D[212] with the notable difference of an internal epoxy group on the macrocycle and are well documented in their biomimetic synthesis[213].

Compound **1** was previously shown to have antimicrobial activity against *Staphylococcus epidermidis*[214], *Klebsiella pneumonia* and *Pseudomonas aeruginosa*[208]. Furthermore, micromolar potency of naturally and synthetically derived **1** was observed when screened against HL-60 cells (IC_{50}: 56.61 µM), A2780 human ovarian carcinoma cells (IC_{50}: >5 µM), PC3 human prostate adenocarcinoma cells (IC_{50}: >25 µM), and LNCaP androgen-sensitive human prostate adenocarcinoma cells (IC_{50}: >50 µM)[11,172].

Compound **2** differs from **1** by a C-7 hydroxy group addition on the isoindole ring[173] and until now had yet to be documented in *A. flavipes*; **2** has been studied much less extensively with no documented cytotoxicity. This report describes the comparative cytotoxic activities of **1** and **2** from a preliminary single-dose (10 µM) assay of two natural products enrolled in the NCI-60 Human Tumor Cell Lines Screen program.



1 **2**

Figure 1. Structures of aspergillin PZ (1) and trichoderone B (2).

2. EXPERIMENTAL

2.1 General Experimental Procedures

For DNA amplification, PCR was performed with Illustra puReTaq Ready-To-Go PCR beads (Cytiva Life Sciences, Marlborough, MA, USA). DNA concentrations and purity were measured with a Take3 2 µL micro-volume plate on a Synergy H1 plate reader with Gen5 software (BioTek,

Winooski, VT, USA). Evolutionary analyses of the ITS1-28S rDNA region were conducted in MEGA X[215]. Prefractionation was performed on an AI-580S flash chromatography system (Yamazen Science, Burlingame, CA, USA) equipped with a 230 nm UV detector (UVD) and tandem evaporative light scattering detector (ELSD). Elution was carried out on an ODS C-18 filled separation column. Semi-preparative and analytical HPLC were performed on an HPLC system equipped with a PrepStar 218 solvent delivery system (Agilent, Santa Clara, CA, USA) and Infinity 1260 diode array detector measuring at 210 nm and 230 nm. HPLC separation occurred on a Zorbax StableBond-Phenyl semi-preparative column (5 µm, 9.4 x 250 mm, Agilent) and analytical HPLC was carried out on a Zorbax StableBond-Aq column (5 µm, 4.6 x 250 mm, Agilent). Purification was guided by cell viability testing against human pancreatic carcinoma MIA PaCa-2 cells (ATCC® CRL-1420™; not presented) measured by the CellTiter-Glo® luciferase luminescence assay (Promega, Madison, WI, USA) 48 hrs after addition of 20 mg/ml semi-purified extract to 2,000 cells/well in triplicate[216] and analyzed on the same Synergy H1 plate reader with Gen5 software. ^{1}H-NMR (300 MHz) on a Fourier 300HD (Bruker, Billerica, MA, USA), Time-of-flight (TOF) ESI-MS was performed on an Agilent 6230 TOF LC/MS. Determination of **2** was aided by comparative MS(n) fragmentation on a LTQ Orbitrap XL Ion Trap Mass Spectrometer (ThermoFisher Scientific, Waltham, MA, USA).

The evolutionary history of isolate 20160726-03F1 was inferred by using the Maximum Likelihood method and Tamura-Nei model[217]. The bootstrap consensus tree (**Appendix C**) was inferred from 1000 replicates[218] and taken to represent the evolutionary history of the taxa analyzed[218]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-

Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter = 0.2454)]. This analysis involved 33 nucleotide sequences taken from the GenBank database.

2.2 Fungal Material

A fungal isolate tentatively identified as *A. flavipes* sp. (lab record no. 20160726-03F1) was isolated from a dry grassy soil sample collected in July 2016 from Concho County in central Texas, USA, and grown on soil extract agar[154].

Fungal species identification was carried out through DNA fingerprinting of the ITS1-28S rDNA region as described[157]. Touchdown PCR was performed in a 200 µL reaction tube containing one Illustra puRe-Taq PCR bead with ITS1-LR3 or LROR-LR6 primer pairs. Size of DNA amplicons was assessed through gel electrophoresis on 1% agarose gel (**Appendix C**) prior to DNA micro-volume quantification.

Sanger sequencing of DNA amplicons was performed with ITS1, LROR, LR3, LR3R, or LR6 primers. An ITS1-28S contig (Figure S3; GenBank accession no. MW522866) was assembled and cleaned using GeneStudio Pro Software and ran through an NCBI-BLASTn search[219].

A. flavipes sp. was tentatively identified based on a 99.82% identity of its ITS1-28S rDNA sequence to that of *A. flavipes* NRRL 302 (**Appendix A**) and an inferred evolutionary history tree (**Appendix C**) was generated.

2.3 Fermentation, Extraction and Purification

A. flavipes sp. was fermented statically in 12 x 1-liter Erlenmeyer flasks each containing 8 g of Cheerios breakfast cereal[131] and 48 mL of sucrose solution (30% w/v), and supplemented with 1 mL of sodium butyrate solution (25 mM) for three weeks at room temperature.

Following fermentation, secondary metabolites were extracted by vigorously agitating the dried fungal mass with ethyl acetate (EtOAc) followed by methanol (MeOH). The solvent extracts were then concentrated separately with a rotary evaporator yielding a total of 8.72 g of crude extract. Crude extracts were resuspended in EtOAc or MeOH and combined into a 50 mL beaker containing 18.0 g of silica gel and allowed to air-dry in a ventilated chemical hood until a fine powder was observed.

In multiple repeated rounds of fractionation, a small injection column (15 x 85 mm) was dry-loaded with approximately 1 g crude extract:silica gel mixture and mounted above a 2L ODS C-18 filled separation column on a flash chromatography system. The column was eluted with a 30-minute MeOH:H_2O gradient from 20% to 100% MeOH followed by 30 minutes of isocratic 100% MeOH elution at a constant flow rate of 10 mL/min. Eluate was monitored by 230 nm UV detection in tandem with ELSD. Each distinct peak on the chromatogram was collected separately and concentrated through centrifugal evaporation followed by lyophilization.

The dried fraction containing **1** and **2** was resuspended in 30% acetonitrile (ACN) and 200 µL per-run was manually injected into an HPLC system and eluted through a Zorbax StableBond-Phenyl semi-preparative column. Elution occurred on an ACN:H_2O gradient from 20% to 80% ACN over 55 minutes. Compounds **1** and **2** were collected separately and further purified by resuspending the dried compound in 30% MeOH and injecting 50 µL per-run into the same HPLC system with

a Zorbax StableBond-Aq analytical column. Elution occurred on a MeOH:H_2O gradient from 30% to 70% MeOH over 55 minutes. Throughout the purification process, the active compounds were tracked by activity-guided purification. For this, 20 mg/mL of each semi-purified extract was measured for cytotoxicity to MIA PaCa-2 pancreatic cancer cells as measured by CellTiter-Glo ATP-luciferase assay. Final yields of **1** and **2** with a greater than 90% purity were 11.8 mg and 11.2 mg, respectively.

Comparison of TOF-ESI-MS (**Appendix C**) and ^{1}H-NMR spectra (**Appendix C**) to historical MS, NMR, and structural data[173,212] identified **1** and **2**, which were then confirmed through stepwise MS(n) fractionation.

2.4 Cytotoxic Activities of Aspergillin PZ and Trichoderone B

Ten mg of each **1** and **2** was further purified through one more round of semi-preparative HPLC as described above. Once each compound was purified to greater than 96% purity, they were subsequently enrolled in the National Cancer Institute's NCI-60 Human Tumor Cell Lines Screen program (https://dtp.cancer.gov/discovery_development/nci-60/). For the screening, 5,000 to 40,000 cells/well depending on doubling time are plated into 96-well microtiter plates and incubated at 37°C and 5% CO_2 for 24 h.

Following compound addition, the cells are incubated further for 48 h in the same conditions prior to growth inhibition assessment. Adherent and non-adherent cells are fixed with TCA (10% and 16%, respectively) incubated for 60 minutes at 4°C then washed and air dried. Sulforhodamine B (100 µl) in 1% acetic acid is added and incubated for 10 minutes at room temperature. Bound stain is solubilized in 10 mM trizma base and absorbance is read at 515 nm[220].

NCI-60 screening is a well-established, reliable, and robust procedure used for testing thousands of samples every year. The initial screening only uses a single dose of 10 μM concentration and is not replicated; therefore, no statistical significance can be generated from the data of the initial screening.

At 10 μM preliminary screening concentration, a greater than 25% reduction in growth was seen in UACC-257 melanoma cells (**2** only), HOP-92 lung adenocarcinoma cells, A498 kidney carcinoma cells, and SNB-75 astrocytoma cells (**Table 1**). The cytotoxic activity of **1** or **2** did not appear to be very potent.

For CCRF-CEM acute lymphoblastic leukemia cells, HL-60 cells, T-47D ductal carcinoma cells and RPMI-8226 plasmacytoma cells, **1** was inactive at 10 μM whereas **2** exerted a >22% reduction of growth (**Table 1**), indicating a certain degree of cytotoxic activity differentiation between **1** and **2.**

The entire panel of NCI-60 human tumor cell lines screening result are presented in Appendix C. Due to their generally weak cytotoxic activities, neither **1** nor **2** advanced to the next step of activity screening at NCI.

Table 1. Percent Cell Growth of Select NCI-60 Cell Lines Following a 10-μM Single Dose of 1 or 2.

Compounds	UACC-257	HOP-92	A498	SNB-75	CCRF-CEM	HL-60	T-47D	RPMI-8226	NCI-60 Mean
1	80.92%	71.33	58.49	48.94	111.65	99.70	98.00	108.59	94.31
2	73.26	65.11	62.06	48.98	84.70	75.89	75.49	86.22	88.33
Δ	7.66	6.22	3.57	0.04	26.95	23.81	22.51	22.37	5.98

*100% denotes no change in cell growth compared to control.
Δ indicates the difference in cell growth percentage between the two tested compounds.

3. CONCLUSIONS

It is not surprising that widespread potent cytotoxic activity was not observed at the 10 μM dosage level as most documented IC_{50} values of **1** in other cell lines have been >25 μM[11,172]. It is however worth noting a >50% reduction of cell growth in SNB-75 cells given the relatively low potency of both compounds in other human tumor cell lines suggesting the potential for selective cell specific activity.

Our NCI-60 results and previous bioactivity comparisons of related aspochalasins[205,211] indicate that there is room for enhancing potency of **1** by assessing structure-activity relationships of the macrocyclic R groups, particularly at C-17, -18, -19, and -20 or C-7 of the isoindole ring (**Figure 1**). While the mechanisms of action for **2** are presently unknown, their presumed precursor, cytochalasan D[213], has been shown to block actin polymerization[158,210,221,222] by binding and capping the elongation site[158,223]. If these aspochalasins work similarly, they could presumably be interrupting the cytokinesis[158], adhesion, motility, or signaling[198] of rapidly dividing cancer cells. Determining the mechanism of action of both molecules would aid in optimizing their activity.

4. ACKNOWLEDGMENTS

We thank Dr. Nigam Mishra and Alshaima'a Qunies (University of North Texas Health Science Center, UNTHSC) for their assistance in procuring and analyzing the NMR data and Dr. Laszlo Prokai (UNTHSC) for assistance in procuring the MS/MS data to aid in finalizing compound identification.

Chapter 6

DISCUSSION AND FUTURE DIRECTIONS

1. DISCUSSION

Perhaps microbial natural products have never been summarized better than Nobel Laureate Satoshi Ōmura's Nobel Lecture in which he referred to natural products as "a splendid gift from the Earth". We have seen historically and through this research project that microorganisms produce a wide array of natural products with innate cytotoxic activities.

While the library has not produced any new metabolites yet, aspergillin PZ and trichoderone B were largely unstudied compounds within the rapidly growing aspochalasin field. Through this library we have expanded knowledge of these two compounds which are not readily available commercially due to their low levels of production in fermentation culture. Through this, we have learned that while these compounds have low levels of cytotoxicity at 10 μM, there was a greater than 50% reduction in cell growth at 10 μM against the glioblastoma cell line SNB-75. Given the aggressiveness and high mortality of this CNS cancer, these are important findings. Further, we have shown through the frequent inactivity at 10 μM, that these compounds have highly selective cell line specific cytotoxicity.

Additionally, C-7-OH appears to be important for cytotoxicity of aspochalasins against leukemia cell lines. No aspergillin PZ anticancer activity was seen in the five leukemia cell lines tested but a reduction in cell growth (albeit never more than 25% at 10 μM) was seen in the trichoderone B inoculated cells. This is an important finding that can assist in the development of anti-leukemic aspochalasin-derivative lead compounds.

This project has also proven that systematic sampling of diverse soils is a viable strategy to create a library of microbial natural products which exhibit cytotoxicity against cancer cell lines. While random sampling is often easier in practice, collecting soil samples in a systematic fashion allows for wider canvassing of soil types such as remote or unremarkable locations which would be largely excluded if a random sampling was undertaken. Because most microorganisms are ungrowable in laboratory conditions or have silent BGCs, casting a wide net through culturing diverse soils allows for increased diversity of the natural product library.

2. LIMITATIONS

A major limitation of this project and perhaps the most important is the simple fact that the library is unable to capture every metabolite produced by a given species. Though efforts were undertaken to elicit expression of silent BGCs, most species will never grow in a laboratory setting absent the use of specialized growth media and conditions. However, if the library is constructed by modifying culture media and conditions, it becomes more specialized, resource consuming, and reduces potential growth of other species.

This is a notably undiscussed part of library construction. To scale up fermentation to allow for detectable or analyzable quantities of secondary metabolite, culture and fermentation media must by necessity be generic and inexpensive if canvasing a wide array of genera. Titer optimization

can be undertaken, and the optimal growth media can be determined if the fermentation is focused on a single species, but this method does not work well in an unspecialized natural product library.

Of the compounds which are produced, this library captures only those which are MeOH or EtOAc soluble. Because of this, compounds which are MeOH insoluble but H_2O soluble as well as those which are non-polar are largely omitted. The latter is not necessarily impactful on the overall success of the library, but H_2O solubility would be a highly desirable trait in a lead compound. Unfortunately, using H_2O for extraction is not preferable due to its high boiling point. Therefore, MeOH remains the best solvent as it has high polarity and low boiling point, making solvent removal a simple process.

The last major limitation of the library design is the use of DMSO as a solvent for the master library. DMSO has countless benefits including being an excellent solvent for a wide array of secondary metabolites, its usefulness as a solvent for long-term storage, and the ability to easily transfer prefraction from the master library to culture plates for library screening. However, once a prefraction is selected for follow-up, removal of DMSO is very difficult to do without causing potential damage to the secondary metabolites given DMSO's high boiling point. Despite these considerations, the new microbial natural product library was constructed in such a way to minimize the impact of these limitations.

Finally, while the sampling and natural product library construction methodologies have been verified through this work, the size of this pilot study (80 fungal isolates) does not allow for interpretation of the value of this library or the concept as a whole. It is supported that diverse soils contain diverse metabolites, but this conclusion cannot currently be applied to this library especially since this pilot study has not produced a previously undiscovered compound. A

significantly larger library compounding a greater screening effort would increase the chances of discovering an undescribed active secondary metabolite and allow for interpretations of the value of our new construction methodology.

3. FUTURE DIRECTIONS

Though this library was constructed in a manner to increase likelihood of identifying new metabolites, whether this library construction method is truly superior to random sampling remains hypothetical. It is supported that soil diversity leads to microbiodiversity and therefore metabolite diversity[121,130] but no comparison has been made to learn whether this library is more diverse than a randomly assembled library. Further understanding of whether systematic versus random soil sampling is more effective at capturing diverse metabolites could aid in future library construction and potentially increase the efficiency of library screening.

The reported bioactivity data from the pilot library study have only been for fungal species. This new natural product library also contains prefractions from Gram-negative and Gram-positive microorganisms. While Gram-negative organisms are typically less successful at producing meaningful metabolites, they are still worthwhile as there have been fewer discoveries of anticancerous compounds from this group of bacteria and therefore a high likelihood of discovering a previously undescribed cytotoxic compounds. Conversely, though Gram-positive organisms such as the streptomycetes are highly screened and have produced countless cytotoxic compounds, the Gram-positive arm of this library is likely still an extremely important source of potentially undiscovered bioactive secondary metabolites.

Of the identified secondary metabolites from the pilot study, aspergillin PZ and trichoderone B still have little information apart from the NCI-60 screening results reported herein. This study has

shown that while these compounds are largely inactive at the 10 μM level, there exists a potential for these compounds to exert selective cytotoxicity. Follow-up of these two compounds using the characteristics of an ideal aspochalasin compiled and presented in **Chapter 4** as well as the cytotoxicity data presented in **Chapter 5** would be a good foundation by which to optimize these two related compounds to enhance their selectivity, determine their mechanisms of action, or increase potency.

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APPENDICES

Appendix A: Contigs from 18S-ITS-28S Genetic Sequencing

Appendix B: Crystallography Data from X-Ray Crystallography of Palmitic Acid

Appendix C: Aspergillin PZ and Trichoderone B Experimental Data

Comparison of 18S rRNA gene contig of 20160113-02F1 to *Aspergillus niger* contig An03c0110

Score	Expect	Identities	Gaps	Strand
2056 bits(1113)	0.0	1131/1139(99%)	6/1139(0%)	Plus/Minus

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Query  2     GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAATAGCACTTTAT  61
             |||||||||||||||||||||||||||||||||||||||||||||||||||  |||||||||
Sbjct  3046  GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAA--GCACTTTAT  2989

Query  62    ACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTA  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2988  ACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTA  2929

Query  122   CATGGATACCTGTGGTAATTCTAGAGCTAATACATGCTGAAAACCTCGACTTCGGAAGGG  181
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2928  CATGGATACCTGTGGTAATTCTAGAGCTAATACATGCTGAAAACCTCGACTTCGGAAGGG  2869

Query  182   GTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTTGGTGAATCATAATAACT  241
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2868  GTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTTGGTGAATCATAATAACT  2809

Query  242   TAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAACTT  301
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2808  TAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAACTT  2749

Query  302   TCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGGTTCGA  361
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  2748  TCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGG-TTCGA  2690

Query  362   TTCCGGAGAAGGGAGCCTGAGAAAACGGCTACCACATCCAAAGGAAGGCAGCAGGCGCGC  421
             |||||||| ||||||||||||| |||||||||||||| |||||||||||||||||||||
Sbjct  2689  TTCCGGAGA-GGGAGCCTGAGAAA-CGGCTACCACATCCAA-GGAAGGCAGCAGGCGCGC  2633

Query  422   AAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACGGGGCTCTTTT  481
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2632  AAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACGGGGCTCTTTT  2573

Query  482   GGGTCTCGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGAACAATTGGAGGGCA  541
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2572  GGGTCTCGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGAACAATTGGAGGGCA  2513

Query  542   AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCA  601
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2512  AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCA  2453

Query  602   GTTAAAAAGCTCGTAGTTGAACCTTGGGTCTGGCTGGCCGGTCCGCCTCACCGCGAGTAC  661
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2452  GTTAAAAAGCTCGTAGTTGAACCTTGGGTCTGGCTGGCCGGTCCGCCTCACCGCGAGTAC  2393

Query  662   TGGTCCGGCTGGACCTTTCCTTCTGGGGAATCTCATGGCCTTCACTGGCTGTGGGGGGAA  721
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2392  TGGTCCGGCTGGACCTTTCCTTCTGGGGAATCTCATGGCCTTCACTGGCTGTGGGGGGAA  2333

Query  722   CCAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATT  781
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2332  CCAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATT  2273

Query  782   AGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTA  841
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2272  AGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTA  2213

Query  842   ATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGA  901
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2212  ATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGA  2153

Query  902   TTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAA  961
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2152  TTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAA  2093

Query  962   CGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCC  1021
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2092  CGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCC  2033

Query  1022  GACTAGGGATCGGACGGTGTTTCTATTATGACCCGTTCGGCACCTTACGAGAAATCAAAG  1081
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2032  GACTAGGGATCGGACGGTGTTTCTATTATGACCCGTTCGGCACCTTACGAGAAATCAAAG  1973

Query  1082  TTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGNTGAAACTTAAAGGAATTGACGGAAG  1140
             |||||||||||||||||||||||||||||||| |||||||||||| ||||||||||||
Sbjct  1972  TTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAG  1914
```

Generated from BLASTn search and comparison of assembled 18S contig of 20160113-02F1 to partial sequence of *Aspergillus niger* contig An03c0110 (Seq. ID: AM260052.1)

Comparison of 28S rRNA gene contig of 20160113-02F1 to *Aspergillus niger* contig An03c0110

Generated from BLASTn search and comparison of assembled 28S contig of 20160113-02F1 to partial sequence of *Aspergillus niger* contig An03c0110 (Seq. ID: AM270052.1)

Comparison of 18S rRNA gene contig of 20160113-01F1 to *Cladosporium* sp. contig Pt-2

Score	Expect	Identities	Gaps	Strand
1849 bits(1001)	0.0	1008/1015(99%)	0/1015(0%)	Plus/Minus

```
Query  1     TCNGANCCCTAGTCGGCATAGTTTATGNTTANGACTACGACGGTATCTGATCGTCTTCGA  60
             ||  || |||||||||||||||||||||  ||| ||||||||||||||||||||||||||||
Sbjct  1028  TCCGATCCCTAGTCGGCATAGTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGA  969

Query  61    TCCCCTAACTTTCGTTCACTGATTAATGAAAACATCCTNGGCAAATGCTTTCGCAGTAGT  120
             ||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  968   TCCCCTAACTTTCGTTCACTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGT  909

Query  121   TAGTNTTCAATCAANCCAAGAATTTCACCTCTGACGATTGAATACTGATGCCCCCGACTA  180
             ||||  |||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  908   TAGTCTTCAATCAATCCAAGAATTTCACCTCTGACGATTGAATACTGATGCCCCCGACTA  849

Query  181   TCCCTATTAATCATTACGGCGGTCCTAGAAACCAACAAAATAGAACCACACGTCCTATTC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  848   TCCCTATTAATCATTACGGCGGTCCTAGAAACCAACAAAATAGAACCACACGTCCTATTC  789

Query  241   TATTATTCCATGCTAATGTATTCGAGCAAAGGCCTGCTTTGAACACTCTAATTTTTTCAA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  788   TATTATTCCATGCTAATGTATTCGAGCAAAGGCCTGCTTTGAACACTCTAATTTTTTCAA  729

Query  301   AGTAAAAGTCCTGGTTCCCCAACACGCCCAGTGAAGGGCATGAGGTTCCCCAGAAGGAAA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  728   AGTAAAAGTCCTGGTTCCCCAACACGCCCAGTGAAGGGCATGAGGTTCCCCAGAAGGAAA  669

Query  361   GGCCCGGCCGGACCAGTACACGCGGTGAGGCGGACCGGCCAGCCAGGCCCAAGGTTCAAC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  668   GGCCCGGCCGGACCAGTACACGCGGTGAGGCGGACCGGCCAGCCAGGCCCAAGGTTCAAC  609

Query  421   TACGAGCTTTTTAACTGCAACAACTTTAATATACGCTATTGGAGCTGGAATTACCGCGGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  608   TACGAGCTTTTTAACTGCAACAACTTTAATATACGCTATTGGAGCTGGAATTACCGCGGC  549

Query  481   TGCTGGCACCAGACTTGCCCTCCAATTGTTCCTCGTTAAGGGATTTAAATTGTACTCATT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  548   TGCTGGCACCAGACTTGCCCTCCAATTGTTCCTCGTTAAGGGATTTAAATTGTACTCATT  489

Query  541   CCAATTACAAGACCCAAAAGAGCCCTGTATCAGTATTTATTGTCACTACCTCCCCGTGTC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  488   CCAATTACAAGACCCAAAAGAGCCCTGTATCAGTATTTATTGTCACTACCTCCCCGTGTC  429

Query  601   GGGATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428   GGGATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCT  369

Query  661   CCCTCTCCGGAGTCGAACCCTAATTCCCCGTTACCCGTTGATACCATGGTAGGCCACTAT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368   CCCTCTCCGGAGTCGAACCCTAATTCCCCGTTACCCGTTGATACCATGGTAGGCCACTAT  309

Query  721   CCTACCATCGAAAGTTGATAGGGCAGAAATTTGAATGAACCATCGCCGGCGCAAGGCCAT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308   CCTACCATCGAAAGTTGATAGGGCAGAAATTTGAATGAACCATCGCCGGCGCAAGGCCAT  249

Query  781   GCGATTCGTTAAGTTATTATGATTCACCAAGGAGCCCCGAAGGGCATTGGTTTTTTATCT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248   GCGATTCGTTAAGTTATTATGATTCACCAAGGAGCCCCGAAGGGCATTGGTTTTTTATCT  189

Query  841   AATAAATACACCCCTTCCGAAGTCGAGGTTTTTAGCATGTATTAGCTCTAGAATTACCAC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  188   AATAAATACACCCCTTCCGAAGTCGAGGTTTTTAGCATGTATTAGCTCTAGAATTACCAC  129

Query  901   GGTTATCCATGTAGTAAGGTACTATCAAATAAACGATAACTGATTTAATGAGCCATTCGC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  128   GGTTATCCATGTAGTAAGGTACTATCAAATAAACGATAACTGATTTAATGAGCCATTCGC  69

Query  961   AGTTTCACCGTATAGTTGCTTATACTTAGACATGCATGGCTTAATCTTTGAGACA  1015
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  68    AGTTTCACCGTATAGTTGCTTATACTTAGACATGCATGGCTTAATCTTTGAGACA  14
```

Generated from BLASTn search and comparison of assembled 18S contig to partial sequence of *Cladosporium* sp. contig Pt-2 (Seq. ID: JQ824844.1)

Comparison of 28S rRNA gene contig of 20160113-01F1 to *Cladosporium cladosporioides*

Score	Expect	Identities	Gaps	Strand
1912 bits(1035)	0.0	1072/1088(99%)	14/1088(1%)	Plus/Plus

```
Query  1     TAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGNCTTCGGGCGGGGGCTC  60
             ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  155   TAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTC  214

Query  61    CGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAAT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  215   CGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAAT  274

Query  121   TAAAACTTTTAACAACGGATCTCTTGGTTTCTGGCATCGATGAAGAACGCAGCGAAATGC  180
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  275   TAAAACTTTTAACAACGGATCTCTTGG-TTCTGGCATCGATGAAGAACGCAGCGAAATGC  333

Query  181   GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  334   GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC  393

Query  241   CCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  394   CCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTT  453

Query  301   GGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  454   GGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCC  513

Query  361   TAAGCGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGGCTACGCCGTAAAACAAACC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  514   TAAGCGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGGCTACGCCGTAAAACAAACC  573

Query  421   CATTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  574   CATTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA  633

Query  481   AGCGGAGGAAAAGAAACCAACAGGGATTGCTCTAGTAACGGCGAGTGAAGCAGCAATAGC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  634   AGCGGAGGAAAAGAAACCAACAGGGATTGCTCTAGTAACGGCGAGTGAAGCAGCAATAGC  693

Query  541   TCAAATTTGAAATCTGGCGTCTTCGACGTCCGAGTTGTAATTTGTAGAGGATGCTTCTGA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  694   TCAAATTTGAAATCTGGCGTCTTCGACGTCCGAGTTGTAATTTGTAGAGGATGCTTCTGA  753

Query  601   GTAACCACCGACCTAAGTTCCTTGGAAACAGGACGTCATAGAGGGTGAGAATCCCGTATG  660
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  754   GTAACCACCGACCTAAGTTCCTTGG-AACAGGACGTCATAGAGGGTGAGAATCCCGTATG  812

Query  661   CGGTCGGAAAGGTGCTCTATACGTAGCTCCCTTCGACGAGTCGAGTTGTTTGGGAATGCA  720
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  813   CGGTCGGAAAGGTGCTCTATACGTAGCT-CCTTCGACGAGTCGAGTTGTTTGGGAATGCA  871

Query  721   GCTCTAAATGGGAGGGTAAAATTTCTTCTAAAAGCTAAATATTTGGCCAGAGACCCGATA  780
             |||||||||||||| |||| |||||||||||| |||||||||| ||||||||| ||||||
Sbjct  872   GCTCTAAATGGGAGG-TAAA-TTTCTTCTAAA-GCTAAATATT-GGCCAGAGA-CCGATA  926

Query  781   GCGCACAAAGTAGAAGTGATCGAAAGATGAAAAAGCACTTTKGRAAAGAGAGTTAAAAAG  840
             |||||||| |||| |||||||||||||||||| ||||||| | ||||||||||||||||
Sbjct  927   GCGCACAA-GTAG-AGTGATCGAAAGATGAAAA-GCACTTTGG-AAAGAGAGTTAAAAAG  982

Query  841   CACGTGAAATTGTTAAAAGGGAAGGGATTGCAACCAGGACTTGCTCGCGGGTGTTCCGCC  900
             |||||||||||||||||||||||||||||||||||| |||||||||| ||||||||||||
Sbjct  983   CACGTGAAATTGTTAAAAGGGAAGGGATTGCAACCA-GACTTGCTCGC-GGTGTTCCGCC  1040

Query  901   GGTCTTCTGACCGGTCTACTCGCCGCGTTGCAGGCCAGCATCGTCTGGTGCCGCTGGATA  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041  GGTCTTCTGACCGGTCTACTCGCCGCGTTGCAGGCCAGCATCGTCTGGTGCCGCTGGATA  1100

Query  961   AGACTTGAGGAATGTAGCTCCCTCGGGAGTGTTATAGCCTCTTGTGATGCAGCGAGCGCC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1101  AGACTTGAGGAATGTAGCTCCCTCGGGAGTGTTATAGCCTCTTGTGATGCAGCGAGCGCC  1160

Query  1021  GGGCGAGGTCCGCGCTTCGGCTAGGATGCTGGCGTAATGGTCGTAATCCGCCCGTCTTGA  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1161  GGGCGAGGTCCGCGCTTCGGCTAGGATGCTGGCGTAATGGTCGTAATCCGCCCGTCTTGA  1220

Query  1081  AACACGGA  1088
             ||||||||
Sbjct  1221  AACACGGA  1228
```

Generated from BLASTn search and comparison of assembled 28S contig to partial sequence of *Cladosporium cladosporioides* contig (Seq. ID: KJ596320.1)

Comparison of 18S rRNA gene contig of 20160726-03F1 to *A. flavipes* NRRL 302

Score	Expect	Identities	Gaps	Strand
2058 bits(1114)	0.0	1118/1120(99%)	0/1120(0%)	Plus/Plus

```
Query  1     TGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCACTTTATACCGTGAAACTGCGA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14    TGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCACTTTATACCGTGAAACTGCGA  73

Query  61    ATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACATGGATACCTGTGG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74    ATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACATGGATACCTGTGG  133

Query  121   TAATTCTAGAGCTAATACATGCTAAAAACCCCGACTTCGGAAGGGGTGTATTTATTAGAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134   TAATTCTAGAGCTAATACATGCTAAAAACCCCGACTTCGGAAGGGGTGTATTTATTAGAT  193

Query  181   AAAAAACCAATGCCCTTCGGGGCTCCTTGGTGATTCATAATAACTTAACGAATCGCATGG  240
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194   AAAAAACCAATGCCCCTCGGGGCTCCTTGGTGATTCATAATAACTTAACGAATCGCATGG  253

Query  241   CCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254   CCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAG  313

Query  301   TGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  314   TGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCC  373

Query  361   TGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  374   TGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACA  433

Query  421   CGGGGAGGTAGTGACAATAAATACTGATACGGGGCTCTTTTGGGTCTCGTAATTGGAATG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434   CGGGGAGGTAGTGACAATAAATACTGATACGGGGCTCTTTTGGGTCTCGTAATTGGAATG  493

Query  481   AGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  494   AGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCG  553

Query  541   CGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  554   CGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTG  613

Query  601   AACCTTGGGTCTGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  614   AACCTTGGGTCTGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTC  673

Query  661   CTTCTGGGGAATCCCATGGCCTTCACTGGCTGTGGGGGGAACCAGGACTTTTACTGTGAA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  674   CTTCTGGGGAATCCCATGGCCTTCACTGGCTGTGGGGGGAACCAGGACTTTTACTGTGAA  733

Query  721   AAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATTAGCATGGAATAATAGAATA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734   AAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATTAGCATGGAATAATAGAATA  793

Query  781   GGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  794   GGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTC  853

Query  841   GGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTAC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  854   GGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTAC  913

Query  901   TGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAA  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  914   TGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAA  973

Query  961   GACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGGCGGTG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  974   GACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGGCGGTG  1033

Query  1021  TTTTTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGA  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1034  TTTTTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGA  1093

Query  1081  GTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAG  1120
             |||||||||||||||||||||||||| ||||||||||||
Sbjct  1094  GTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAG  1133
```

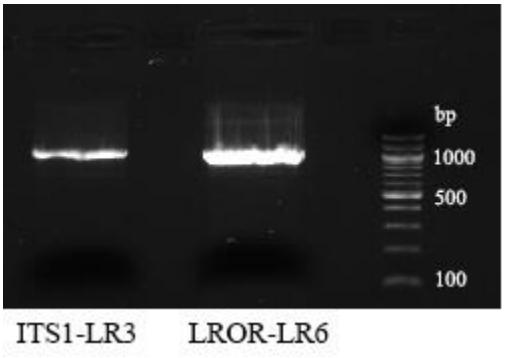
NCBI-BLASTn[219] comparison showed a 99.82% sequence identity of the ITS1-28S rDNA region of a fungal isolate (lab record: 20160726-03F1) to that of *A. flavipes* NRRL 302

Table A1. 20160113-01F1-R8-6
Fractional Atomic Coordinates

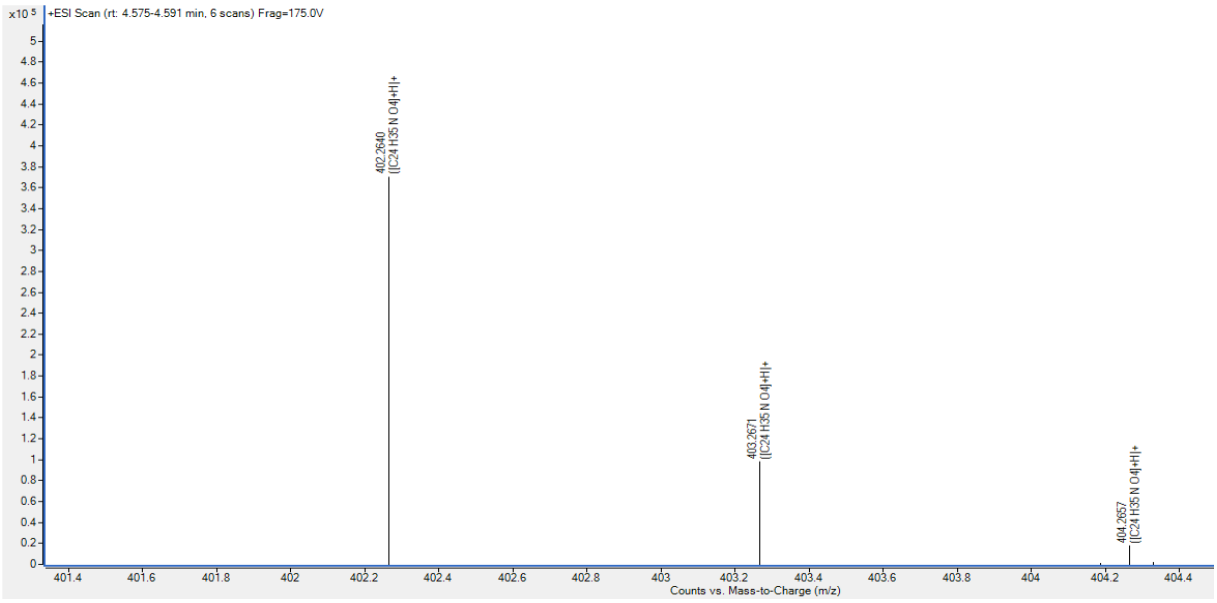
Atom	x	y	z
O1	4578.1(7)	3820(5)	705(3)
O2	4944.4(7)	7331(5)	1308(3)
C1	4657.8(9)	5802(6)	1494(3)
C2	4418.0(10)	6577(7)	2727(4)
C3	4062.4(10)	4937(7)	2905(4)
C4	3852.1(10)	5864(7)	4203(4)
C5	3474.4(10)	4492(7)	4427(4)
C6	3276.5(10)	5566(7)	5740(4)
C7	2891.2(10)	4352(7)	5971(4)
C8	2698.8(10)	5538(7)	7279(4)
C9	2311.0(10)	4352(6)	7531(4)
C10	2118.7(10)	5572(6)	8833(4)
C11	1731.7(10)	4381(7)	9094(4)
C12	1539.6(10)	5602(7)	10403(4)
C13	1151.9(10)	4415(7)	10643(4)
C14	958.5(11)	5589(8)	11955(4)
C15	570.2(11)	4418(9)	12186(5)
C16	381.2(13)	5536(10)	13524(5)

Table A2. 20160113-01F1-R8-6
Hydrogen Fractional Atomic Coordinates

Atom	x	y	z
H2	5094.47	6554.12	760.4
H2A	4344.75	8494.74	2600.24
H2B	4574.29	6444.87	3641.49
H3A	3896.68	5113.69	2016.79
H3B	4129.81	3007.23	3024.47
H4A	3807.88	7831.67	4111.32
H4B	4016.03	5572.37	5087.41
H5A	3308.47	4760.25	3546.32
H5B	3516.15	2526.92	4549.46
H6A	3439.79	5226.55	6620.83
H6B	3248.13	7546.04	5635.24
H7A	2918.75	2377.71	6108.47
H7B	2727.89	4653.99	5086.57
H8A	2863.02	5235.39	8160.98
H8B	2672.85	7512.93	7140.34
H9A	2337.01	2379.39	7681.27
H9B	2147.21	4637.16	6645.72
H10A	2283.41	5296.44	9716.58
H10B	2091.62	7542.93	8679.45
H11A	1758.65	2409.79	9246.02
H11B	1566.76	4660.95	8212.08
H12A	1702.73	5304.46	11288.13
H12B	1514.27	7575.51	10256.78
H13A	1177.37	2437.36	10773.77
H13B	988.5	4730.2	9759.53
H14A	1120.8	5260.14	12839.86
H14B	934.78	7568.33	11828.24
H15A	592.85	2432.56	12285.17
H15B	405.77	4792.75	11313.02
H16A	342.4	7484.95	13408.32
H16B	543.28	5194.85	14394.3
H16C	136.84	4644.46	13628.17

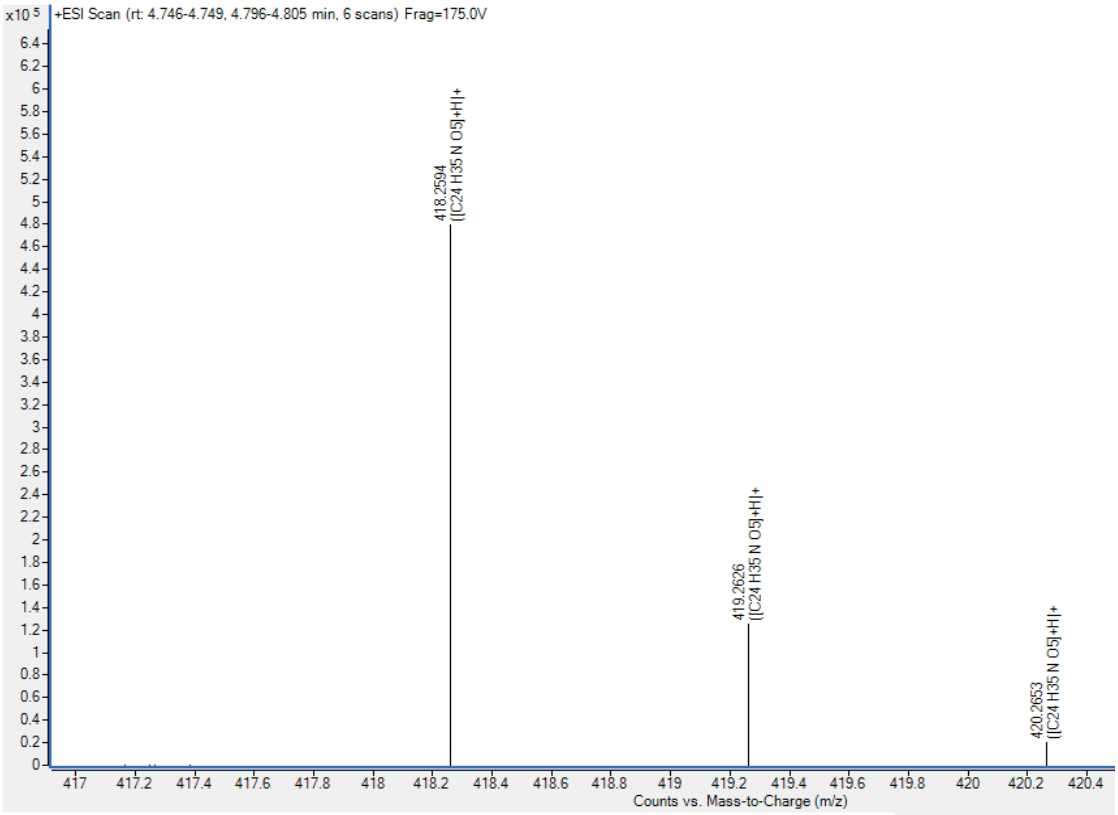


EtBr-stained gel electrophoresis image of two DNA amplicons generated by touchdown PCR with primer pairs ITS1-LR3 and LROR-LR6, respectively.



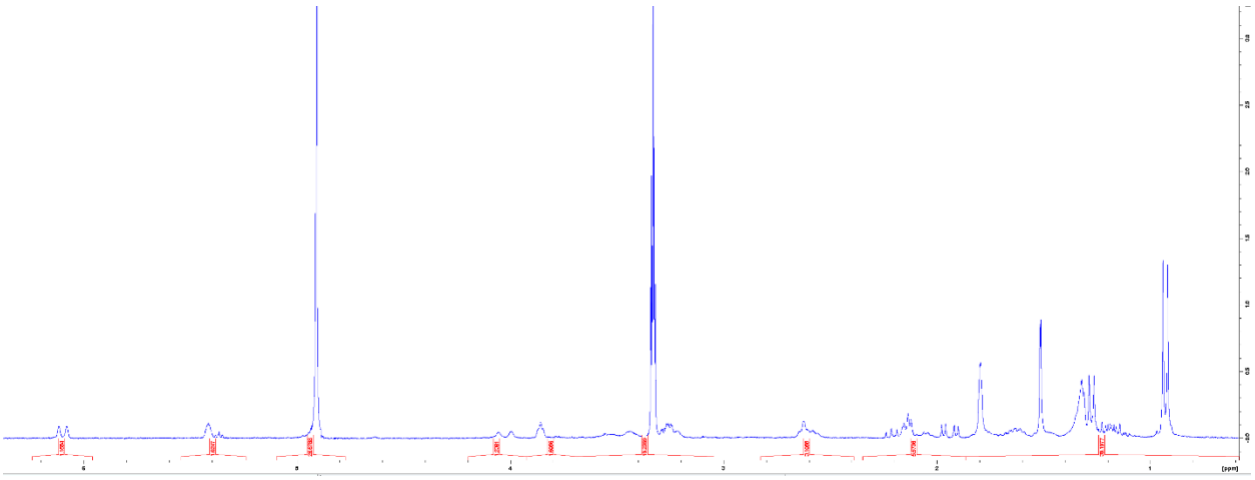
Species	m/z (Calc)	m/z (Observed)	Isotope Distribution (Calc)	Isotope Distribution (Observed)
((C24 H35 N O4) + H) +	402.264	402.264	76.2%	76.1%
	403.267	403.267	20.5%	20.2%
	404.27	404.266	3.3%	3.6%

TOF-ESI-MS and HRMS comparison table of observed to calculated isotope abundances and masses of a purified secondary metabolite from *Aspergillus flavipes* sp. collected from the Concho County in central Texas, USA determined to be aspergillin PZ (**1**) [*m/z* 402.2643 $(M + H)^+$; calculated M.W. 402.264 for $C_{24}H_{35}NO_4 + H^+$].

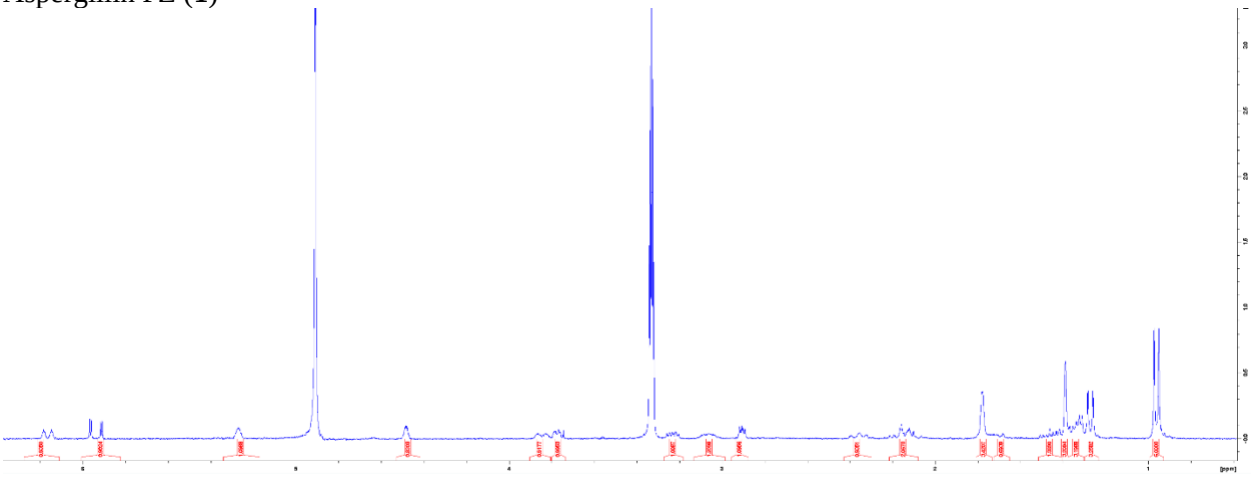


Species	m/z (Calc)	m/z (Observed)	Isotope Distribution (Calc)	Isotope Distribution (Observed)
((C24 H35 N O5) + H)+	418.259	418.259	75.8%	76.3%
	419.262	419.263	20.4%	20.0%
	420.265	420.265	3.4%	3.2%
	421.268	421.267	0.4%	0.5%

TOF-ESI-MS and HRMS comparison table of observed to calculated isotope abundances and masses of a purified secondary metabolite from *Aspergillus flavipes* sp. collected from the Concho County in central Texas, USA determined to be trichoderone B (**2**) [*m/z* 418.2594 $(M+H)^+$; calculated M.W. 418.259 for $C_{24}H_{35}NO_5 + H^+$].



Aspergillin PZ (**1**)

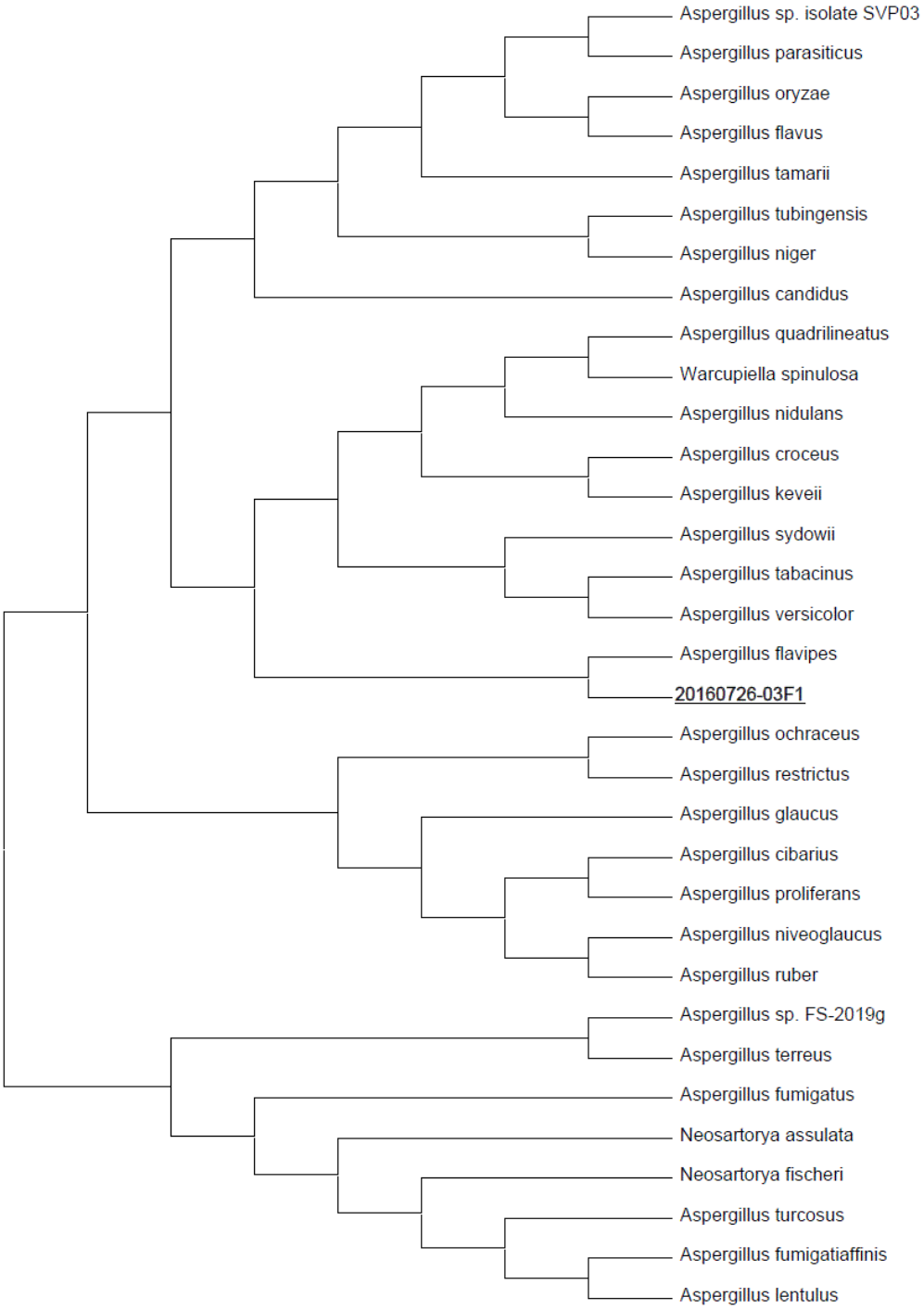


Trichoderone B (**2**)

Aspergillin PZ (**1**): White amorphous powder. ^{1}H NMR (MeOD, 300 MHz) δ 6.10 (d, J = 10.76 Hz, 1H), 5.41 (m, 1H), 5.36 (t, J = 4.70 Hz, 1H), 4.02 (d, J = 18.09 Hz, 1H), 3.86 (m, 1H), 3.30 – 3.18 (m, 1H), 2.67 – 2.53 (m, 2H), 2.24 -2.18 (m, 1H), 2.18 – 2.10 (m, 1H), 1.94 (dd, J = 17.73, 5.49 Hz, 1H), 1.79 (s, 4H), 1.63 (m, 2H), 1.51 (d, J = 1.32, 3H), 1.32 (m, 4H), 1.27 (d, J = 6.92 Hz, 3H), 1.19 (m, 2H), 0.93 (d, J = 6.57 Hz, 6H).

Trichoderone B (**2**): White amorphous powder. ^{1}H NMR (MeOD, 300 MHz) δ 7.27 (dd, J = 15.30, 2.40 Hz, 1H), 6.16 (m, 1H), 5.96 (d, J = 2.39 Hz, 1H), 5.91 (d, J = 2.39 Hz, 1H), 5.27 (m, 1H), 4.28 (m, 1H), 3.85 (m, 1H), 3.80 – 3.75 (m, 1H), 3.23 (m, 1H), 3.06 (m, 1H), 2.93 – 2.88 (m, 1H), 2.36 (m, 1H), 2.23 – 2.08 (m, 2H), 1.78 (m, 4H), 1.50 – 1.42 (m, 2H), 1.39 (s, 3H), 1.37-1.31 (m, 3H), 1.27 (d, J = 7.28 Hz, 3H), 0.96 (d, J = 6.57 Hz, 6H).

^{1}H-NMR spectra for **1** and **2** obtained in MeOD at 300MHz.

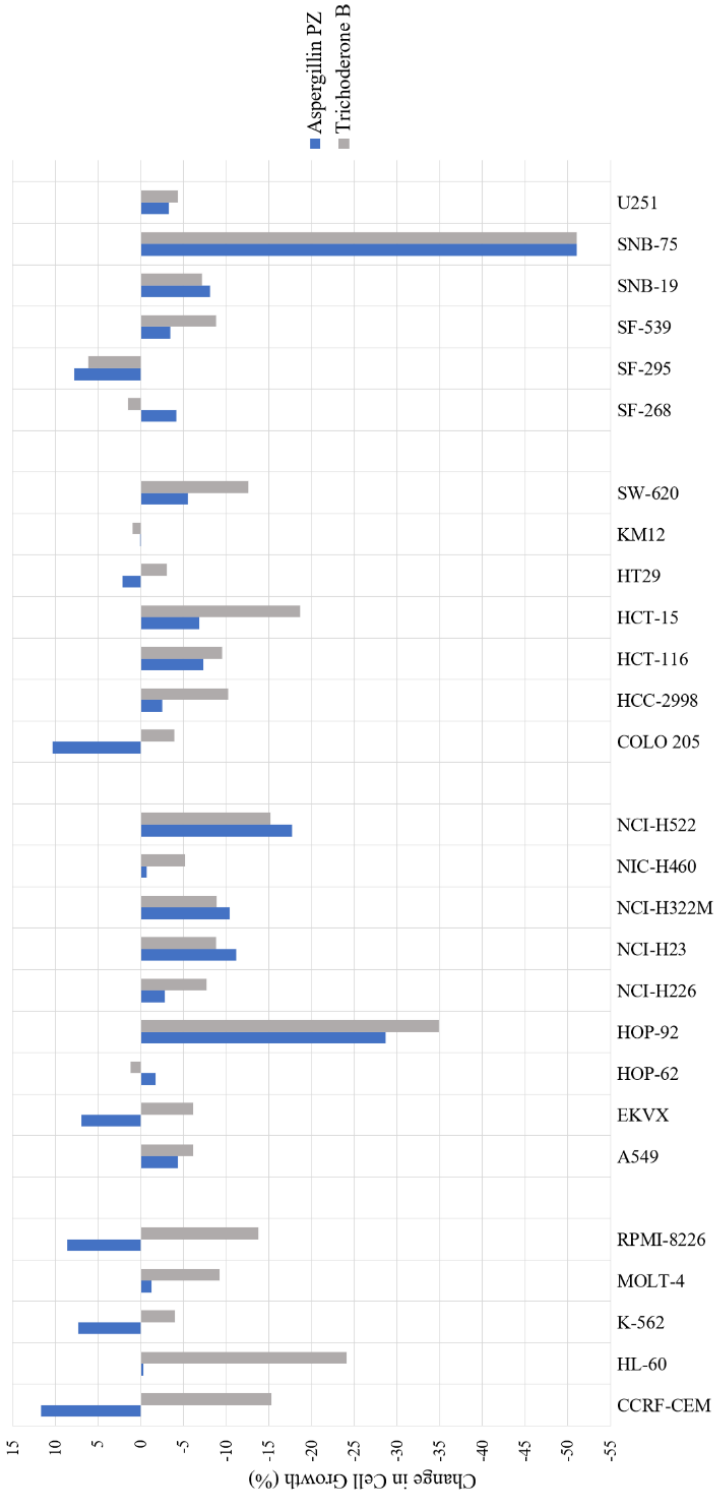


Inferred evolutionary history tree for the ITS-28S rDNA region of a fungal isolate (lab record no. 20160726-03F1) from the Concho County in central Texas, USA, which produced aspergillin PZ (**1**) and trichoderone B (**2**) (MEGA X) [215,217,218].

NCI-60 µM Single Dose Screening (% Cell Growth)

				Breast		
	MCF7	MDA-MB-231	HS 578T	BT-549	**T-47D**	MDA-MB-468
Compound 1	94.27	103.65	88.52	84.83	98	90.75
Compound 2	76.79	99.3	84.85	77.17	75.49	72.84
Δ	17.48	4.35	3.67	7.66	22.51	17.91
				CNS		
	SF-268	SF-295	SF-539	SNB-19	**SNB-75**	U251
Compound 1	95.85	107.8	96.51	91.89	48.94	96.72
Compound 2	101.48	106.12	91.19	92.81	48.98	95.67
Δ	-5.63	1.68	5.32	-0.92	-0.04	1.05
				Colon		
	COLO 205	HCC-2998	HCT-116	HCT-15	HT29	KM12
Compound 1	110.33	97.48	92.72	93.19	102.12	100.09
Compound 2	96.11	89.75	90.46	81.3	96.93	100.93
Δ	14.22	7.73	2.26	11.89	5.19	-0.84
	Colon			Leukemia		
	SW-620	**CCRF-CEM**	**HL-60(TB)**	K-562	MOLT-4	**RPMI-8226**
Compound 1	94.47	111.65	99.69	107.34	98.77	108.59
Compound 2	87.37	84.7	75.89	95.96	90.81	86.22
Δ	7.1	26.95	23.8	11.38	7.96	22.37
				Non-Small Cell Lung Cancer		
	HOP-92	NCI-H226	NCI-H23	NCI-H322M	NCI-H460	NCI-H522
Compound 1	71.33	97.15	88.82	89.63	99.3	82.32
Compound 2	65.11	92.26	91.16	91.08	94.79	84.8
Δ	6.22	4.89	-2.34	-1.45	4.51	-2.48
	Non-Small Cell Lung Cancer				Melanoma	
	A549/ATCC	EKVX	HOP-62	SK-MEL-5	**UACC-257**	UACC-62
Compound 1	95.67	106.91	98.26	99.1	80.92	96.42
Compound 2	93.84	93.86	101.19	96.01	73.26	89.75
Δ	1.83	13.05	-2.93	3.09	7.66	6.67
				Melanoma		
	LOX IMVI	MALME-3M	M14	MDA-MB-435	SK-MEL-2	SK-MEL-28
Compound 1	95.15	94.06	91.12	98.39	94.25	91.68
Compound 2	96.83	92.44	88.4	97.65	94.22	93.45
Δ	-1.68	1.62	2.72	0.74	0.03	-1.77
				Renal		
	786-0	**A498**	ACHN	CAKI-1	RXF 393	SN12C
Compound 1	99.54	58.49	100.46	89.53	90.87	95.43
Compound 2	96.06	62.06	82.08	85.52	77.84	91.28
Δ	3.48	-3.57	18.38	4.01	13.03	4.15
	Renal			Ovarian		
	TK-10	UO-31	IGROV1	OVCAR-3	OVCAR-4	OVCAR-5
Compound 1	95.27	76.32	101.3	96.91	93.67	93.58
Compound 2	85.45	77.19	87.76	91.69	85.4	95.35
Δ	9.82	-0.87	13.54	5.22	8.27	-1.77
		Ovarian			Prostate	
	OVCAR-8	NCI/ADR-RES	SK-OV-3	PC-3	DU-145	Mean
Compound 1	90.29	98.66	102.01	94.13	103.31	94.31
Compound 2	87.1	97.7	105.28	80.88	93.96	88.33
Δ	3.19	0.96	-3.27	13.25	9.35	5.98

Percentages of cell growth of 60 cell lines following exposure to a single dosing (10 µM) of aspergillin PZ (**1**) or trichoderone B (**2**). Δ indicates the difference in cell growth percentage between the two tested compounds.



Graph of growth percentages from NCI60 human cell lines screening following single dosing (10 µM) of aspergillin PZ (**1**) or trichoderone B (**2**).