OYEKUNLE, OLALEKAN SAHEED, M.S. Growth Media Studies Approach to Enhance Production of the Bioactive Fungal Metabolite Viridicatumtoxin A. (2018) Directed by Dr. Nicholas H. Oberlies. 60pp.

Viridicatumtoxin A is a unique tetracycline-like aromatic polyketide first isolated as a mycotoxin from *Penicillium viridicatum* in 1973. Along with its 5-oxo derivative, viridicatumtoxin B, discovered in 2008 from the liquid fermentation cultures of another *Penicillium sp*. (strain FR11) has exhibited cytotoxicity against the NCI-H460 human lung carcinoma cell line, KB3-1 human cervix carcinoma cell line, and SW620 human colon carcinoma cell line with reported IC_{50} values of 1.0 μM, 2.5 μM and 1.0 μM respectively. Based on the scarcity and the more bioactive properties of viridicatumtoxin B, Nicolaou and coworkers in 2013 achieved the total synthesis of viridicatumtoxin B; albeit through a lengthy protocol which would not be scalable. In this context, and as part of our ongoing research centered on the optimization of drug leads for the treatment of cancer, my project is focused on the enhanced production of viridicatumtoxin A from fungal cultures which would provide materials for synthesis of more bioactive analogues.

GROWTH MEDIA STUDIES APPROACH TO ENHANCE PRODUCTION OF THE BIOACTIVE FUNGAL METABOLITE VIRIDICATUMTOXIN A

by

Olalekan Saheed Oyekunle

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2018

Approved by

_____
Committee Chair

APPROVAL PAGE

This thesis written by OLALEKAN SAHEED OYEKUNLE has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____
Nicholas H. Oberlies

Committee Members _____
Nadja B. Cech

_____
Mitchell P. Croatt

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGEMENTS

My gratitude to Dr. Nicholas Oberlies for his all-round mentorship and the opportunity to work in his laboratory. My appreciation to my committee members, Dr. Nadja Cech and Dr. Mitchell Croatt, for their valuable inputs.

I like to thank Dr. José Rivera-Chavez for his positive contributions towards the success of this project. I also appreciate Mr. Tyler Graf, Dr. Huzefa Raja, Dr. Mohammed Al-Huniti, and Dr. Laura Bocanegra for lending their effective assistance whenever needed. My thanks to every member of the Oberlies research group for making my experience in the group very pleasant.

I am very grateful to my wife, Yolanda, and children: Phebe, and David for their generous sacrifice. I'm also thankful indeed to my parents for their consistent support. Most importantly, all the glory to God for His unspeakable faithfulness through Jesus Christ.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1.1 Overview of Cancer

Cancer is a group of diseases in any part of the body that results from unregulated division and growth of randomly mutated cells possessing metastatic capabilities. Unlike normal cells, cancer cells are not subject to cell regulatory mechanisms like confinement to a specific space within a tissue as well as apoptosis. Cancer diagnosis is often associated with panic, apprehension, and despondence.

Historical records date the existence of different kinds of cancer as early as the discovery of bone tumors on autopsies of primitive Egyptian mummies.[1] Also, the earliest documentation of cancer (not called cancer at the time) was found on the Edwin Smith Papyrus, where the treatment of eight characteristic cancer cases were described.[2] It was reported that ancient Chinese and Arabic medical writings described conditions that are closely related to cancer in signs and symptoms.[1]

In the 4th century, the "Father of Medicine", Hippocrates coined carcinos (non-ulcer forming) and carcinoma (ulcer-forming) derived from the Greek word Karkinos (meaning crab), to describe the finger-like growth pattern and the tenacity with which cancer intrudes on surrounding cells.[2] Cancer, which is the Latin word for crab, was first used by Celsus, an early Roman Physician.[2]

Classification of cancer is along the line of their cell of origin. Carcinomas constituting 80 % of all human cancer are of epithelial origin, while cancer of the connective or supportive tissues like bones, muscles, cartilage, tendons, and fibrous tissues are known as sarcomas.[3] The buildup of immature and abnormal blood cells in the body due to cancer originating in hematopoietic stem cells is called leukemia. Cancers of the lymphatics and immune cells are called lymphomas and myeloma respectively.

Interest in cancer reportedly gained momentum in the late 18th century to the early 19th century after the scourge of infectious diseases was curtailed.[1] This has resulted in breakthroughs in early detection and quality of treatment at patients' disposal, but in spite of this, cancer is still a leading cause of death and continues to persist as a prime public health concern all across the globe.[4] For example, the number of deaths due to cancer in first and developing countries was estimated to be about 7.4 million in 2004 and about 8.2 million deaths were registered in 2012.[5] These deaths are estimated to hit 11.5 million by 2030.[5-6] In the United States alone, where cancer is the second leading cause of death (except for American Indians or Alaska Natives and Asians or Pacific Islanders) after heart related diseases, it has been projected that over 1.7 million new cancer cases and over 600, 000 cancer deaths will be recorded in 2018.[7] Thus, it is imperative to find new therapeutics to combat this deadly disease.

1.2 History of Cancer Chemotherapy

Discovery of new leads in anticancer drug development is the central focus of our research laboratory.[8] In the US alone, organizations like the National Cancer Institute (NCI) and a number of pharmaceutical companies have spent several billions of dollars in anticancer drug development studies.[9]

Herbal preparations were the first reported cancer treatment administered in history.[10] Up until the 1960s, cancer treatments, which mostly combined surgical excision with radiotherapy, resulted in a low survival rate of 33% ascribable to metastases from undetected and minuscule cancer cells.[11] This occurrence brought supportive chemotherapy into the fray, where drugs were coupled with surgery and/or radiation treatments.[11] This mode of treatment has become the effective standard of care for cancer patients with various types and stages of cancer the world over.[11]

As with many scientific discoveries, the earliest effective anticancer therapeutics came by way of knowledge-based and/or chance discoveries for example, nitrogen mustard exposure in World War I soldiers caused suppression of lymphoid and myeloid cells, and removal of ovaries in women often minimized breast cancers.[10]

Sources of anticancer therapeutics would however, have to undergo a series of evaluations, which may take several years to decades before becoming a drug.[10] These sources include nature, which has often times provided compounds as new leads in the discovery of novel anticancer agents as well as other drugs.[12]

1.3 Importance of Nature to Drug Discovery

Nature has been responsible for the emergence of wide-ranging therapeutic drugs for years.[13] For a fact, natural sources have demonstrated beyond doubt that they are the most abundant source of novel chemical entities for biological studies and a valuable source for drug discovery.[14] Natural products have contributed immensely to the world of drug discovery, especially over the past 30 years, with 79% of new small molecule anticancer drugs and 68% of the antiinfectives.[15] Natural products account for 5% of the 1,031 new chemical entities approved as drugs by the US Food and Drug Administration (FDA) between 1981 and 2002, and natural-product-derived compounds made up another 23%.[16] Those contributions include inspiration for many therapeutic agents for regulation of immune response, cancer, infectious diseases (both bacterial and fungal), and lipid disorders. For example, between the 1940s to the end of 2014, 83% of approved anti-cancer drugs were either natural products per se/based/mimicked natural products in one form or another.[17]

They have been successful because of their function-first approach as opposed to the target-first approach, which some pharmaceutical companies employ.[16] These natural products are often used as starting points for drug discovery in combinatorial chemistry, where highly desired analogues can then be prepared.[18]

Natural product chemistry pertains to a group of low molecular weight compounds, known as secondary metabolites, with potent physiological activities.[6] The depth of chemical diversity of natural products stem from the fact that they have evolved over a significant period of time.[18] A number of important anticancer drugs like: taxol,

camptothecin, romidepsin and etoposide (Figure 1), which are naturally derived or inspired, demonstrate this chemical diversity.[19]

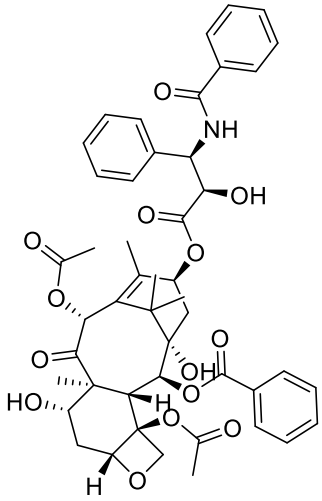
1.4 Fungi are a Rich Source of Natural Products

The fungal kingdom is said to be the second largest among the eukaryotes, with a recently proposed estimate of 1.5 to 5.1 million species[20] but with only about 135 000 are classified in literature and a fewer number analyzed chemically.[21] Many fungal species are sources of bioactive secondary metabolites which may be produced to neutralize competitions from bacteria, algae, and other fungi in their intricate environment.[22] These secondary metabolites serve as useful leads for drug discovery and have culminated in pharmaceutical successes (Figure 2) such as penicillin (antibiotic), griseofulvin (antifungal), compactin (cholesterol-lowering agent), fingolimod (receptor-modulating agent), ergometrine (uterine vasoconstrictor) and psilocybin (anxiolytic).

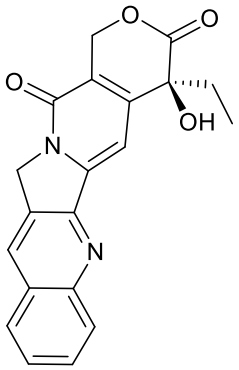
Terrestrial plants were the sole source of natural products-derived drugs until the discovery of penicillin in the early 20th century (1940), which shifted focus to other natural sources such as fungi.[19] The availability of molecular tools such as DNA barcoding for fungal identification has aided the popularity of the use of fungi in natural products research.[20]

Fungal secondary metabolites are built from simple materials including: organic acids, sugars, amino acids, terpenes, and nitrogenous bases such as purines and pyrimidines. They are categorized into polyketides, non-ribosomal peptides, terpenes, and indole terpenes, among others.[6]

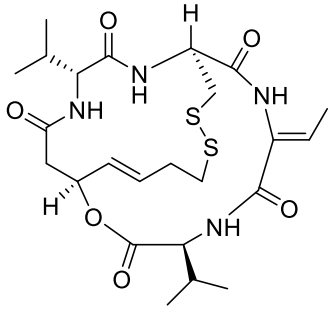
Figure 1. **Nature-Derived Anticancer Drugs.**



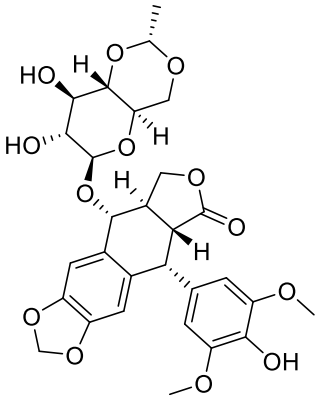
Taxol



Camptothecin

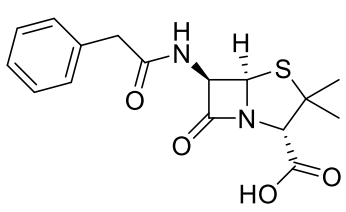


Romidepsin

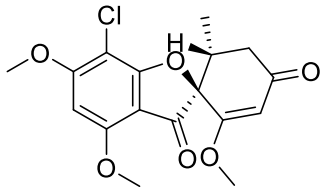


Etoposide

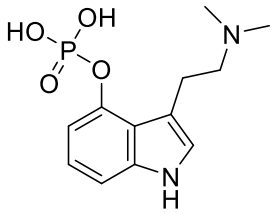
Figure 2. Pharmaceutical Successes from Fungi.



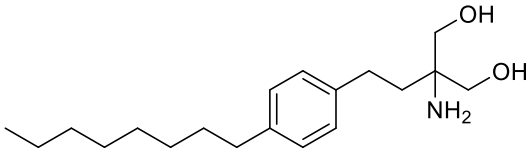
Penicillin



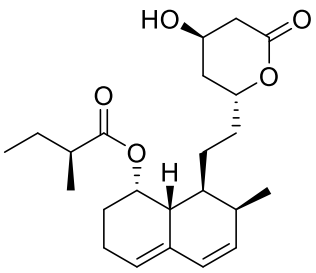
Griseofulvin



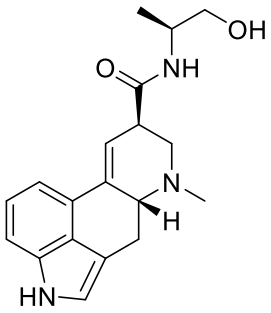
psilocybin



Fingolimod



Compactin



Ergometrine

Fungi are a proven source of natural products because it is well documented that they produce diverse secondary metabolites that display a wide range of biological activities.[23] While true, it is believed that they are yet to be explored fully because studies into fungal metabolites have yet to culminate in the development of a blockbuster anticancer drug.[24]

1.5 History of Viridicatumtoxins

The fungal metabolite, viridicatumtoxin A has potential cytotoxic and antibacterial activities. In 2005, investigation of secondary metabolites from *Aspergillus unilateralis* by Capon and co-workers revealed viridicatumtoxin A as having both these activities.[25]

Studies on the cause of swine hepatotoxicity from feed contaminated by the fungus, *Penicillium viridicatum*, led to further studies resulting in the isolation of viridicatumtoxin (as it was called then) in 1973 as a toxic compound.[26] Cultures of several isolates of *P. viridicatum* grown on moist maize proved toxic to guinea pigs producing characteristic renal, hepatic and splenic lesions microscopically.[26]

Viridicatumtoxin A is a tetracycline-like (containing the naphthacenedione core) aromatic polyketide. While it was first isolated as a mycotoxin from *Penicillium viridicatum,*[26] it has since been reported to be produced by several species of *Penicillium.*[27]

Its color is bright yellow when newly isolated, and its molecular formula of $C_{30}H_{31}NO_{10}$ was determined by elemental analysis and high-resolution mass spectroscopy.[26] It differs from tetracycline in the presence of a spirobicyclic ring, forming an additional two rings, the absence of the 4-dimethylamino moiety, and its fungal origin as opposed to the largely bacterial origin of the tetracyclines.[27] Its first structure elucidation was done by X-ray crystallographic methods in 1976.[28]

In 2013, Nicolaou et al. revised crystal structure of viridicatumtoxin and renamed it viridicatumtoxin A (Figure 3).[27] Perhaps the most significant analogue to date is a 5-oxo derivative which was termed viridicatumtoxin B and was discovered in 2008.[29] In early 2012, Yi Tang and co-scientists identified a new analogue, previridicatumtoxin while studying the gene clusters involved in the biosynthesis of viridicatumtoxin A in *Penicillium aethiopicum*.[30] Later the same year, Tomoda et al. reported the isolation of viridicatumtoxin A alongside a new analogue, spirohexaline, from a liquid culture of *Penicillium brasilianum*.[31] Capon and co-researchers isolated the latest analogues (Figure 4), viridicatumtoxins D−F and previously isolated compunds like spirohexaline and previridicatumtoxin from scaled up cultivation of a *Paecilomyces sp*. (CMBMF010) in 2015.[27]

The pursuit of promising cytotoxic drug leads from the perspective of natural products with emphasis on fungal secondary metabolites is the longest running project of the Oberlies group till date. Our interest in viridicatumtoxin A is a product of this search.[8]

1.6 Previous Isolations of Viridicatumtoxin A in Literature

The first isolation of viridicatumtoxin A was from cultures of a strain of *P. viridicatum*, CSIR 1029, grown on 3 kg of wet sterile maize meal.[26] The extractions were done exhaustively with an undisclosed ratio of $CHCl_3$ to MeOH resulting in extracts weighing 240 g upon drying.[26] The extracts were partitioned with an undisclosed ratio of $CHCl_3$ to H_2O mixture.[26] The organic ($CHCl_3$) layer, which was evaporated to dryness weighing 82 g, was subjected to further partitioning, this time, with an undisclosed ratio of hexane to MeOH (90%) mixture.[26] The hexane layer was evaporated to yield 35 g of a nontoxic sample and the 90% MeOH layer was evaporated to yield 46 g of toxic sample.[26] The 90% MeOH layer was purified to yield 11 g of griseofulvin and 4 g of viridicatumtoxin A via column chromatography on formamide-impregnated cellulose powder using hexane and benzene mixtures as the mobile phase.

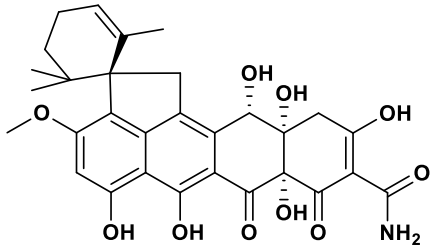
In 2008, cultures of *Penicillium sp.*, strain FR11, were grown on 80 mL of yeast extract peptone sucrose (YPS) liquid medium in a 500 mL Erlenmeyer flask.[29] The culture flask was place on a rotary shaker (150 rpm) for 3 days at 28°C.[29] From the culture, 15 mL were inoculated into a 1000 mL Erlenmeyer flask to enhance the growth of the fungus and therefore, the production of active compounds.[29] These cultures were placed on a rotary shaker (150 rpm) at 28°C for 7 days and were extracted comprehensively with 50% acetone with the resulting extracts dried *in vacuo*.[29] The extract, after being reduced to a combined aqueous solution, was extracted three times with the same volume of EtOAc.[29] Initial fractionation of the EtOAc extract (5.0 g) was done using an YMC s-150 mm octadecyl-silica (ODS) column chromatography.[29]

The gradual elution followed the method gradient of MeOH to H_2O with the active fractions coming out with the 80 : 20 and 90 : 10 gradients. These active fractions were combined and dried *in vacuo*.[29] This combined active portion was then dissolved in MeOH and purified using YMC C-18 reverse phase high performance liquid chromatography (HPLC) system.[29] Viridicatumtoxin A and B eluted at retention times of 32.1 and 38.2 mins, respectively, with MeOH to H_2O at 85 : 15 gradient.[29]

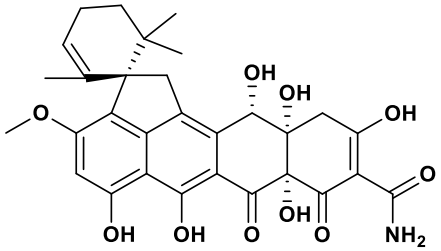
The third isolation of virdicatumtoxin A reported in 2015 was cultured both on peptone yeast extract glucose (PYG) agar and rice solid media.[27]

For the Agar study, PYG plates (n = 100) were inoculated with a lone colony of the fungal strain CMB-MF010 (*Paecilomyces sp*.) and were incubated at 26.5 °C for 25 days. These samples were harvested and diced (~$1cm^3$) after comprehensively extracting with EtOAc.[27] The resulting extract weighed 1173 mg after drying *in vacuo*.[27] Partitioning with a series of soluble solvents yielded: hexane fraction (153.9 mg), CH_2Cl_2 fraction (700.0 mg), and MeOH fraction (144.0 mg).[27] SPE (C-18) chromatography of the CH_2Cl_2 fraction with a 70% H_2O/MeOH to MeOH gradient elution yielded fractions 1 and 2 weighing 38.7 mg and 96.3 mg respectively.[27] Preparative HPLC of both fractions using a C-8 column and gradient elution from 35% - 52% CH_3CN to H_2O over 5 mins, followed by 52% - 57% CH_3CN to H_2O over 10 mins yielded in order of retention time, viridicatumtoxin F (2.6 mg), viridicatumtoxin E (7.5 mg) and viridicatumtoxin D (8.2 mg) (for fraction 1) and viridicatumtoxin A (14.1 mg) and viridicatumtoxin B (0.6 mg) for fraction 2.[27]

Figure 3. **Previous (left) and Revised (right) Structures of Viridicatumtoxin A.**



VCT A_Previous



VCT A_Revised

For the rice solid media study, fermentation was performed by using an inoculum of CMB-MF010 from one of the PYG agar plates above and transferring into a sterile solid rice medium in a 1000 mL Erlenmeyer flask, which was then incubated at 26 °C for 25 days.[27] The resulting fungal culture was extracted with EtOAc completely and dried *in vacuo*, weighing 673.0 mg.[27] Partitioning with a series of soluble solvents yielded: hexane fraction (375.9 mg), CH_2Cl_2 fraction (161.1 mg), and MeOH fraction (22.1 mg).[27] Starting with 90% H_2O/MeOH to MeOH, SPE (C-18) chromatography of the CH_2Cl_2 fraction yielded 10 subfractions.[27] Subfractions at 50%, 40% and 30% H_2O/MeOH were combined and preparative HPLC of the combined fractions in a C-3 column using isocratic elution of 45% CH_3CN to H_2O over 20 min generated viridicatumtoxin A (7.4 mg) and viridicatumtoxin C (2.9 mg), while a fraction at 10% H_2O/MeOH generated spirohexaline (0.3 mg) and previridicatumtoxin (0.4 mg) with preparative HPLC gradient elution of 50% - 65% CH_3CN to H_2O.[27]

1.7 Organic Synthesis of Viridicatumtoxin Analogues

Nicolaou and co-workers, inspired by the potent antibiotic properties, the scarcity, structural complexity, and the incomplete structural assignment of viridicatumtoxin B, achieved its total synthesis (3.5 mg), albeit through a lengthy protocol involving 39 steps.[32] The design of the total synthesis was based on retrosynthetic analysis through which they were able to transform viridicatumtoxin B into four simpler building blocks each of which was accessed using the same method.[33] The pursuit of the total synthesis began with the construction of the four main building blocks: an allylic bromide, a cyclic anhydride, a quinone monoketal, and an isoxazole phenyl ester.[32] The four building blocks were then successfully connected together through convergent synthetic mechanisms to form viridicatumtoxin B.[32]

The successful total synthesis of viridicatumtoxin B enabled its structure to be revisited, its earlier assigned epoxy hemiacetal structure to be correctly reassigned as hydroxy ketone.[33] Therefore, the absolute configuration of viridicatumtoxin B was confirmed.[33] Application of the scientific knowledge acquired in the total synthesis above led them to synthesize less demanding (lacking the C4a hydroxyl) analogues of viridicatumtoxin B, namely: (±)-V2, (±)-V3, (±)-V4, (±)-V5, and (±)-V6.[33]
As a whole, their commendable achievement has set the tone for overall advances in the area of design, synthesis, and biological evaluation of analogues of viridicatumtoxin A as potential leads for drug discovery.

Figure 4. Structures of Viridicatumtoxin A and Known Naturally Occurring Analogues.

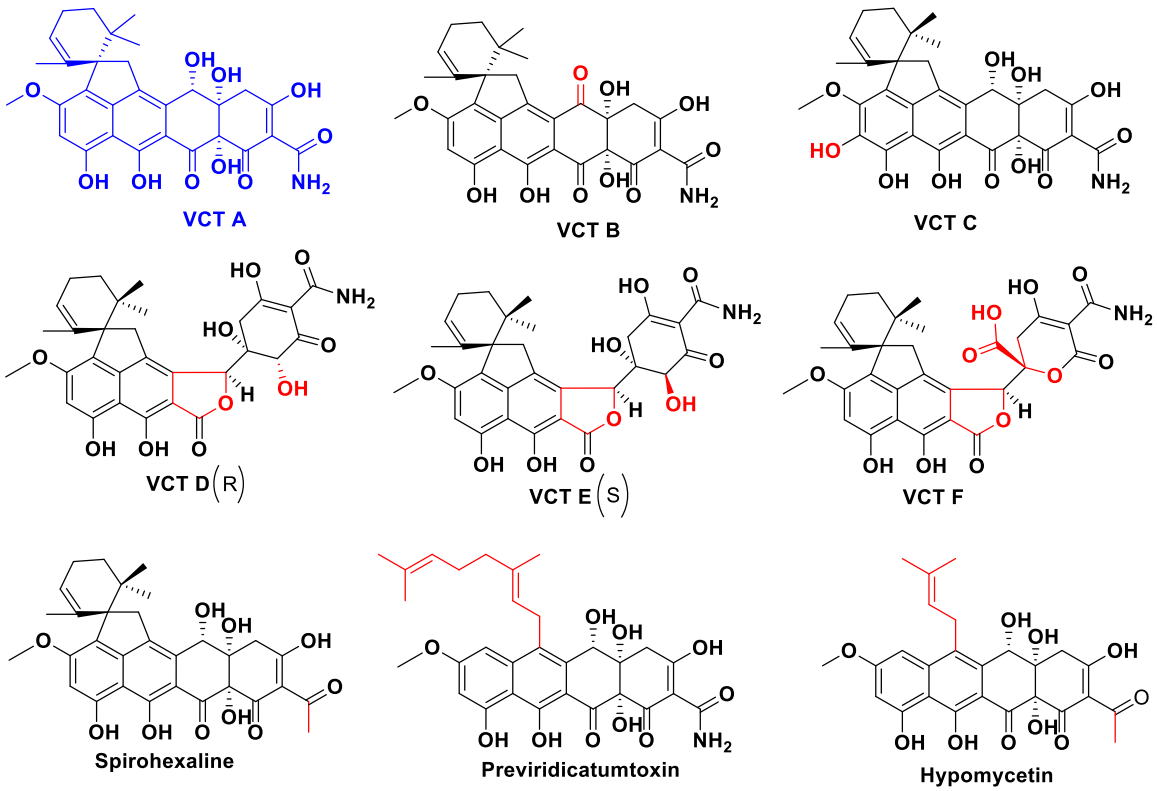


Table 1. Abbreviations of Viridicatumtoxins.

VCT A	**Viridicatumtoxin A**
VCT B	**Viridicatumtoxin B**
VCT C	**Viridicatumtoxin C**
VCT D	**Viridicatumtoxin D**
VCT E	**Viridicatumtoxin E**
VCT F	**Viridicatumtoxin F**

CHAPTER II

GENERAL PROCEDURE

2.1 Fungal Culture

The seed culture of MSX54809 was grown in a Petri dish with a malt extract agar, made up of 10 g malt extract, 10 g glucose, and 20 g agar per 1 L of H_2O. When adequate growth was observed, an inoculum of the fungus from the seed culture was transferred into Erlenmeyer flasks containing the media of interest. The flask cultures are left in static conditions and allowed to grow for three to four weeks at room temperature. Afterwards, the fungus was killed by spraying the culture with MeOH and extracted immediately or stored at -18 ˚C until the time of extraction.

2.2 Solvent Extraction

In the Oberlies lab, we work with organic compounds, which are generally more soluble in organic solvents like chloroform than in water, an aqueous solvent. Shaking a fungal culture in a solution of chloroform and water gives a two-layer partition i.e. the organic (non-aqueous) and aqueous layers. Organic compounds will, therefore, be in the non-aqueous (organic) layer and vice versa. Finally, this non-aqueous layer is removed and is ready for purification to obtain the compound of interest.

Extractions for this study, unless otherwise stated, follow the Oberlies laboratory extraction protocol, which begins by adding 60 mL 1:1 $CHCl_3$-MeOH mixture to each fungal culture and shaking overnight at 100 rpm. The next day each flask is filtered via

Buchner funnel. 90 mL $CHCl_3$ and 150 mL H_2O were added to the organic filtrate to obtain a 4:1:5 $CHCl_3$-MeOH-H_2O mixture. This extraction mixture is stirred for half-hour with a magnetic stirrer and partitioned in a separatory funnel, and the organic fraction was dried down *in vacuo*. The dried organic fraction is then reconstituted in 100 mL CH_3CN-MeOH to 100 ml hexanes producing a 3:3:4 CH_3CN-MeOH-hexane mixture and a second partition is carried out to remove fatty materials from the sample. The defatted crude extract, which contains the compound of interest, is then dried *in vacuo*.

2.3 Flash Chromatography

The first step in the Oberlies laboratory isolation protocol of defatted fungal extracts is initial fractionation. Flash chromatography is used for the initial fractionation of the mixture of compounds in crude exacts.

The initial fractionation in this study is carried out with a flash chromatography instrument, Teledyne ISCO CombiFlash Rf, set-up in normal phase with a silica gel stationary phase column using hexane, chloroform, and methanol solvent mixture as mobile phase. Empty sample cartridges available in various sizes are packed with solid samples by means of Celite (a sorbent) and then mounted unto the column. A UV-Vis detector and an external evaporative light scattering detector (ELSD) are connected to the ISCO system. The flash chromatography uses a medium pressure pump and yields fractions based on polarity.

2.4 High Performance Liquid Chromatography (HPLC)

Our pure fractions were obtained using a Varian Prostar HPLC system comprising of a photodiode array (PDA) detector, a reciprocating two-head pump, a fraction collector, an evaporative light scattering detector (ELSD) connected to a computer to read the generated data. The HPLC technique for this project involves an Atlantis T3 column packed with C-18 sorbent molecules (stationary phase) and a CH_3CN-H_2O with 0.1% formic acid solvent mixture as mobile phase. In contrast to column chromatography, the mobile phase is powered through the column by high pressure delivered by the pump. After dissolving the sample in a solvent of about 300 µL, the homogenous solution is injected into the flow path of the mobile phase. The analyte is separated into distinct peaks on a generated chromatogram using optimum elution method as it passes through the HPLC column and each peak represents a pure compound. The pure compounds are collected as fractions in test tubes arranged in the fraction collector. HPLC columns also include, Gemini (C-18), Synergi (C-12), and PFP (C-18) columns to mention a few. These columns are used for analytical, semi preparative, or preparative application to obtain pure compounds.

2.5 Ultra Performance Liquid Chromatography (UPLC)

UPLC analytical separation technique operates on similar principles to HPLC but with increased speed, sensitivity & resolution. UPLC columns are packed with fine particles of < 2.0µm thereby resulting in lower flow rate and higher peak capacity, reduced run times and minimal solvent consumption. UPLC columns includes, BEH C-18, BEH C-8, HSS C-18, and HSS T3 among others. An Acquity UPLC system equipped

with a BEH C-18 (1.7 mm; 50 × 2.1 mm) column and a flow rate of 0.2 -0.4 mL/min was used to confirm the purity of viridicatumtoxin A. The Acquity UPLC system has a binary solvent manager that uses two individual pumps to deliver two solvents in parallel gradient, sample manager that draws samples from 96-well 1 mL plates and injects into the flow stream of the mobile phase, the column heater controlled by the sample manager for temperature of 5°C - 65°C, detectors consisting of an ultraviolet (UV) detector called PDA that operates between 190 and 500 nm λ, and an ELSD to detect the relative abundance of analytes.

2.6 Mass Spectrometry

The Q Exactive Plus mass spectrometer coupled to Water's Acquity UPLC system at the Triad Mass Spectrometry laboratory of UNCG department of chemistry and biochemistry was mainly used for precise mass analysis during the course of this study. The mass spectrometry (MS) analytical technique is used to determine the amount of an analyte in a sample by measuring the mass-to-charge ratio. MS has evolved as a key analytical tool in almost all levels of drug discovery due to its high sensitivity and speed in the identification of analytes in complex mixtures.

The MS is made up of three main components: an ionization source, a mass analyzer, and a detector. The UPLC was used as the delivery mechanism for sample introduction. The electrospray ionization (ESI) method was employed in the study where charged droplets are produced at the tip of the ESI capillary, desolvation of the droplets by nebulizing gas and, the production of gas phase ions from the highly charged small droplets. Mass analyzers vary in the fundamental way in which they separate species on a

mass-to-charge basis. The quadrupole (single or triple) separates ions according to their mass-to-charge ratio as they pass through the central axis of electric fields of four parallel equidistant rods. The signals from the mass analyzer are received and displayed by the detector as a readable spectrum on the computer.

2.7 Nuclear Magnetic Resonance

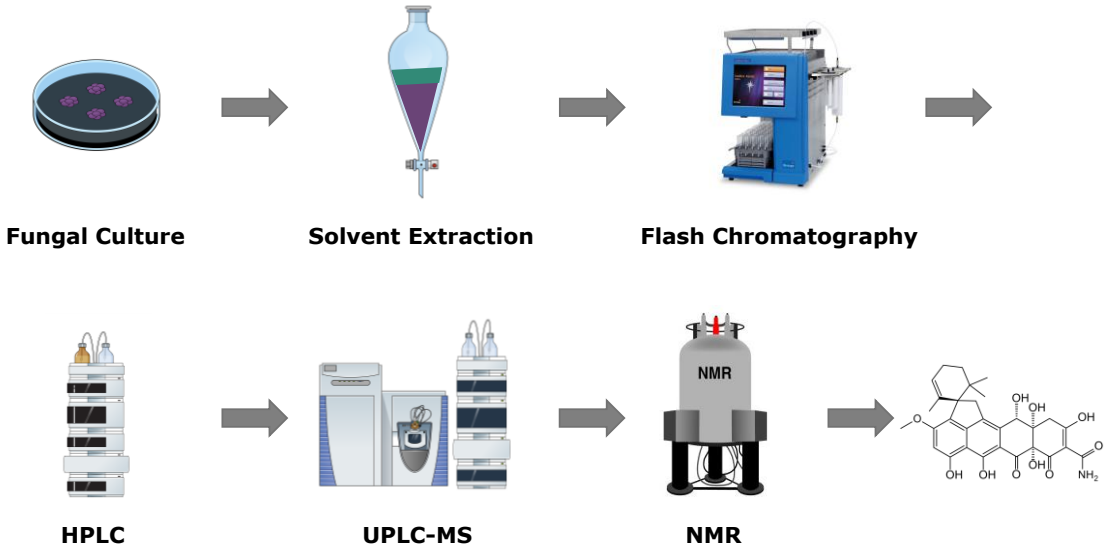
Nuclear Magnetic Resonance (NMR) spectroscopy generate distinct signals that when interpreted provide structural information on the number and position the atoms in of an organic compound. These distinct signals are generated from the magnetic nuclear spin of atoms when they experience dissimilar milieu in an applied magnetic field. The frequency of each signal on the NMR spectrum, relative to tetramethylsilane (TMS), the reference standard, is called chemical shifts. NMR spectroscopy is very useful in structure elucidation of organic compounds especially for drug discovery purposes.

For ^{1}H NMR, the absence or presence of electronegative atom around a proton increases or decreases respectively the electron density around the proton. A deshielded proton has decreased electron density in an applied magnetic field, thereby shifting its signal downfield (left) of the spectrum, while a shielded proton by virtue of its intact electron density is seen upfield (right) of the spectrum. The intensities of the signal produced is a ratio of the number of equivalent protons generating it. Protons that experience comparable magnetic environment are called chemically equivalent protons and therefore, are seen as the same signal (singlet) in the NMR spectrum.

NMR signals spectrum usually have peaks that appear as groups of peaks due to interaction (coupling) with neighboring proton(s) are usually labeled as singlet doublets (2 peaks), triplets (3 peaks), quartets (4 peaks)… multiplet (complex peaks). The distance within each group of peaks are called coupling constants, denoted by J.

^{13}C NMR is used for carbon NMR because ^{12}C, the most abundant natural carbon isotope has a net spin of zero in NMR. The number and position of discrete signals are the most important pieces of information conveyed by ^{13}C NMR as there is no correlation between each NMR signal and the number of atoms producing it.

Figure 5. Process Flow Chart



CHAPTER III

EXPERIMENTAL

3.1 Bioactivity Directed Fractionation

Extracts of the filamentous fungus MSX71909 exhibited cytotoxicity (Table 2) with the following IC_{50} values; 1.2 µM against MDA-MB-435 (human melanoma cancer cell lines), 10.8 µM against MDA-MB-231 (human breast cancer cell lines) and 11.1 µM against OVCAR3 (human ovarian cancer cell lines). Next, we isolated viridicatumtoxin A by bioactivity directed fractionation. Through dereplication protocol by matching full-scan high-resolution mass spectra and MS/MS spectra from both the positive- and negative-ionization modes coupled with UV-absorption maxima and retention times with in-house database of over 450 compounds constructed based on cytotoxic fungal secondary metabolites, we identified the bioactive compound as viridicatumtoxin A.

Three viridicatumtoxin A producing fungal strains from Mycosynthetix were utilized in the present study and were identified via ITS sequencing and molecular phylogenetic methods.[20] Our lab has been systematically investigating the vast Mycosynthetix library for anti-cancer drug leads for years.[34] These three strains, MSX71909, MSX72658, and MSX54809 were identical and can be described as *Purpureocillium lavendulum*.[35]

Due to the cytotoxic activities exhibited by viridicatumtoxin A, we set up an experiment to scale up its production. In addition, we wanted more materials for the organic synthesis of the bioactive but scarce analogue, viridicatumtoxin B which we would attempt in collaboration with the Croatt lab.

Table 2. Cytotoxicity (IC_{50} Values) Data for Viridicatumtoxin A.

Compound	MDA-MB-435 (melanoma)	MDA-MB-231 (breast)	OVCAR3 (ovarian)
VCT A	1.2 µM	10.8 µM	11.1 µM

The solid-state rice media has been the adopted media for drug-lead screening projects based on previous studies.[36] The Oberlies-Mycosynthetix drug-lead discovery collaboration involves growing fungal samples in solid-state rice media for routine chemical and biological analysis.[34]

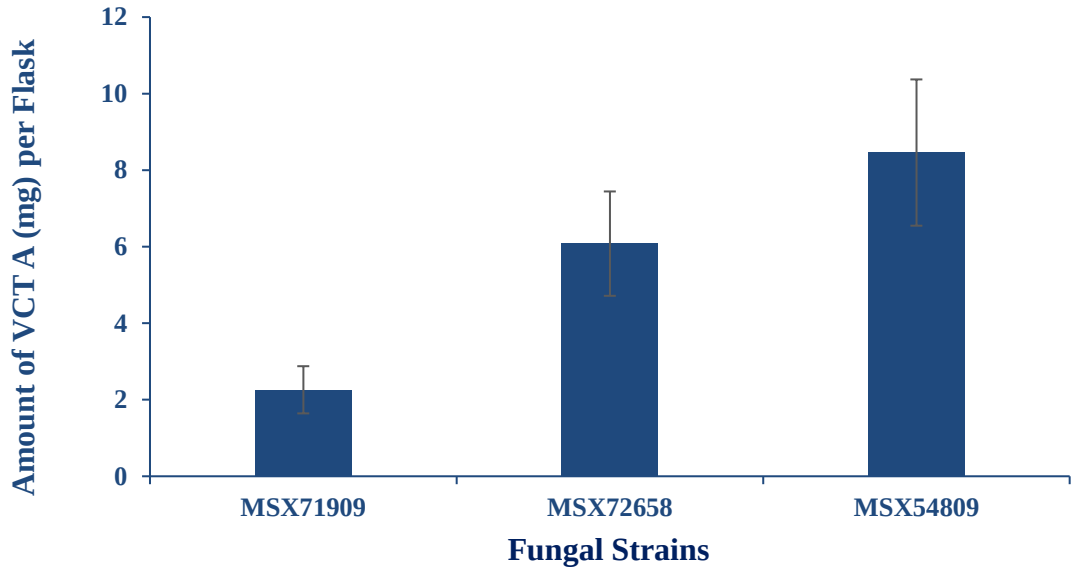
Therefore, the three in-house fungal strains of *Purpureocillium lavendulum*, namely: MSX72658, MSX71909, and MSX54809, which are known to produce viridicatumtoxin A were fermented on solid-state rice media within the same period and conditions. The cultures were incubated for four weeks at room temperature. They were all extracted at the same time after fermentation using our conventional extraction protocol described earlier. After extraction, the five replicates of the extracts were

to flash chromatography on our Teledyne ISCO CombiFlash Rf, set-up in normal phase using hexane, chloroform, and methanol. The fraction containing our compound of interest was then purified using preparative High-Performance Liquid chromatography.

Table 3. **Reported IC_{50} Values (μM) for Viridicatumtoxin A and Related Analogues.**[27]

Compound	SW620 (colon)	NCI-H460 (lung)	KB3-1 (cervix)
VCT A	**1.0**	**1.0**	**2.5**
VCT B	**0.6**	**0.6**	**1.6**
VCT C	**17.4**	**9.2**	**11.0**
VCT D	**20.7**	**24.2**	**>30**
VCT E	**>30**	**>30**	**>30**
VCT F	**>30**	**>30**	**>30**
Spirohexaline	**16.7**	**11.3**	**6.3**
Previridicatumtoxin	**6.0**	**5.3**	**4.1**

Figure 6. **Viridicatumtoxin A Production on Rice Media**. A comparison of the amount of pure viridicatumtoxin A isolated from the three fungal strains. Data plotted are the averages ± SE for five replicate cultures per fungal strain.



After statistical analysis of the pure HPLC fractions (Figure 6), MSX54809 was determined to be the best viridicatumtoxin A producer. The research, therefore, progressed from this point forward with MSX54809.

3.2 Optimizing Production of Viridicatumtoxin A via Media Study

The next stage in the project was to optimize the production of viridicatumtoxin A from MSX54809 via media study. Solid-state rice media has hitherto been established as a functional growth medium in which most fungi thrive and produce active compounds,

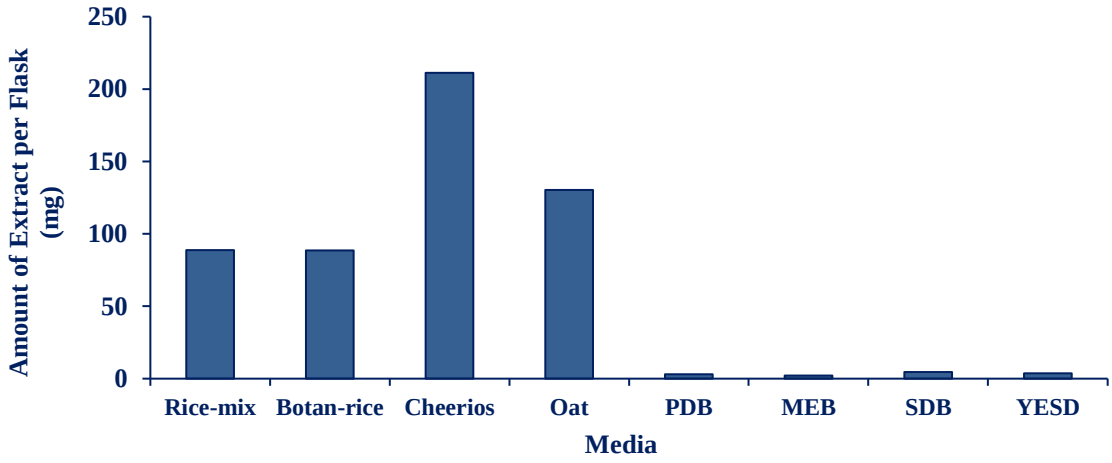
but we hypothesized that it may not be the most functional media for some fungi to produce a secondary metabolite of interest.[36]

To determine the most functional growth media to optimize the production of viridiactumtoxin A by MSX54809, the fungus was grown in eight media, including the established rice media, which are divided into two groups. These two groups are the solid and the liquid media groups. Their names and composition are as follows: Rice mix (Botan and Basmati variety), Botan Rice (Botan variety), Cheerios (Multigrain variety), Oats (regular breakfast Oats), PDB (Potato Dextrose Broth composed of potato infusion and dextrose with an organic acid), MEB (Malt Extract Broth composed of malt extract and peptone with an organic acid), SDB (Sabouraud Dextrose Broth composed of peptone and dextrose with an organic acid for low pH), YESD (Yeast Extract Soy Peptone made up of 1% yeast extract, 2% soy peptone, and 2% dextrose). Peptone serves as utilizable source of nitrogen, vitamins, minerals, amino acids, and growth factors while dextrose serves as the carbon source. The organic acid favors fungal growth and inhibits bacteria growth.

The growth of MSX54809 started in a Petri dish by using a malt extract agar, made up of 10 g malt extract, 10 g glucose, and 20 g agar per 1 L of H_2O. When it is considered that the fungus has attained adequate size of growth, then an inoculum of the fungus, MSX54809, was transferred into a separate 250 mL Erlenmeyer flask containing one of the above listed media.

The cultures were fermented in static conditions for four weeks, after which they were killed by spraying with MeOH. The cultures were either extracted immediately following step-by-step the protocol stated above or stored at -18 °C until the time of extraction (Figure 7).

Figure 7. **Fungal Growth on Various Media.** Comparison of the amount of fungal extracts produced by MSX54809 on eight different media in a flask each.

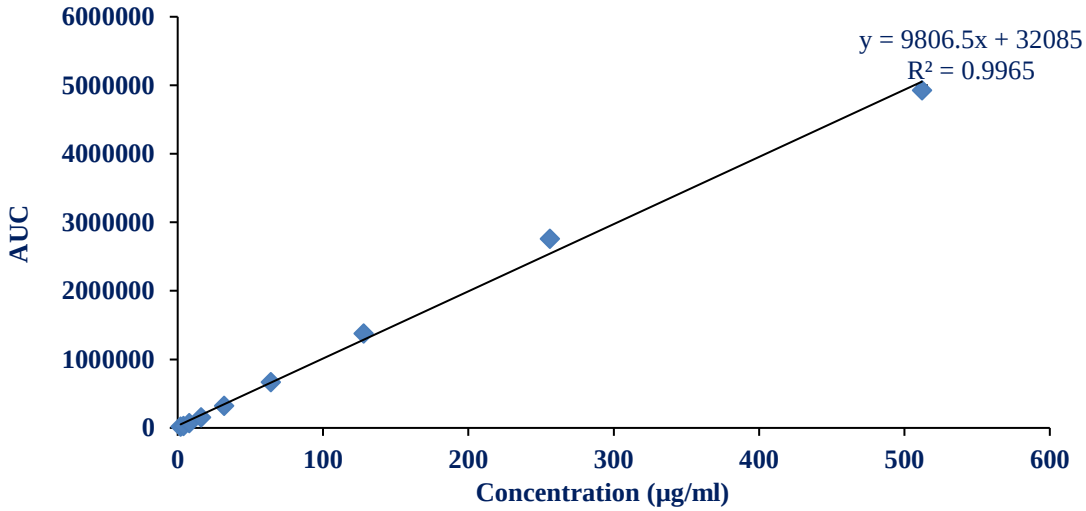


To determine the concentration (amount) of viridicatumtoxin A in each media, a quantitation experiment was designed. Concentrations of ten standard solutions was set up to adequately cover the expected concentration range in the extract solutions which are the test samples. Starting with 1024 µg/mL and by sequential dilutions: 512 µg/mL, 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL and 2 µg/mL evenly spread out standard solutions were prepared.

The test solutions were prepared to a concentration of 1000 µg/mL each. Both the standard solutions and the test samples were prepared in MeOH and 100 µL of each solution was pipetted into a 1 mL 96 well plate. A blank with zero concentration of analyte was put into the plate to run directly before and directly after the standard solutions as controls.

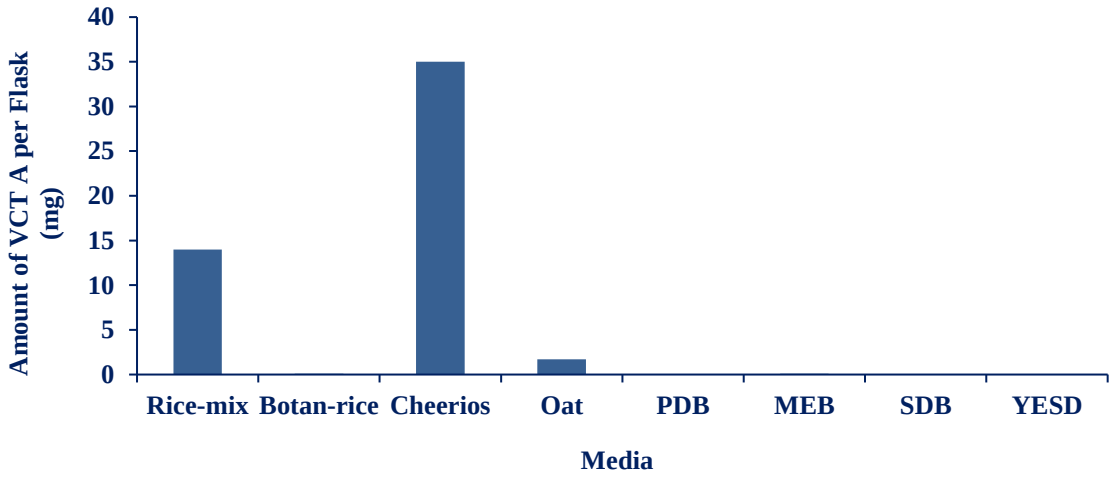
For the quantitation studies, a UPLC Acquity system equipped with a BEH C-18 (1.7 mm; 50 × 2.1 mm) column, using a flow rate of 0.3 mL/min, column and sample manager temperatures set at 40 ^{0}C and 10 ^{0}C respectively. Starting with a gradient of 45:55% CH_3CN – H_2O (acidified with 0.1 % formic acid) rising to 100% CH_3CN between 0 – 8 min and holding 100% CH_3CN for 2 mins; 8.0 – 10 min, returning to starting conditions and holding it for 10 secs.

Figure 8. Calibration Curve 1 for Viridicatumtoxin A. Area Under Curve (AUC) at 254 nm vs eight standard concentration levels in MeOH which ran in triplicate.



This study was done in experimental triplicates, and the standard samples were run from lowest to highest concentration. The data obtained was graphed as UV absorption (254 nm) against concentration. In accordance with Beer's law, a good linear relationship between detector response (UV absorption) and concentration (of standard and test solutions) quantified as R^2 came up. Hence, the linear fit ($y= mx + b$) was determined for the calibration curve (Figure 8), with y as UV absorption, x as concentration, y axis intercept b as zero (i.e. the concentration was zero when the absorption was zero), and m as the slope (i.e. the molar absorptivity times the path length of 1 cm). Thus, the concentration of viridicatumtoxin A in each media was calculated. The cheerios media was determined to have the highest concentration with a 2.5-fold increase over the established solid-rice media (Figure 9).

Figure 9. **Amount of Viridicatumtoxin A in Extract.** Comparison of the amount of pure viridicatumtoxin A isolated from extracts of MSX54809 on eight different media in a flask each.



Flash chromatography was used to fractionate each of the extracts. The extracts for fractionation were first prepared by dissolving the samples in $CHCl_3$/MeOH mixture and then, pipetting onto evenly spread-out diatomaceous earth known as Celite (a sorbent) in a mini bowl. The Celite and the extract samples are mixed thoroughly in the mini bowls and left to sit in the laboratory hood for some time to allow the $CHCl_3$/MeOH mixture to evaporate, leaving the extract solidly adsorbed onto the Celite. The dried extract-Celite complex was broken up with a mortar and pestle and then packed into the preselected cartridges. The packed cartridge and a RediSep Rf Si-gel Gold column were fitted into position according to manufacturer's directives.

The initial fractionation of the extracts was carried out using our UV and ELSD connected Teledyne ISCO CombiFlash Rf flash chromatography instrument. The instrument switches between hexane, chloroform and methanol as mobile phase with a flow rate of 18 mL/min. The method gradient of hexane-$CHCl_3$-MeOH started with 100% hexane which rapidly mixes with $CHCl_3$ until the gradient reaches 100% $CHCl_3$. The 100% $CHCl_3$ gradient is sustained for a while before it is mixed in a stepwise approach with MeOH until 100% MeOH gradient is attained. This 100% MeOH gradient is held till the end of the run.

The flash chromatography (Figure 10) for each sample yielded four fractions based on polarity. The fractions were then dried *in vacuo* and transferred by means of solvents ($CHCl_3$ and MeOH) into scintillating vials to dry under nitrogen gas lines. After drying the fractions, they were analyzed using HRMS to determine which fraction contains the mass (based on m/z of 564.1866, $[M - H]^-$) of our compound of interest. This was further confirmed with prep-HPLC and ^{1}H-NMR.

Preparative and semi preparative HPLC were utilized to purify the compound of interest. Sample preparation involves first analyzing the ISCO fractions to optimize the preparative conditions. After optimal preparative conditions have been obtained, the samples were dissolved in ~300 mL of tetrahydrofuran (THF) and loaded into a 500 µL Hamilton syringe.

Reverse-phase preparative HPLC with 40 – 100% CH_3CN to H_2O (with 0.1% formic acid) gradient over 30 mins using Atlantis T3 (C-18) column was utilized for the purification of the desired compound which, is viridicatumtoxin A. The initial gradient of 40% CH_3CN – 60% H_2O climbed to 100% CH_3CN over 30 mins. The 100% CH_3CN was held for 2 mins before returning to starting conditions in 30 secs in readiness for the next run. After each run, the pure sub-fractions (Figure 11) obtained were also dried *in vacuo* and transferred by means of solvents into scintillating vials to dry under nitrogen.

The pure fractions were further analyzed by a UPLC/PDA/MS method whereby the retention time, UV spectrum, HRMS, and MS/MS were compared with an authentic standard. The HRESIMS data (Figure 12) was collected in a Thermo Fisher Scientific Q Exactive Plus instrument. This HRMS analysis produced a molecular formula of $C_{30}H_{30}NO_{10}$ [M - H]- based on m/z of 564.1866. Additionally, the ^{1}H-NMR (Figure 13) of the pure compound was acquired in the JEOL ECA-500 NMR spectrometer to determine the authenticity of the compound. The amount of the pure viridicatumtoxin A tallied with the amount calculated in the extract from the calibration curve (Figure 14).

Figure 10. **Flash Chromatography of Extracts of MSX54809 (*Purpureocillium lavendulum*)**. Above is chromatogram of extracts from Cheerios and below is for extracts from Rice-Mix.

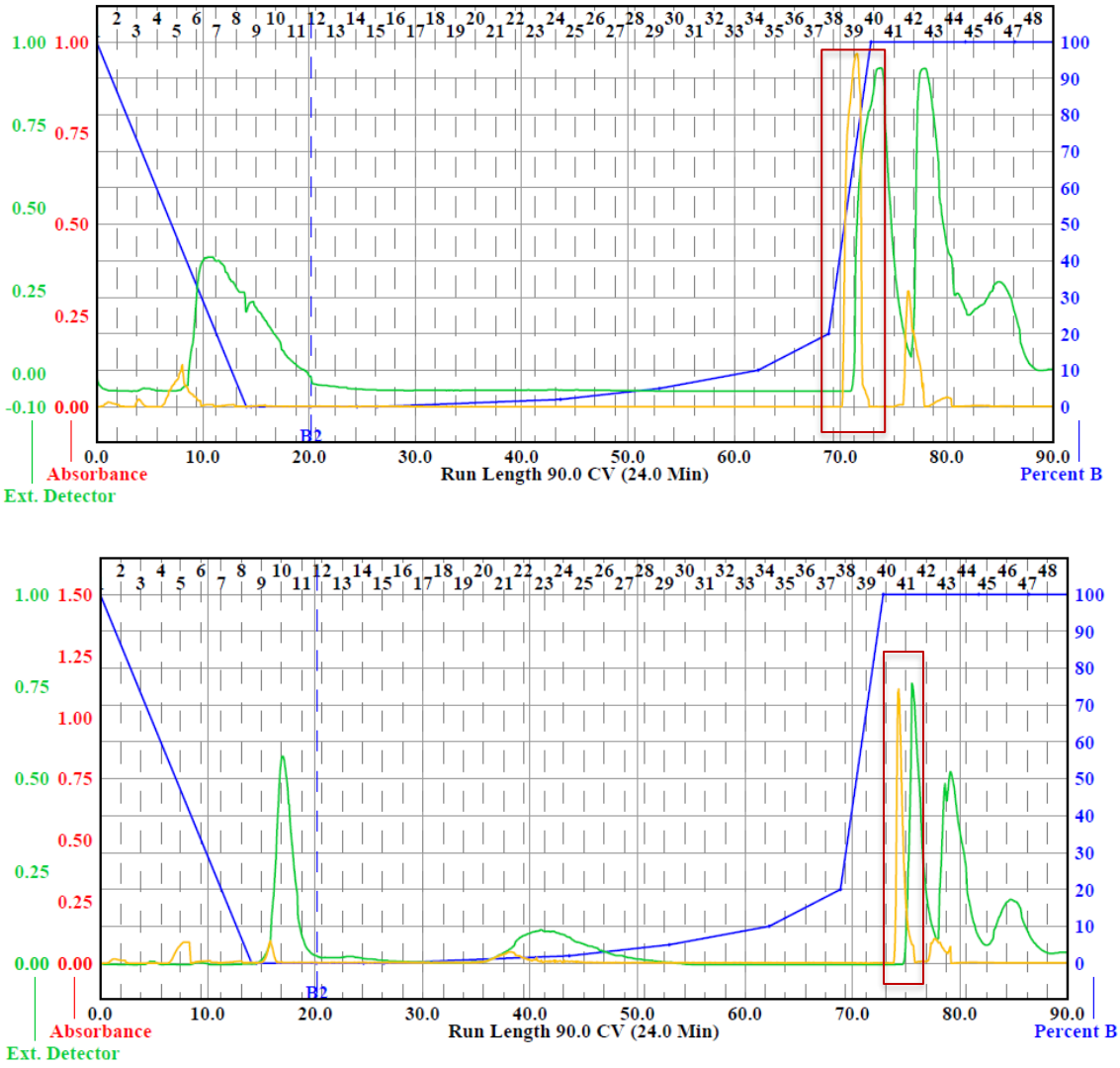
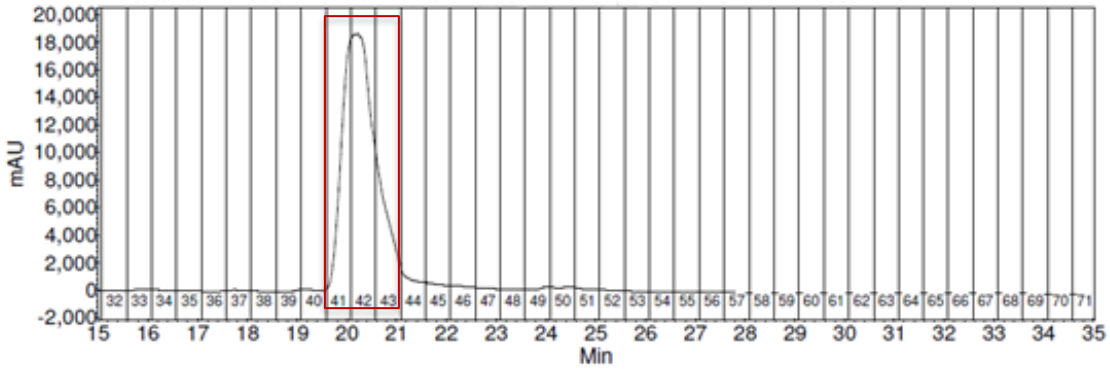


Figure 11. **Preparative HPLC of Flash Chromatography Fractions**. Above is chromatogram of flash chromatography fractions of *Purpureocillium lavendulum* from Cheerios (Top) and below is for Rice-Mix.



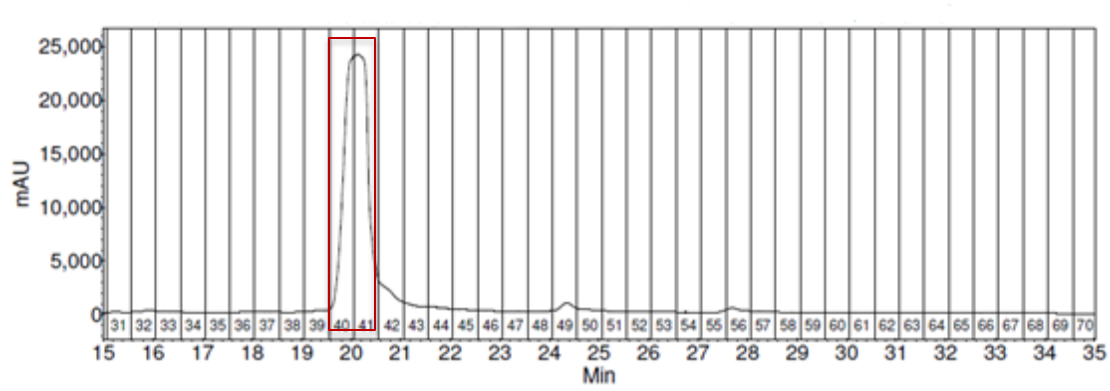


Figure 12. **MS Analysis Data of Viridicatumtoxin A**. Top is viridicatumtoxin A isolated from the Cheerios Media, Middle is from the Rice-Mix Media and Bottom is the Pure Standard.

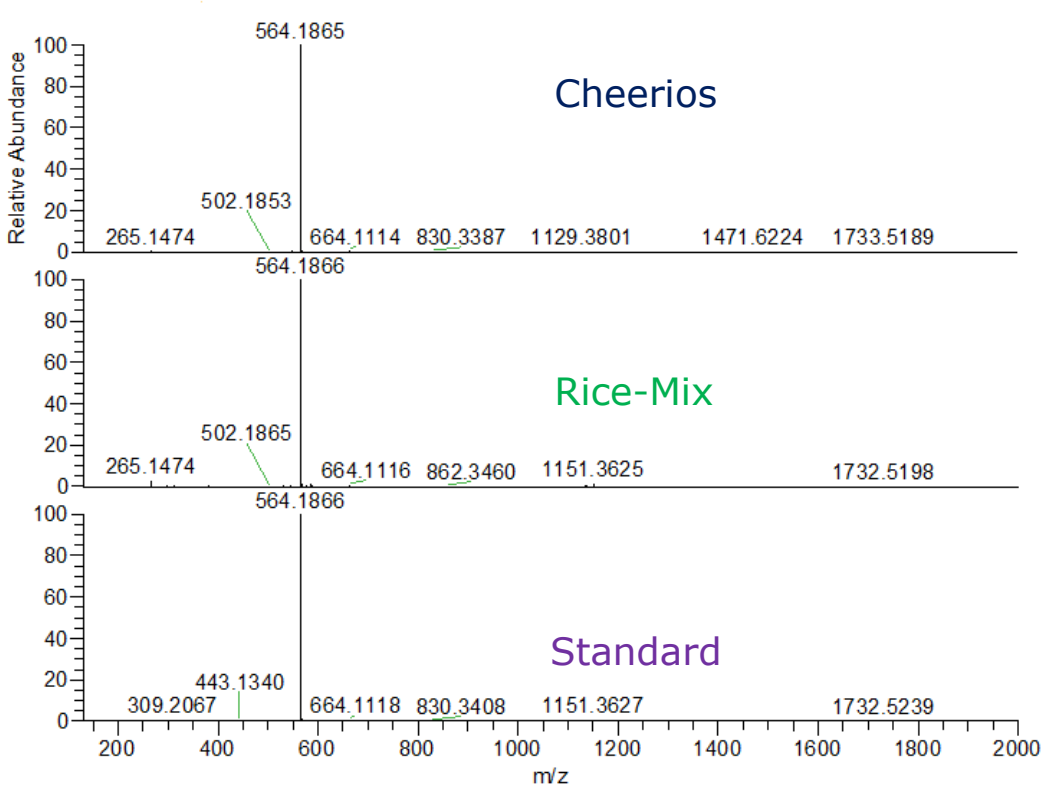


Figure 13. ^{1}H-NMR Data of Viridicatumtoxin A from Cheerios Media.

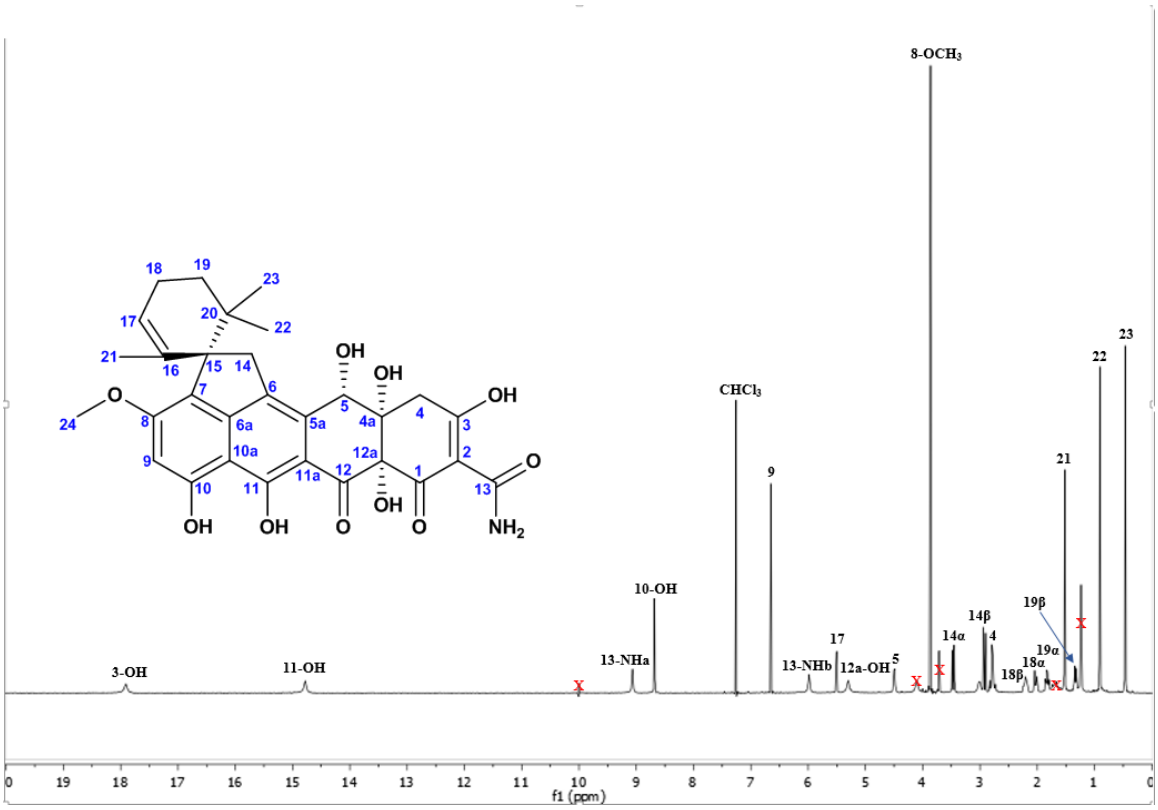
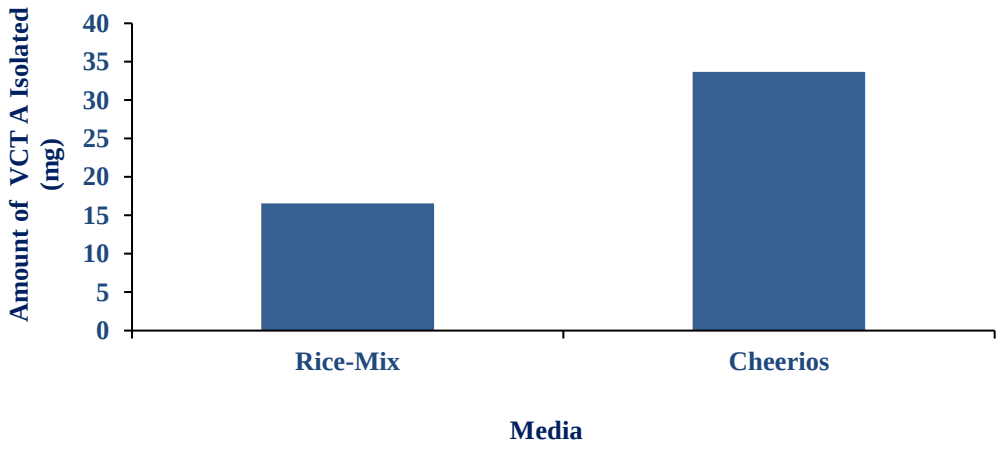


Table 4. Pure Viridicatumtoxin A Isolated from *Purpureocillium lavendulum* in Cheerios and Rice-Mix Media.

Media	Notebook Code	Amount (mg)
Cheerios	MSX54809-01062-22-2	29.9
Cheerios	MSX54809-01062-22-3	3.7
	Total	33.7
1:1 Rice-Mix	MSX54809-01062-23-2	8.6
1:1 Rice-Mix	MSX54809-01062-23-3	0.5
1:1 Rice-Mix	MSX54809-01062-29-2	0.7
1:1 Rice-Mix	MSX54809-01062-29-3	5.7
1:1 Rice-Mix	MSX54809-01062-29-4	1.2
	Total	16.6

Figure 14. Amount of Pure VCT A Agrees with Quantitation Studies.



After the quantitation, 77 250 mL Erlenmeyer flasks, 1 2800 mL Erlenmeyer flask and 1 large bag of the fungus MSX54809 were cultured and extracted in five batches throughout this project.

The first batch (Table 5) involved 10 250 mL Erlenmeyer flasks. These were cultured following the same protocol described above. After the cultures have grown for four weeks, they were combined and extracted using a different protocol. This started by adding 150 mL of acetone to the sample, which was then chopped with spatula and allowed to sit for 30 mins at room temperature. The samples were filtered in a vacuum via Buchner funnel, and evaporated to an aqueous solution. Twice to the aqueous solution, 100 mL of $CHCl_3$ was added. The biphasic solution was stirred for about half-hour with a magnetic stirrer and partitioned in a separatory funnel, and the organic fraction dried down in vacuo. The dried organic fraction is then reconstituted in 100 mL of hexane and 100 mL of 1:1 MeOH – CH_3CN. A second partition was carried out to remove fatty materials from the sample. The defatted crude extract is then dried in vacuo and transferred by means of $CHCl_3$ and MeOH into scintillating vials to dry under nitrogen gas lines.

The second batch of cultures (Table 5) comprised of 24 250 mL Erlenmeyer flasks, 1 2800 mL Erlenmeyer flask and 1 large bag of the fungus MSX54809. The 250 mL flasks were combined and extracted. The 2800 mL Erlenmeyer flask and the large bag (each ~8 250 mL Erlenmeyer flasks) were extracted separately. These extractions were according to our conventional extraction protocol described earlier.

The third batch (Table 5), which also followed our conventional extraction protocol, involved 7 250 mL Erlenmeyer flasks. This was the first attempt to probe the growth of MSX54809 on other varieties of cheerios. Two flasks of each variety of berry, frosted, honey nut, and multigrain cheerios were used as culture media to ferment MSX54809, but the fungus failed to grow in a flask of the berry cheerios media.

Batch four (Table 5) was a repeat of batch three experiment but in this case, 4 250 mL Erlenmeyer flasks of each variety of berry, frosted, honey nut, and multigrain cheerios were cultured to ferment MSX54809 and were extracted after four weeks of growth. The results showed no significant difference in the growth of the fungus and amount of viridicatumtoxin produced across the varieties of cheerios tested.

Table 5. **Production of Viridicatumtoxin A across Batches**. Amount of extracts and isolated viridicatumtoxin A diminished drastically up to batch 4 despite using other varieties of cheerios (varied cheerios).

Batches	Media	Extract per Flask (mg)	VCT A per Flask (mg)
0	Multigrain Cheerios	211	35
1	Multigrain Cheerios	69	7.1
2	Multigrain Cheerios	70	1.5
3	Varied Cheerios	79	4.2
4	Varied Cheerios	58	1.2
5	Multigrain Cheerios	117	15

3.3. **Production of Viridicatumtoxin A Attenuated**

By the time prep HPLC of the fourth batch was completed, the production of viridicatumtoxin A had waned by about 92% of expected yield. This occurrence is similar to what has been reported in subcultures of endophytic fungal strains, where metabolite production becomes attenuated after a series of subculturing exercises.[37]

Following the recommendation of our resident mycologist, we decided to carry out quantitation studies to determine which of our 7 in-house slants of MSX54809 still retained the best growth potentials.

As described above ten calibration standards: 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL to 0.125 µg/mL were prepared. The test samples (from the seven plates of MSX54809) were prepared to a concentration of 1000 µg/mL each. Both the calibration standards and the test samples were prepared in DMSO, and 200 µL of each solution was pipetted into a 1 mL 96 well plate. A blank with zero concentration of analyte was put into the plate to run directly before and directly after the standard calibrations as control. For this quantitation study, a UPLC Acquity system equipped with a BEH C-18 (1.7 mm; 50 × 2.1 mm), a flow rate of 0.4 mL/min, column and sample manager temperatures of 40 ^{0}C and 10 ^{0}C respectively was used. The gradient utilized consisted of CH_3CN – H_2O (with 0.1 % formic acid) rising from 45 to 100 % CH_3CN between 0 –5 min and holding 100% CH_3CN for 2 mins; 5.0 –7.0 min, returning to starting conditions and holding it for 10 secs.

The study was done in biological and experimental triplicates. The data obtained was graphed (Figure 15) as instrument's response (Area under the Curve) and concentration of standard solutions. Thus, a good linear relationship quantified as R^2 emerged. Quantitation of the analyte, viridicatumtoxin A, was based on comparing the instrument's response acquired from the analyte in each test sample with instrument's response from a set of calibration standards of known concentration. Thus, the concentration of viridicatumtoxin A in each test sample was calculated (Figure 16).

Figure 15. **Calibration Curve 2 for Viridicatumtoxin A**. Area Under Curve (AUC) measured by UPLC-ESIMS vs eight standard concentration levels in DMSO which ran in triplicate.

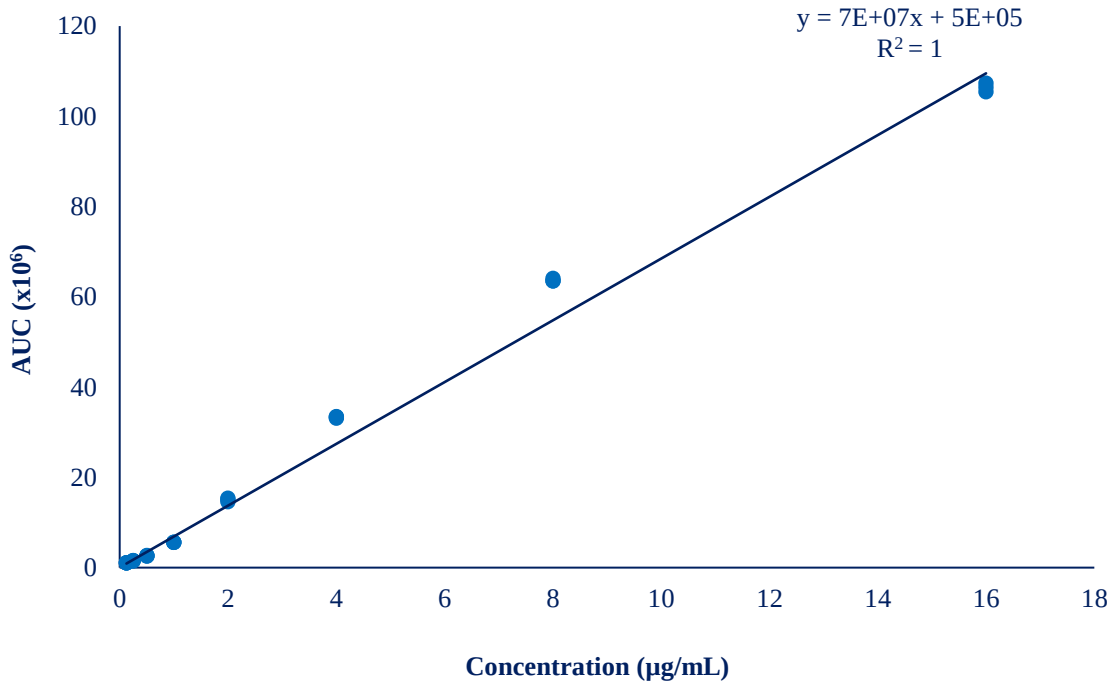
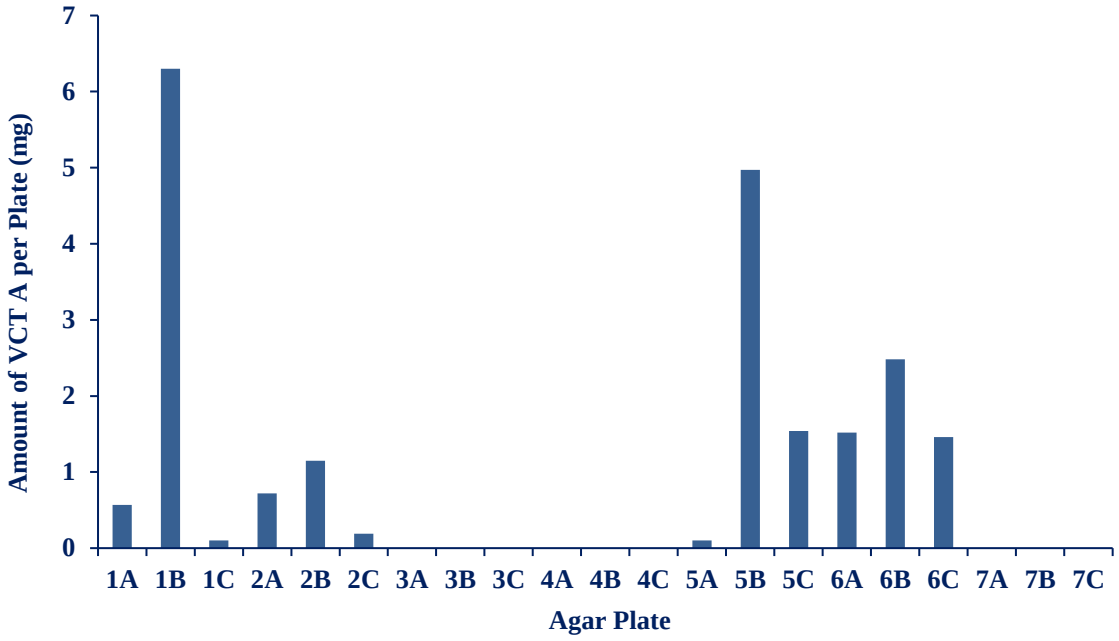
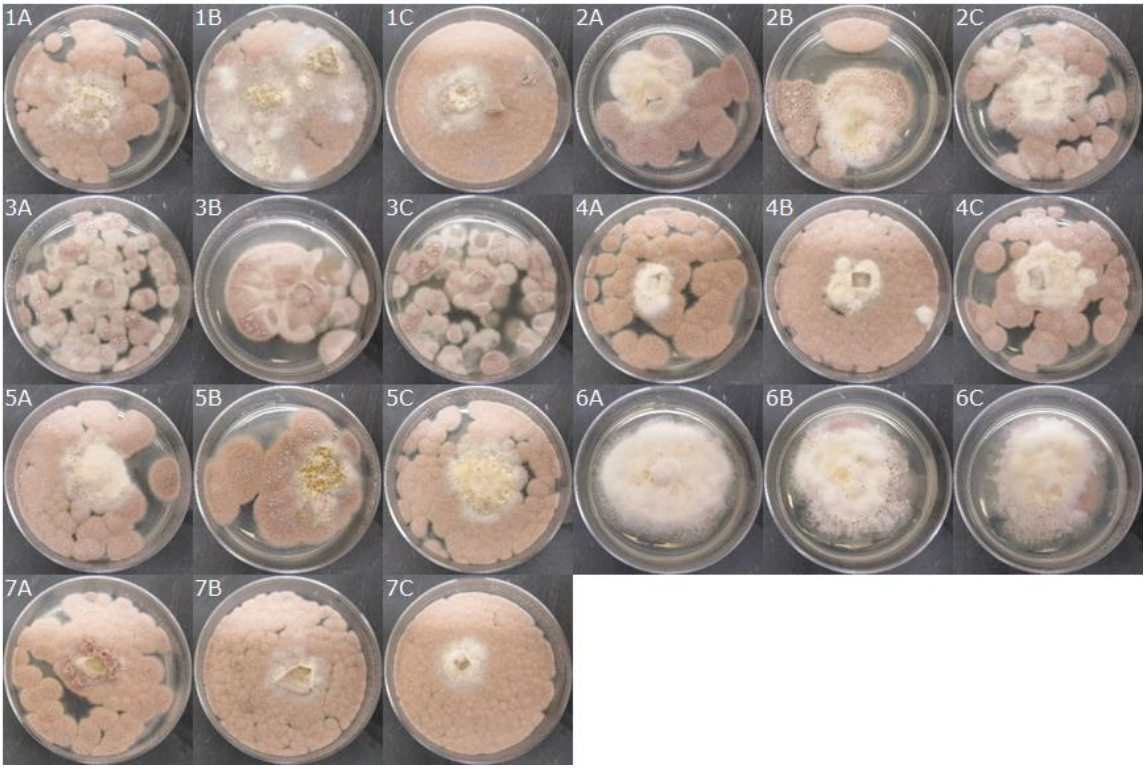


Figure 16. **Quantitation of VCT A in Agar Extract**. A, B and C are replicates of the same fungal colony.



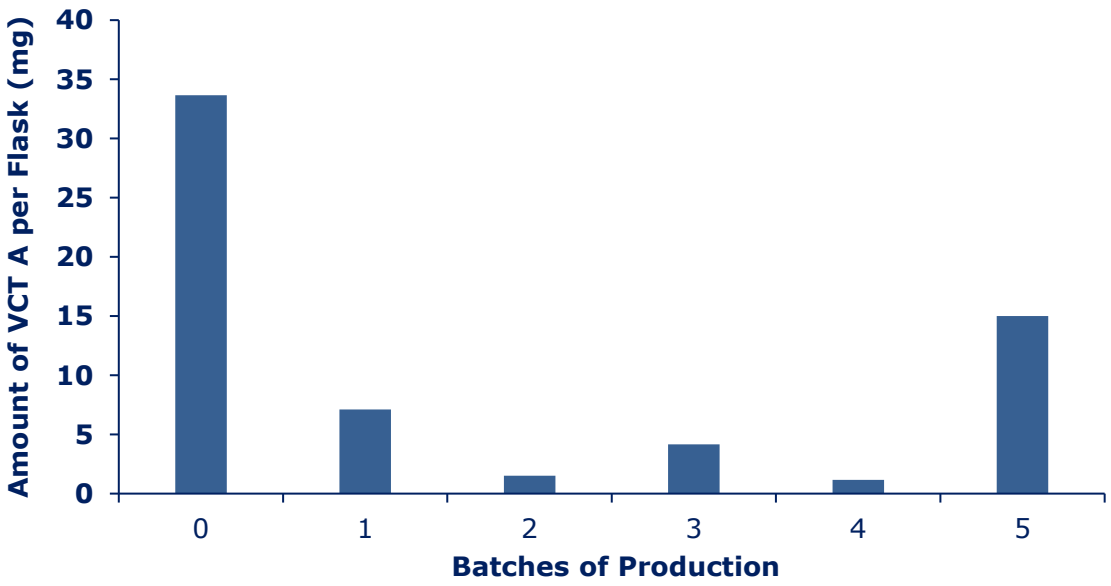
The appearance of the plate cultures of the *Purpureocillium lavendulum*, MSX54809, after four weeks of growth clearly showed variations in color and morphology across and within replicate plates (Figure 17). The results showed that plate six replicates were the most consistent replicates used for this study. It was also observed across replicate plates that guttate formation, which was present in plate six replicates, corresponded to increased viridicatumtoxin A production.[38] Therefore, we concluded to proceed with the next round of culturing with an inoculum of *Purpureocillium lavendulum* from plate six.

Figure 17. **Plates Showing Growth of *Purpureocillium lavendulum* on Agar Media.** Variations of morphology within and across plates can be seen.



The last batch (Table 5) was made up of 8 250 mL Erlenmeyer flasks in which the fungus was grown for four weeks and the cultures were combined and extracted in line with our conventional extraction protocol. The results showed a rise in the yield of viridicatumtoxin A from an average of ~ 2 mg per flask in the last round of extraction to ~ 15 mg representing an 86% increase.

Figure 18. Summary of Viridicatumtoxin A Production per Flask.



The amount of viridicatumtoxin A quantitated in extract via calibration curve and the amount obtained from preparative HPLC tallied well when figures 6 and 11 above are compared. Hence, the attenuation of viridicatumtoxin A production suffered by *Purpureocillium lavendulum*, MSX54809, in the course of this studies is attributable to domestication.[37]

Fungal metabolites, like viridicatumtoxin A, are produced to enhance the competitiveness of a fungus in its complex and hostile habitats. When this aggressiveness is quenched by taking the fungus away from its natural habitat to the serene atmosphere of the laboratory, the fungus' competitive nature is suppressed. Hence, the ability of the fungi to produce secondary metabolites, is therefore hampered.

CHAPTER IV

CONCLUSION

4.1. Biosynthesis of Viridicatumtoxin A

The isolation of a secondary metabolite with a tetracyclic framework from fungi other than the bacterial group called *Streptomyces* elicited investigations into the biosynthesis of viridicatumtoxin A.[39] Isotopic incorporation of ^{13}C, ^{14}C and ^{18}O from different sources into viridicatumtoxin A by supplying the isotopes into a culture of *Penicillium exparzsum* was the first experiment aimed at understanding the biosynthesis of viridicatumtoxin A.[40]

In 2010, by means of DNA pyrosequencing, guided gene deletions and RNA silencing of the genome of a viridicatumtoxin A producing fungus, *Penicillium aethiopicum*, the gene clusters involved in the biosynthesis were located.[30] These gene clusters, named as "vrt", are fascinating examples of a hybridized polyketide-isoprenoid biosynthetic pathway in fungi.[30]

A pathway based on results from the acetate labelling and the gene cluster studies was put forward for the biosynthesis of viridicatumtoxin A. It is initiated by an asparagine-derived malonamoyl-CoA starter unit culminating in the formation of viridicatumtoxin A at step 14.[30]

The finding that viridicatumtoxin A is the last product of the proposed biosynthetic pathway also supported our earlier consideration of attenuation due to domestication. This prompted us to affirm that the decline in the production of viridicatumtoxin A in the course of this study from *Purpureocillium lavendulum*, MSX54809, may have been caused by diminution in secondary metabolism due to domestication or sub-culturing (as described above).[37]

4.2. Mechanism of Antibacterial Activities

Viridicatumtoxin A, along with viridicatumtoxin B possess antibacterial activities by reason of their tetracyclic naphthacenedione core. The mechanism of action for their antibacterial properties was reported by Tomoda et al. to be through the inhibition of undecaprenyl pyrophosphate (UPP) synthase. UPP synthase is an enzyme essential to the biosynthesis of peptidoglycan in bacteria.

The assay involved calculating the amount of phosphate liberated from inorganic pyrophosphatase (IPP) obtained from *Saccharomyces cerevisiae* when incubated with UPP synthase and viridicatumtoxin A at 37˚C. Viridicatumtoxin A inhibited UPP synthase activity with an IC_{50} value of 4.0 µM. This result further differentiated viridicatumtoxin A from the tetracycline family, which inhibits protein synthesis in bacteria by binding to the 30s subunit of ribosome.

4.3 Mechanism of Anticancer Activities

Viridicatumtoxin A and B have demonstrated anticancer properties in cytotoxicity assays. The IC_{50} values obtained from these tests have raised the prospects of viridicatumtoxin A becoming a cancer therapeutic agent in the foreseeable future. The realization of this lofty goal depends among others on determining the actual mechanism by which viridicatumtoxin A inhibits cancer cell lines.

4.4 Semi-synthesis Challenge

The oxidation of the hydroxyl group on C-5 of viridicatumtoxin A to a ketone, thereby forming viridicatumtoxin B, which is the second part of this project remains a work in progress. The main challenge of this experiment is the ability to efficiently and selectively oxidize only the hydroxyl group on C-5 of viridicatumtoxin A while avoiding over oxidation to carboxylic acid and oxidation of other adjacent hydroxy groups. The development of highly chemo-selective synthetic conditions is overcoming this challenge.

4.5. Toxicity

Reports of toxicity in rats and mice that were dosed with viridicatumtoxin A have raised concerns about the toxicity of this compound to humans.[26] These concerns need to be resolved through appropriate and adequate in vivo evaluations in experimental animals before any application for clinical trials can be considered.

4.6 Future Considerations

Viridicatumtoxin A and analogues constitute indeed an interesting class of compounds that nature has given us. Their antibacterial properties, which currently favors the Gram-positive bacteria are well-documented but investigations into their cytotoxic activities is beginning to gather momentum.

Preliminary results of the semi-synthesis of viridicatumtoxin B, our goal number two (in collaboration with the Croatt lab), have shown great progress towards the development of a novel synthetic protocol that affords optimum yields of pure viridicatumtoxin B. The possibility of making nontoxic synthetic analogues with surpassing cytotoxicity is also within the area of consideration.

More screening of the vast Mycosynthetix library for additional viridicatumtoxin A producing fungi with the aim of further raising the optimization bar which will serve as production factory to conduct more successful synthetic experiments. It is suggested that these fungal strains when discovered should be tested on cheerios to determine their levels of output in terms of viridicatumtoxin A production.

REFERENCES

1. dos Santos Silva, I., *Cancer Epidemiology: Principles and Methods*. International Agency for Research on Cancer: 1999.

2. Society, A. C. Early History of Cancer. https://www.cancer.org/cancer/cancer-basics/history-of-cancer/what-is-cancer.html (accessed 3/26/2018).

3. Alberts, B.; Johnson, A.; Lewis, J.; Morgan, D.; Raff, M.; Roberts, K.; Walter, P., *Molecular Biology of the Cell, Sixth Edition*. Taylor & Francis Group: 2014.

4. Kinghorn, A. D.; De Blanco, E. J. C.; Lucas, D. M.; Rakotondraibe, H. L.; Orjala, J.; Soejarto, D. D.; Oberlies, N. H.; Pearce, C. J.; Wani, M. C.; Stockwell, B. R.; Burdette, J. E.; Swanson, S. M.; Fuchs, J. R.; Phelps, M. A.; Xu, L.; Zhang, X.; Shen, Y. Y., Discovery of Anticancer Agents of Diverse Natural Origin. *Anticancer Research* **2016,** *36* (11), 5623-5637.

5. Organization, W. H. Cancer. www.who.int/nmh/publications/fact_sheet_cancers_en.pdf (accessed 3/26/2018).

6. Keller, N. P.; Turner, G.; Bennett, J. W., Fungal secondary metabolism [mdash] from biochemistry to genomics. *Nat Rev Micro* **2005,** *3* (12), 937-947.

7. L., S. R.; D., M. K.; Ahmedin, J., Cancer statistics, 2018. *CA: A Cancer Journal for Clinicians* **2018,** *68* (1), 7-30.

8. El-Elimat, T.; Figueroa, M.; Raja, H. A.; Graf, T. N.; Swanson, S. M.; Falkinham, J. O.; Wani, M. C.; Pearce, C. J.; Oberlies, N. H., Biosynthetically Distinct Cytotoxic Polyketides from Setophoma terrestris. *European Journal of Organic Chemistry* **2015,** *2015* (1), 109-121.

9. Narang, A. S.; Desai, D. S., Anticancer Drug Development. In *Pharmaceutical Perspectives of Cancer Therapeutics*, Lu, Y.; Mahato, R. I., Eds. Springer US: New York, NY, 2009; pp 49-92.

10. Baguley, B. C.; Kerr, D. J. Anticancer drug development. http://www.sciencedirect.com/science/book/9780120726516.

11. DeVita, V. T.; Chu, E., A History of Cancer Chemotherapy. *Cancer Research* **2008,** *68* (21), 8643.

12. Feher, M.; Schmidt, J. M., Property Distributions: Differences between Drugs, Natural Products, and Molecules from Combinatorial Chemistry. *Journal of Chemical Information and Computer Sciences* **2003,** *43* (1), 218-227.

13. Cragg, G. M.; Newman, D. J., NATURAL PRODUCTS: A CONTINUING SOURCE OF NOVEL DRUG LEADS. *Biochimica et biophysica acta* **2013,** *1830* (6), 3670-3695.

14. Newman, D. J.; Cragg, G. M., Natural Products as Sources of New Drugs over the Last 25 Years. *Journal of Natural Products* **2007,** *70* (3), 461-477.

15. Newman, D. J.; Cragg, G. M., Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010. *Journal of Natural Products* **2012,** *75* (3), 311-335.

16. Clardy, J.; Walsh, C., Lessons from natural molecules. *Nature* **2004,** *432* (7019), 829-837.

17. Newman, D. J.; Cragg, G. M., Natural Products as Sources of New Drugs from 1981 to 2014. *Journal of Natural Products* **2016,** *79* (3), 629-661.

18. Hong, J., Natural product diversity and its role in chemical biology and drug discovery. *Current opinion in chemical biology* **2011,** *15* (3), 350-354.

19. Khazir, J.; Mir, B. A.; Mir, S. A.; Cowan, D., Natural products as lead compounds in drug discovery. *Journal of Asian Natural Products Research* **2013,** *15* (7), 764-788.

20. Raja, H. A.; Miller, A. N.; Pearce, C. J.; Oberlies, N. H., Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *Journal of Natural Products* **2017,** *80* (3), 756-770.

21. Rivera-Chavez, J.; Raja, H. A.; Graf, T. N.; Gallagher, J. M.; Metri, P.; Xue, D.; Pearce, C. J.; Oberlies, N. H., Prealamethicin F50 and related peptaibols from Trichoderma arundinaceum: validation of their authenticity via in situ chemical analysis. *RSC Advances* **2017,** *7* (72), 45733-45741.

22. Chiang, Y.-M.; Lee, K.-H.; Sanchez, J. F.; Keller, N. P.; Wang, C. C. C., Unlocking Fungal Cryptic Natural Products. *Natural product communications* **2009,** *4* (11), 1505-1510.

23. Bills, G. F.; Gloer, J. B., Biologically Active Secondary Metabolites from the Fungi. *Microbiology Spectrum* **2016,** *4* (6).

24. Evidente, A.; Kornienko, A.; Cimmino, A.; Andolfi, A.; Lefranc, F.; Mathieu, V.; Kiss, R., Fungal metabolites with anticancer activity. *Natural Product Reports* **2014,** *31* (5), 617-627.

25. Capon, R. J.; Ratnayake, R.; Stewart, M.; Lacey, E.; Tennant, S.; Gill, J. H., Aspergillazines A-E: novel heterocyclic dipeptides from an Australian strain of Aspergillus unilateralis. *Organic & Biomolecular Chemistry* **2005,** *3* (1), 123-129.

26. Hutchison, R. D.; Steyn, P. S.; van Rensburg, S. J., Viridicatumtoxin, a new mycotoxin from Penicillium viridicatum westling. *Toxicology and Applied Pharmacology* **1973,** *24* (3), 507-509.

27. Shang, Z.; Salim, A. A.; Khalil, Z.; Quezada, M.; Bernhardt, P. V.; Capon, R. J., Viridicatumtoxins: Expanding on a Rare Tetracycline Antibiotic Scaffold. *The Journal of Organic Chemistry* **2015,** *80* (24), 12501-12508.

28. Raju, M.; Wu, G. S.; Gard, A.; Rosazza, J., Errata -"Microbial Transformation of Natural Antitumor Agents. 20. Glucosylation of Viridicatumtoxin". *Journal of Natural Products* **1982,** *45* (5), 654-654.

29. Zheng, C.-J.; Yu, H.-E.; Kim, E.-H.; Kim, W.-G., Viridicatumtoxin B, a new anti-MRSA agent from Penicillium sp. FR11. *J Antibiot* **2008,** *61* (10), 633-637.

30. Chooi, Y.-H.; Cacho, R.; Tang, Y., Identification of the Viridicatumtoxin and Griseofulvin Gene Clusters from Penicillium aethiopicum. *Chemistry & Biology* **2010,** *17* (5), 483-494.

31. Inokoshi, J.; Nakamura, Y.; Hongbin, Z.; Uchida, R.; Nonaka, K.-i.; Masuma, R.; Tomoda, H., Spirohexalines, new inhibitors of bacterial undecaprenyl pyrophosphate synthase, produced by Penicillium brasilianum FKI-3368. *J Antibiot* **2013,** *66* (1), 37-41.

32. Nicolaou, K. C.; Nilewski, C.; Hale, C. R. H.; Ioannidou, H. A.; ElMarrouni, A.; Koch, L. G., Total Synthesis and Structural Revision of Viridicatumtoxin B. *Angewandte Chemie International Edition* **2013,** *52* (33), 8736-8741.

33. Nicolaou, K. C.; Hale, C. R. H.; Nilewski, C.; Ioannidou, H. A.; ElMarrouni, A.; Nilewski, L. G.; Beabout, K.; Wang, T. T.; Shamoo, Y., Total Synthesis of Viridicatumtoxin B and Analogues Thereof: Strategy Evolution, Structural Revision, and Biological Evaluation. *Journal of the American Chemical Society* **2014,** *136* (34), 12137-12160.

34. Ayers, S.; Graf, T. N.; Adcock, A. F.; Kroll, D. J.; Matthew, S.; Carcache de Blanco, E. J.; Shen, Q.; Swanson, S. M.; Wani, M. C.; Pearce, C. J.; Oberlies, N. H., Resorcylic Acid Lactones with Cytotoxic and NF-κB Inhibitory Activities and Their Structure–Activity Relationships. *Journal of Natural Products* **2011,** *74* (5), 1126-1131.

35. Perdomo, H.; Cano, J.; Gené, J.; García, D.; Hernández, M.; Guarro, J., Polyphasic analysis of Purpureocillium lilacinum isolates from different origins and proposal of the new species Purpureocillium lavendulum. *Mycologia* **2013,** *105* (1), 151-161.

36. VanderMolen, K. M.; Raja, H. A.; El-Elimat, T.; Oberlies, N. H., Evaluation of culture media for the production of secondary metabolites in a natural products screening program. *AMB Express* **2013,** *3*, 71-71.

37. Li, J. Y.; Sidhu, R. S.; Ford, E. J.; Long, D. M.; Hess, W. M.; Strobel, G. A., The induction of taxol production in the endophytic fungus—Periconia sp from Torreya grandifolia. *Journal of Industrial Microbiology and Biotechnology* **1998,** *20* (5), 259-264.

38. Figueroa, M.; Jarmusch, A. K.; Raja, H. A.; El-Elimat, T.; Kavanaugh, J. S.; Horswill, A. R.; Cooks, R. G.; Cech, N. B.; Oberlies, N. H., Polyhydroxyanthraquinones as Quorum Sensing Inhibitors from the Guttates of Penicillium restrictum and Their Analysis by Desorption Electrospray Ionization Mass Spectrometry. *Journal of Natural Products* **2014,** *77* (6), 1351-1358.

39. Chooi, Y.-H.; Wang, P.; Fang, J.; Li, Y.; Wu, K.; Wang, P.; Tang, Y., Discovery and Characterization of a Group of Fungal Polycyclic Polyketide Prenyltransferases. *Journal of the American Chemical Society* **2012,** *134* (22), 9428-9437.

40. de Jesus, A. E.; Hull, W. E.; Steyn, P. S.; van Heerden, F. R.; Vleggaar, R., Biosynthesis of viridicatumtoxin, a mycotoxin from Penicilium expansum. *Journal of the Chemical Society, Chemical Communications* **1982,** (16), 902-904.