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Natural products have proven to be a good source of anticancer drugs since the 1940's. In fact, they have influenced the creation of 65% of all 246 anticancer drugs since that time. Fungi in particular provide an almost untapped source of compounds with promising bioactivity. To access compounds from natural products, an extraction and purification process using flash chromatography, and high performance liquid chromatography is needed. Characterization of those compounds is done using high-resolution mass spectrometry and NMR spectroscopy. These methods were used to isolate and characterize six known compounds that were added to an in-house database. Leporin B, leporin C, austdiol and 7-*epi*-austdiol were all isolated from *Scytalidium cuboideum* strain 68345. Leporin B has specifically proven to be effective in different aspects of research including, its effect on hexokinase II and it has shown potential as a cytotoxic compound towards cancer cells. The re-isolation of leporin B has increased the in-house supply for use in future experimentation. Through the re-isolation of leporin B, leporin C was found. Leporin C is new to natural product; however, the compound has been previously synthesized, and it only differs from the structure of leporin B by one oxygen atom. Austdiol and 7-*epi*-austdiol were also isolated during the search for leporin B. Fungal strain MSX74367 was also studied, and yielded, 9-*O*-methylbostrycoidin and 8-*O*-methylfusarubin. The benzoisoquinoline structure, the core structure of 9-*O*-methylbostrycoidin and 8-*O*-methylfusarubin, is new to the current in-house database of over 300 compounds isolated from fungi. Mass defect analysis and high-resolution mass

spectrometry were used in an effort to isolate other benzoisoquinoline type compounds through mass guided fractionation; however, the process was unsuccessful in finding additional analogues. In total, six compounds were found from two different strains of fungi, all of which were new to an in-house database. The fungus *Scytalidum cuboideum,* in particular, produced leporin B and leporin C, which was isolated for the first time from a natural source.

ISOLATION OF FUNGAL SECONDARY METABOLITES: LEPORIN B, BENZOISOQUINOLINE AND RELATED COMPOUNDS

by

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APPROVAL PAGE

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Cancer

Cancer is one of the leading causes of death in the world today. People have come to fear the very name, because it is known to be an extremely difficult disease to treat. But how far back has cancer been affecting life? According to the American Cancer Society, cancer was first described in an Egyptian textbook called the Edwin Smith Papyrus around 3000 BC (1). The book expressed the existence of a few different types of cancer and how to remove them using surgery, however it also expresses that there is not a treatment for cancer. The word cancer was not portrayed until Hippocrates, somewhere between 460 and 370 BC (1). At the time, Hippocrates used *carcino* and *carcinoma* to describe different types of cancer. The words were later translated into Latin, which became the word cancer, which is the descriptive word for crab, the reference to how the cancer appeared with its protruding features. Since the first discovery of cancer, people have tried to understand it and a number of theories were expressed in order to describe the disease. When Hippocrates studied cancer, he explained it as the result of an imbalance in the four humors of the body, which included: blood, phlegm, yellow bile and black bile (2). That theory was later replaced by the Lymph theory, which said that the formation of cancer was the result from fermenting

and degenerating lymph that flows through the body (2). The Blastema theory was next, fermenting and degenerating lymph that flows through the body (2). The Blastema theory was next, expressing cancer was made up of cells and not lymph and that those cells were created from other cells (2). The chronic irritation theory followed, explaining that cancer was due to chronic irritation and that it spread through the body as a liquid (2). A German surgeon, who expressed the spread of cancer through malignant cells, later disproved aspects of this theory. Other theories included the trauma theory and the infectious disease theory.

The trauma theory was based on the idea that trauma caused cancer, but was later disproved through animal testing (2). The infectious disease theory expressed cancer was contagious (2). As of today, we know that there is a long list of things that can be the cause of cancer. Some of those compounds include: coal tars, hydrocarbons, benzene, aniline, asbestos, ionizing radiation and some viruses (3). These aspects explain the initiation; it is what they do to the cells in the body that has been the unanswerable question until the past century. Cancer is the proliferation of cells that have damaged DNA (3). Cells in the body have a specific mechanism that causes apoptosis in cells that have damaged DNA (3). Cancer occurs where these cells do not perform apoptosis (3). The rapid growth of these cells with damaged DNA can invade into various areas of the body and affect the function of vital organs, leading to death. One reason for the difficulty in finding a treatment for cancer is that a number of cells can mutate and copy itself, therefore, cancer can start anywhere in the body. This aspect leads to cancers of the skin, bones, lungs, breast, colon, blood and other areas of the body. There are three

different treatments that can be used in the fight against cancer including surgery, chemotherapy and radiation (4). Surgery is a method used when trying to remove tumors, the cancerous cells. Chemotherapy is a method where compounds that are toxic to rapidly dividing cells are infused into the body in the hopes of killing the cancer cells. Radiation is a method that uses x-rays to target a specific area of the body and kill the cells in that area (5). Based on the type of cancer and the extent to which it has grown, these methods can be used in varying combinations. However, these methods are not always effective.

In terms of statistics, cancer is one of the leading causes of death worldwide. In 2012 there were approximately 14 million new cases of cancer worldwide and 8.2 million deaths related to cancer (6). As of 2016, it is believed that there will be 1,685,210 cancer cases and 595,690 cancer related deaths in the United States alone (7). Even with great strides to reduce the effects of cancer, death rates are still increasing in the US in cancers of the liver, pancreas and uterine corpus. With the reduction of deaths due to heart disease, cancer is also becoming the leading cause of death in a number of states. Finding a potential cure or treatment for this disease has become increasingly significant as time goes on, and one front that may lead to active drug compounds for use as chemotherapeutics is natural products.

Natural Products

Natural products chemistry can produce active compounds for drug development. Of the three approaches to cancer treatment, chemicals that can be infused in the body are effective at attacking cancer cells that have metastasized. The interaction that nature's compounds have with enzymes and proteins of living tissue allow those chemicals to be

effective active ingredients for different drugs. From a statistical perspective, of 1562 newly approved drugs from 1981-2014, 4 % come from unaltered natural products, 21 % from natural product derivatives, and 1 % from botanical drugs (8). Looking at the 246 anticancer drugs ranging from the 1940's to 2014, 12 % come from unaltered natural products and 25 % from natural product derivatives (8). Looking at best selling drugs today, natural products makes up nearly 45 % (9). Nature has provided the chemistry that can aid in future drug development, but it needs to be further studied.

Fungi

Fungi are diverse in species and they can be grown with relative ease. Therefore, isolation of compounds from fungal samples is a good start in the search for potential anticancer drugs. Fungi produce interesting chemistry that have proven useful in the field of drug discovery and development, and there is such a large variety of fungal samples around the world to be sampled that the possibility for new drugs is almost endless. There are nearly 135,000 species of fungi that have been described out of the believed 1.5-5.5 million species in the world (9). From the research done on those fungi, secondary metabolites have provided compounds such as penicillins and cephalosporin, cyclosporine A, statins, echinocandin B, fingolimod, lentinan, methergin, and strobilurins. These compounds are the active ingredients that make up some antibiotics, immunosuppressants, cholesterol-lowering agents, antifungals, multiple sclerosis treatments, anticancer drugs, and treatments for hemorrhage and fungicides, respectively (10). These secondary metabolites can be affected by the environment, leading to fungi around the world producing different secondary metabolites to respond to environmental

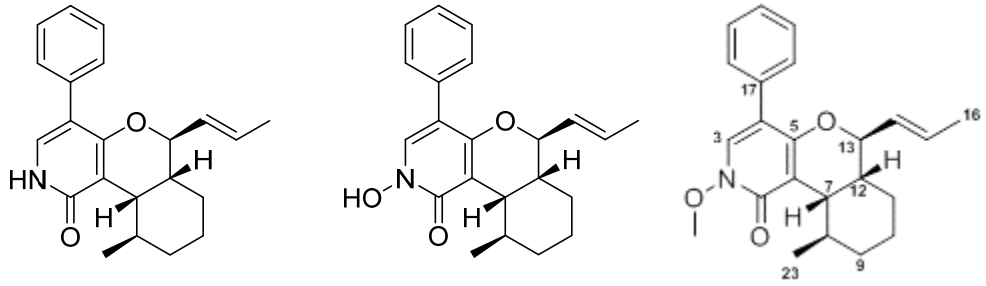
changes. This is where close work with Mycosynthetix comes in. Mycosynthetix is a small business that collects and grows various strains of fungi from around the world. Dr. Cedrick Pierce, Mycosynthetix CEO, has provided the ability to access a library of over 55,000 species of filamentous fungi, each of which have the potential of producing an average of three unique compounds (11). To put that number in comparison, literature shows less than 24,000 metabolites reported from fungi. Natural products chemistry has only scratched the surface of fungal metabolites and further study is needed for the advancement of medicinal chemistry.

History of Leporin

Leporin A was first isolated from sclerotia of *Aspergillus leporis* that was growing on white-tailed jackrabbit dung in 1991 (12). The compound was tested and showed moderate activity against corn earworm, helicoverpazea, and mild activity as an antibacterial compound. The compound was fully synthesized in 1996 (13). Later on in 2003, leporin B was isolated from a fungal strain in Victoria Mexico (14). It was tested initially as a compound that would decrease the activity of Hexokinase II, an enzyme that alters sugars as they enter the cell (14). This can be a problem with diabetic patients that have too much sugar in their cells and low blood sugar. Therefore, leporin B decreased the activity of Hexokinase II so that the sugar molecules can enter back into the blood stream. Leporin A did not show activity in this regard, showing the importance in changing a structure by one side chain. Leporin A and B have the same core structure and side substituents with the exception being the side chain on the nitrogen, shown in (Fig. 1). Leporin A has a methoxy group on the nitrogen and leporin B has an alcohol group on

the nitrogen. Leporin B was later isolated from the fungus *Scytalidium cuboideum* (15). The compound was tested for its activity against breast, lung, and astrocytoma cancer cell lines. The activity of leporin B against these three cancer cell lines was only an order of magnitude lesser from the activity of the positive control camptothecin (15). Therefore, we hypothesized further study of leporin B could lead to more active analogues.

Figure 1. Structures of Leporin A, Leporin B, and Leporin C Respectively (13)



CHAPTER II

METHODOLOGY

Flash chromatography is an effective tool for the initial separation of compounds. This method uses normal phase silica columns to separate compounds based on polarity. This process takes an extract that can be anywhere from 20 mg to one g, and it creates a number of fractions in less time than it would take using other methods (16). The solutions used start with 100 % hexane and go to 100 % chloroform and finally to 100 % methanol. The small scale (10 g of rice and 20 mL of H_2O) fungal samples typically use four gram columns that allow for 20 mg-400 mg of sample to be injected. The stationary phase is based on silica gel that allow the attachment of polar or nonpolar groups, allowing for normal or reverse phase columns to be made. Silica is also used due to its ability to refine the separation between closely eluting compounds, decreased back pressure on the instrument, and allow higher sample concentrations which reduces the solvent needed and therefore reducing solvent evaporation time. High performance liquid chromatography preparative columns, on the other hand, can only handle approximately and it takes time and solvent to clean. Therefore, flash chromatography is a useful first stage separation of an extract.

HPLC is a liquid chromatography technique that can separate compounds based on size, shape, and charge. The difference between flash chromatography and HPLC is efficiency HPLC has more selectivity and separates compounds using retention time more completely, because the columns are packed more tight and the columns are longer, which allows for more interaction between the compound and the stationary phase of the column (17). This process is used to more accurately purify compounds from fractions that are created by flash chromatography. In relation to fungal sample purification, HPLC typically uses reverse phase columns. Reverse phase chromatography uses a non-polar stationary phase, made with silica beads coated in long carbon chains, which allow polar compounds to elute off the column first and nonpolar compounds to elute at the end of a chromatogram. The HPLC system is also made to allow for higher pressures to be used for different samples. HPLC is the main method used for purification in fungal extract analysis, and when the sample is pure, ultra-performance liquid chromatography can be used to confirm purity. UPLC operates similarly to HPLC and flash chromatography, but it uses less sample. Once all purification is performed and a pure sample is obtained, analysis can be done using mass spectrometry and nuclear magnetic resonance.

Analytical methods such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) are used for their high sensitivity and ability to provide essential aspects of a compound for identification. Mass spectrometry takes a sample, ionizes that sample, sends it through a mass analyzer, and finally the ions made are sent to a detector to provide a spectrum for analysis. There are a number of mass spectrometer injection methods, such as electron ionization, chemical ionization, electrospray ionization and

matrix assisted laser desorption ionization. Each ionization method has its advantages and disadvantages, but electrospray ionization is the ionization of choice for most natural product compound analysis (18, 19). The main idea of a mass analyzer is the rotation of ions in a chamber using magnetic poles. Ions are selected based of magnitude of rotation created by the magnetic field. The magnetic poles can be in groups of 4, a quadrupole, or 8, an octupole. Mass analyzers can also include ion collision cells that produce MS/MS results and an orbitrap that produces separation of signal that hits a detector. Finally there is the detector that produces the spectrum that is used for analysis. Mass data can be used to determine the molecular weight of a compound and MS/MS data that can provide structural data for use in dereplication, a method for filtering data showing known compounds. The accurate mass obtained can be compared to NMR data to allow the characterization of compounds found from purified organic extracts of fungal samples.

A nuclear magnetic resonance spectrometer (NMR) is an instrument that uses a magnet and radio frequency detector to monitor the signal produced from protons of different chemical environments as they relax through energy states. The data created provides the number of protons in a compound through the use of integrals, the chemical environment of protons using chemical shifts, connectivity of protons using coupling constants and the relation of protons through space using relaxation effects. The combination of data from mass spectrometry, molecular mass, and NMR, proton environments, can provide almost everything needed to solve the structure of a pure compound.

MS and NMR provide a large portion of the data needed to solve the structure of a compound, but there are other pieces of data that allow for structure elucidation such as: ultraviolet spectroscopy (UV), infrared spectroscopy (IR), and x-ray crystallography (17). Ultraviolet spectroscopy provides data about conjugation, or pi-bond character and ring structures. IR spectroscopy data are used to determine the presence of certain functional groups. X-ray crystallography is the final piece of data that essentially gives a picture of the molecule. The combination of all of these analytical methods with NMR and MS allows for accurate structure elucidation of a compound.

Mass spectrometry is an effective tool when it comes to determining the structure of specific compounds found in a sample. There are cases where a specific type of compound or structure is being isolated, and mass spectrometry can be used to determine the presence of that compound or not. Therefore, mass spectrometry data can be used at various stages of the purification process in order to obtain the compound of interest through specific fractionation. There are methods for narrowing the search of the complicated mass spectrometry data, including the use of precursor ion and neutral loss scanning techniques, however, these techniques are not completely effective in highlighting both the common and uncommon metabolites of various drug compounds. Therefore, in 2003, mass defect filtering was expressed as a potential method for the determination of both common and uncommon metabolites from compounds of interest (20). It was determined that a specific algorithm could analyze the non-integral portion of the m/z value and reduce the cluttered high resolution mass spectrometry data to

metabolites that are more related structurally. This method is important due to its ability to provide masses that correlate in structure through metabolic pathways.

Mass defect filtering uses a set of parameters to sift through complex liquid chromatography high resolution mass spectrometry data to isolate metabolite ions from a biological matrix while at the same time, excluding the interference ions that do not correlate to the parent molecule in terms of mass defect values (20). This process reduces the background noise that is present in complex samples, thus allowing for the data of interest, analogues of a specific molecule, to be isolated without time consuming and tedious manual interpretation. To put this technique in perspective of a real world situation, say that the compound of interest was glucose, $C_6H_{12}O_6$ with a mass of 180.063385 Da. Glycolysis of glucose leads to the production of glucose-6-phosphate, $C_6H_{11}O_9P$ with a mass of 258.015167 Da. The mass difference of these compounds is 77.951782 Da, which correlates to the difference in HPO_3. The two compounds have the same core structure, but differ by the addition of a side substituent. If the mass spectrum of glucose-6-phosphate was being analyzed, the mass for glucose could be found in the mass spectrum due to the fact that it is an analogue of glucose-6-phosphate. However, searching for analogues of the parent compound in the complex data would take time and there is the potential for human error. This is where mass defect analysis comes in. The algorithm can analyze previously obtained mass data and select the specific peaks that correlate to a mass defect range to obtain potential analogues. The first step in mass defect analysis is to determine the mass defect, which is defined by the subtraction of the closest integer value from the exact mass. In the case mentioned above, that number is

defined as the change in mass minus the closest integer value, or 77.951782 – 78 = -0.048218 Da. That number is converted to mDa, which would be 48.218 mDa. Even though the difference in the masses of the compounds was approximately 78 Da, the mass defect difference in the molecules changed by less than 50 mDa. This shows that an analogue of a parent molecule can have a large mass difference; however, the mass defect is relatively small. Based on this analysis, parameters can be set to search for analogues of a parent molecule. Mass defect parameters can be set once, and for the purpose of this paper, the mass defect was set to less than 50 mDa. This parameter was chosen due to the various metabolite ions that fall within that range. Examples of this include: "hydroxylation changes the mass defect by -5 mDa, dehydrogenation by -16 mDa, demethylation by -23 mDa, glucuronidation by +32 mDa, and sulfation by -43 mDa" (20).

Mass defect filtering is a different approach to drug metabolite screening; however, it is complementary to the existing methods of precursor ion and neutral loss analysis (20). The advantage of mass defect over these methods is the use of instrumentation. Mass defect analysis uses a common accurate mass analysis method, whereas the other techniques require the use of a triple-quadrupole or a hybrid triple-quadrupole-linear ion trap mass spectrometer and some prior knowledge of the parent molecule (20). Mass defect analysis can take previously acquired high resolution mass spectrometry data and analyze it using a mathematical approach to obtain the compound masses of interest.

CHAPTER III

EXPERIMENTATION

Organic compounds were extracted from filamentous fungi that were grown on rice media. Fungal strains MSX68345 and MSX74367 had 60 mL 1:1 $CHCl_3$: CH_3OH added to it, and the mixture was cut up into small protions (21). The fungal samples were shaken at 100 rpm in their flask overnight, approximately 16 hr (21). The mixture was filtered using vacuum filtration and 90 mL $CHCl_3$ and 150 mL deionized H_2O were added (21). The mixture was stirred for 30 min and then transferred to a separatory funnel. The bottom organic layer was drawn off and dried down under vacuum. An aqueous sample was taken from the top layer of the separatory funnel and dried down under N_2 gas. The aqueous fraction was stored for further analysis. The organic fraction was reconstituted in 100 mL of 1:1 MeOH : MeCN and 100 mL of hexanes (21). The solution was transferred to a separatory funnel and shaken approximately 20 times. The bottom organic layer and top hexane layer were separately dried under vacuum, transferred to pre-weighed vials, and dried under N_2 gas lines.

Dried organic fractions were sent off for cytotoxicity against cancer cell lines, using approximately 0.5 mg of sample. The samples were tested against MDA-MB-435, MDA-MB-231 and OVCAR3, which correspond to human melanoma cancer cells, human breast cancer cells and human ovarian cancer cells respectively (22). Data from cytotoxicity testing was reported as percent survival in concentrations of 20 μg/mL and 2 μg/mL for each of the three cell lines. Samples showing less than 50 % survival were chosen to be further purified, prioritizing the smaller percent survivals. Cytotoxicity values can be reported as IC_{50} values when the compound is pure. This value represents the concentration of sample needed to kill or inhibit the growth of half of the cells in an assay. Therefore, lower IC_{50} values show better cytotoxicity results. Chosen cytotoxic samples were analyzed through dereplication, in order to focus resources on samples with promising new chemistry.

Dereplication uses ultra-performance liquid chromatography-photodiode array-high-resolution tandem mass spectrometry (UPLC-PDA-HRMS-MS/MS) with samples at a concentration of 2 mg/mL and compares the MS/MS data in positive and negative mode (23). To compare new mass data with previously identified natural products, a database was used that includes natural product structures, retention times, UV data, accurate mass and ten MS/MS peaks. Mass data from organic extracts of fungal samples can be compared with previously isolated compound mass data to determine the presence of know structures. Organic fractions with little to no correlation with the in house database of over 300 compounds were further prioritized over other fractions. This process allows for faster analysis of compounds in order to find new structures as opposed to re-isolating

known structures. Dereplication is also used to determine the existence of specific compounds for future study, such that if a specific compound was being searched for, the mass data from the organic fraction would show the presence of that compound in the sample. This technique is an effective tool in determining which samples should be further studied.

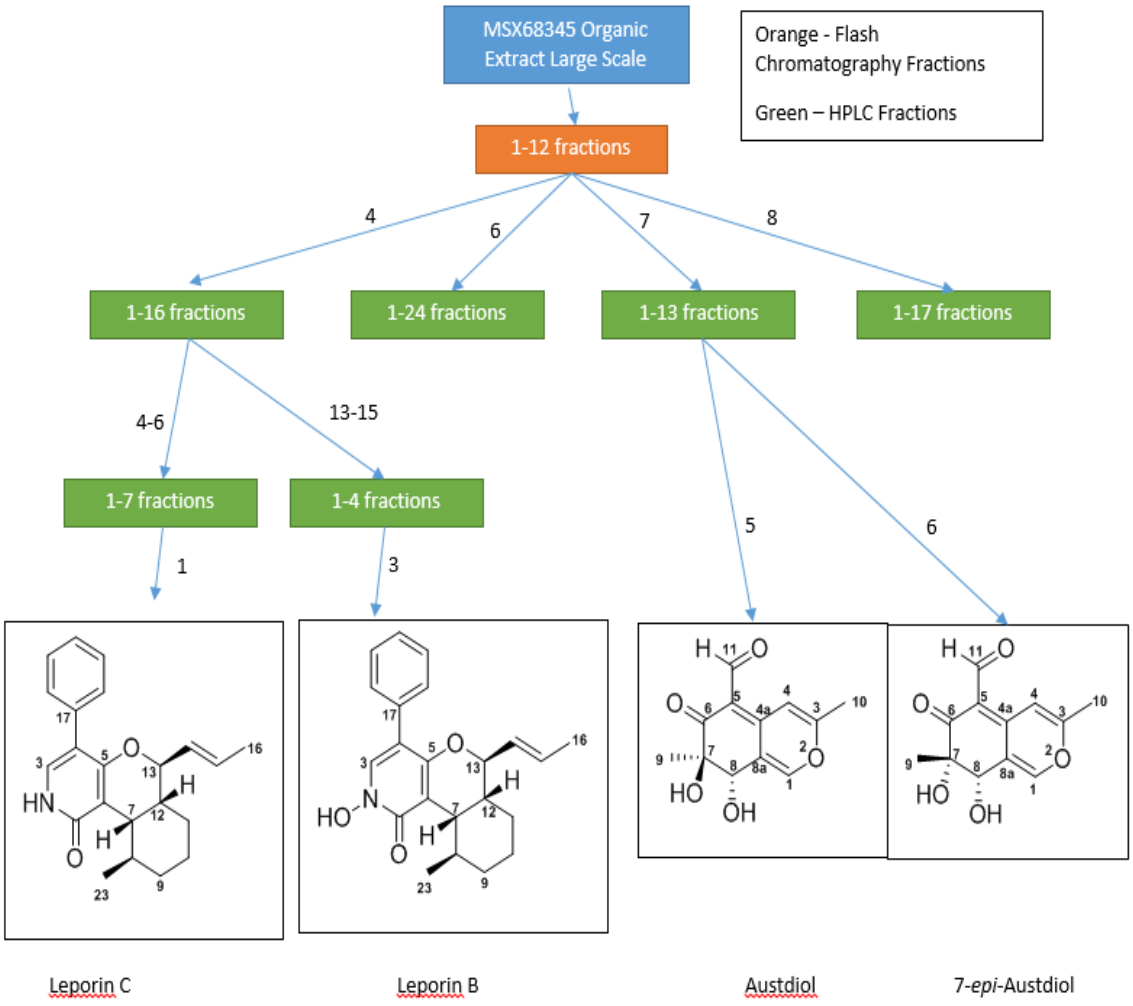
Organic extracts that showed good cytotoxicity and the potential for new natural products were pushed forward. The crude organic extract of MSX68345 and MSX74367 fungal strains were purified using normal phase flash chromatography using mobile phase that was changed from hexane to chloroform to methanol. The large scale organic fraction from MSX68345 was dissolved in a mixture of $CHCl_3$:MeOH and adsorbed on Celite. The sample was fractionated using flash chromatography on a 24 g silica column with a flow rate of 35 ml/min. The flash chromatography analysis was done using 1908 mg of sample with a hexane to chloroform to methanol gradient over a 25 minute chromatogram. The chromatogram provided 12 fractions (Fig. 2), with the addition of a fraction that was created when a compound could only be dissolved using water from the last fraction. From this flash chromatography analysis, four of the fractions were further purified using HPLC. Fraction four from the flash chromatography of the large scale MSX68345 was analyzed through HPLC using the C-18 reverse phase Atlantis T3 column using a 60 % isocratic gradient of acetonitrile water over the course of 30 minutes with a 17.06 mL/min flow rate. That purification chromatogram produced 16 fractions (Fig. 2). All 16 fractions were analyzed through high resolution mass spectrometry. The data collected from the mass spectrometry analysis allowed for the

identification of two fractions that may have contained leporin B, due to the mass being 352.19083 and 352.19080 amu for the two fractions. UPLC data showed that the fractions were pure enough to analyze via NMR. The NMR data was compared to the NMR data previously published for leporin B, which proved to be a match. When comparing the NMR data for fractions 13 through 15, it was determined that they were both leporin B. However this was a problem because the HPLC chromatogram showed the fractions as different peaks. Peaks in the HPLC chromatogram were all tested using NMR, and it was determined that the guard filter for the T3 column was not clean. The contaminant on the guard filter for the column caused the peaks to split where they should not have. Therefore the fractions were tested with NMR to confirm which peaks had the same compound. Fractions with the same compound based on NMR data were combined and further purified. Thus, fractions 13, 14 and 15 were combined to produce 18 mg of sample, which was purified using the HPLC with the C-18 reverse phase Atlantis T3 column. The gradient used was 65-100% acetonitrile water over 30 minutes, and four fractions were collected with a flow rate of 17.06 mL/min. Pure leporin B was obtained in the third fraction and confirmed with NMR data. Therefore, an additional 7 mg of pure leporin B was added to the current supply of less than 3 mg. Looking back at the HPLC purification from fraction four of the large scale, NMR data of fractions four through six showed similarities to each other and to the proton NMR of leporin B. Mass data for the fractions showed masses of 336.195 amu, which is a difference from leporin B by an oxygen. Therefore, the fractions were combined and further purified to produce seven fractions. This purification used the Atlantis T3 reverse phase C-18 column with a

55 % acetonitrile water isocratic gradient over 70 minutes. Proton and carbon NMR experiments were performed on fraction one to provide the structure of leporin C, which is new to natural products, but it is a known compound (13). This data showed the need for further analysis of fractions from MSX68345 due to the potential for additional analogues of leporin B.

MSX68345 Purification

Figure 2. Purification Fractions for MSX68345



Further analysis of the fungus was done by looking at fraction six from the large scale flash chromatography. The fraction was analyzed through HPLC on the C-18 reverse phase Gemini column with a 55-90 % acetonitrile water gradient over 30 min. A flow rate of 21.24 mL/min was used to create 24 fractions. NMR data of the fractions did not show signs of the leporin core structure, and the fractions were set aside. Fraction

seven from the same large scale flash chromatography analysis was purified on HPLC using the reverse phase C-18 Gemini column with a 5-30 % acetonitrile water gradient over 20 min, and then 30-100 over the last 10 minutes of the chromatogram. There were 13 fractions produced using a flow rate of 21.24 mL/min. Analysis of the fractions showed interesting UV data from the HPLC chromatogram, and some fractions formed crystals as they dried down. The crystals were analyzed to show the presence of austdiol and 7-epi-austdiol through x-ray crystallography. Finally, fraction 8 from the same large scale was purified on the HPLC using the C-18 reverse phase Gemini column with a gradient of 20-100 % acetonitrile water over the course of 20 min in order to produce 17 fractions using a flow rate of 21.24 mL/min. Proton NMR and mass data did not show the presence of the leporin core structure, and therefore the fractions were set aside.

Mycosynthetix fungi 74367 was another fungus of interest. The screener scale sample was fractionated using flash chromatography on a 4 g silica column with a flow rate of 18 ml/min. The flash chromatography analysis was done using 135 mg of sample with a hexane to chloroform to methanol gradient over an 18 minute chromatogram to create three fractions (Fig. 3). The third fraction was fractionated further using the HPLC. The purification used the Gemini C-18 reverse phase column with a 20-100 % acetonitrile water gradient over 20 minutes with a flow rate of 21.24 mL/min to create nine fractions. High resolution mass spectrometry and proton NMR experiments were performed on the fractions created. Fraction three was determined to be 9-*O*-methylbostrycoidin, a known compound confirmed through the use of the dictionary of natural products. This led to the search for benzoisoquinoline type structures. Therefore,

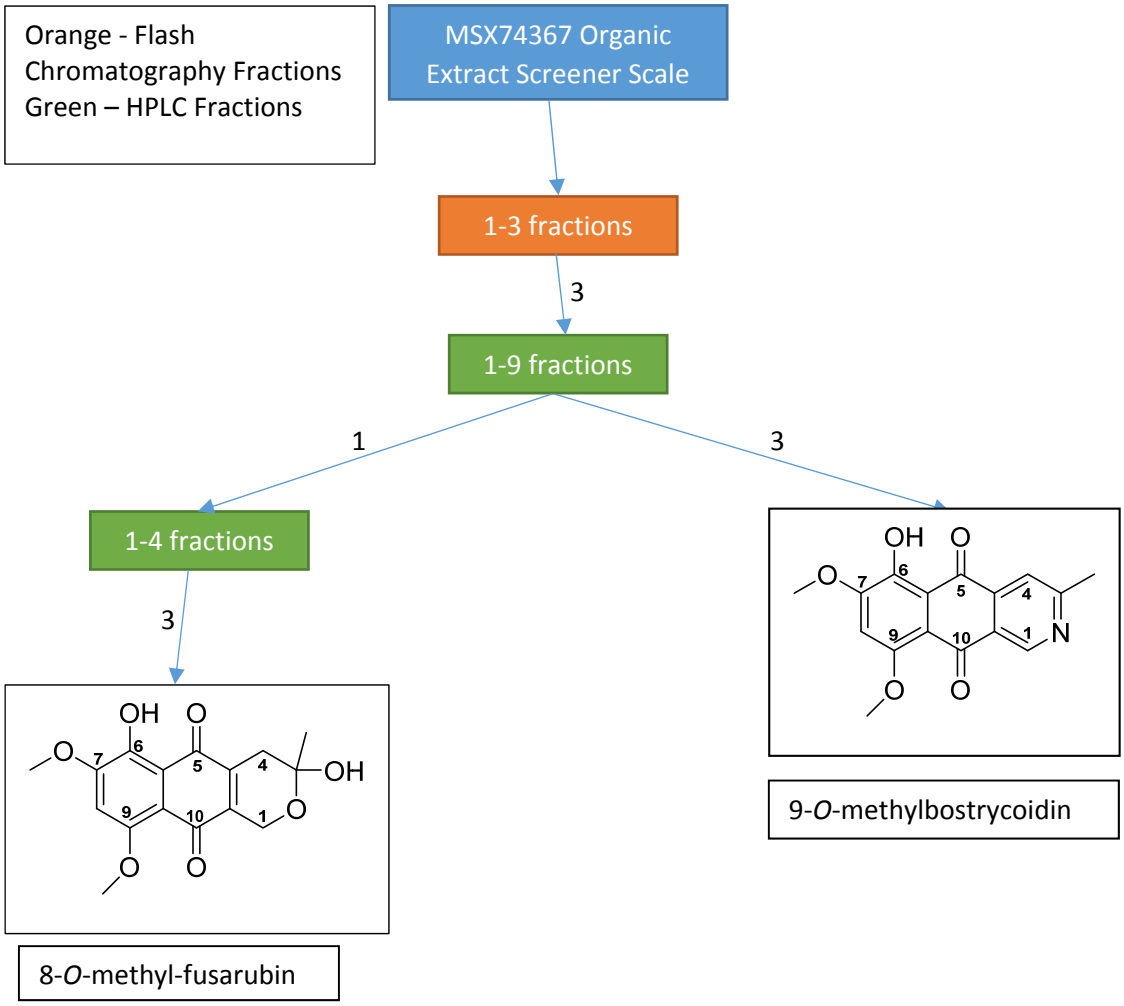
fraction one was further purified due to its similar proton NMR. Fraction one was purified on the HPLC using the Luna PFP reverse phase semi-prep column using a 30-55 % acetonitrile water gradient over 20 min with a 4.6 mL/min flow rate. The purification produced four fractions, which were analyzed using HRMS and proton NMR data to show the presence of 8-*O*-methylfusarubin in fraction three. The compound is known and does not contain a pyridine ring in the structure. Further analysis of the remaining fractions was needed to determine if other pyridine ring structures were present in MSX74367, however material was too low for further purification. Therefore, a large scale growth of MSX74367 was extracted and analyzed, looking for the benzoisoquinoline structure.

The large scale MSX74367 growth was fractionated using flash chromatography on a 24 g silica column with a flow rate of 35 ml/min. The flash chromatography analysis was done using 1243.12 mg of sample with a hexane to chloroform to methanol gradient over a 40 minute chromatogram to create 10 fractions (Fig. 4). The fifth and sixth fractions were fractionated further using the HPLC due to mass analysis of the fractions showing a potential for benzoisoquinoline structures. Fraction five was purified using the Gemini C-18 reverse phase column with a 40-100 % acetonitrile water gradient over 30 min with a 21.24 mL/min flow rate to create nine fractions. Fraction six was purified using the Gemini C-18 reverse phase column with a 20-100 % acetonitrile water gradient over 20 min at a flow rate of 21.24 mL/min to create 11 fractions. HRMS and NMR analysis showed the presence of 9-*O*-methylbostrycoidin in fraction four. Other

analogues of the benzoisoquinoline structure were not found in any of the fractions, and the sample was set aside.

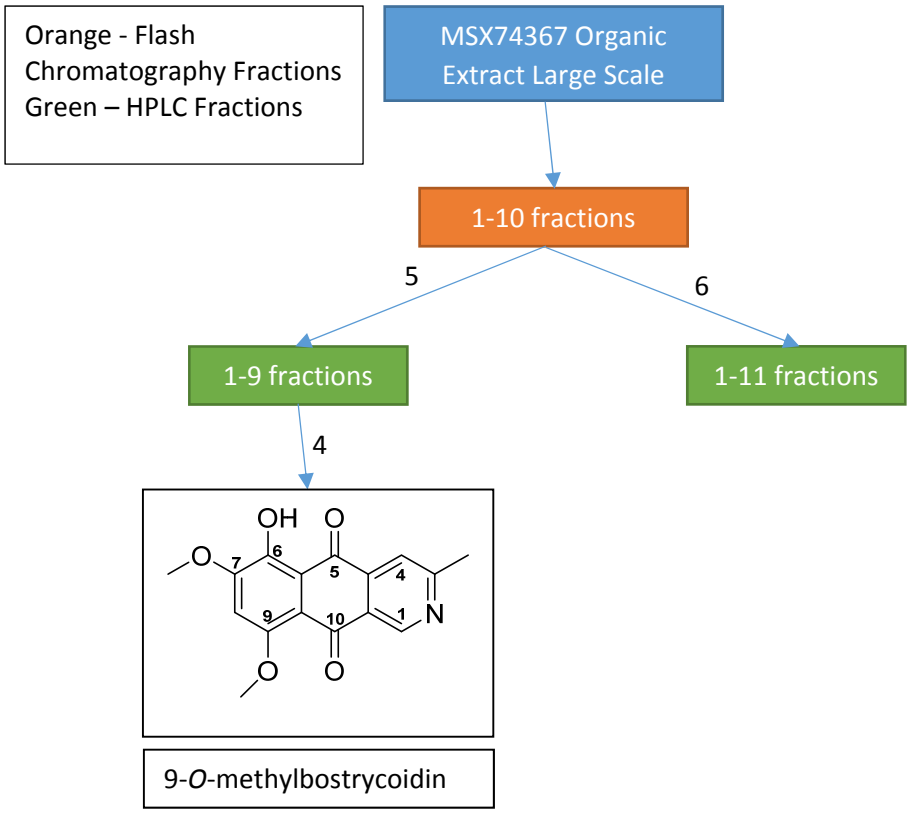
MSX74367 Purification

Figure 3. Purification Fractions for MSX74367



Large Scale MSX74367 Purification

Figure 4. Purification Fractions for MSX74367



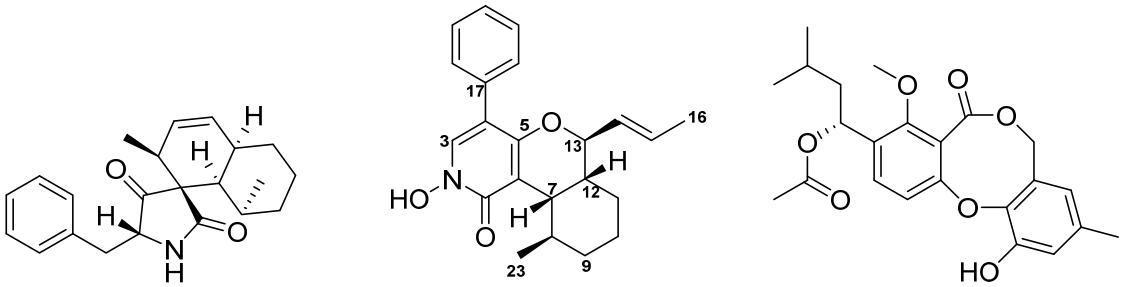
CHAPTER IV

LEPORIN

Introduction/Past Isolation

Scytalidium cuboideum (MSX 68345) is a filamentous fungus that was studied and determined to produce leporin B (2) and purpactin A (3), along with a new compound spiroscytalin (1) (15). Of these compounds, compound (2) and (3) were found to be active against a panel of cancer cell lines. One of those compounds was purpactin A (3). The second one was leporin B (2), which is a N-hydroxy-2-pyridone alkaloid. The interesting aspect of leporin B is the bioactivity data obtained when tested against human breast carcinoma, MCF-7, human large cell lung carcinoma, NCI-H460, and human astrocytoma, SF-268. The IC_{50} values in μM for leporin B were 0.2, 0.1, and 0.2 respectively. This data showed that leporin B is active against these three cancer cells and further study could lead to a potential drug compound. However, the challenge found from producing leporin B was the small supply. Therefore, optimizing production and obtaining potential analogues of leporin B was the goal.

Figure 5. Structures of Spiroscytalin, Leporin B, and Purpactin A (15)



1 **2** **3**

Table 1. IC_{50} Values for Previously Isolated Compounds from MSX68345 (15)

Compound	IC_{50} values (in µM)		
	MCF7 (breast)	H460 (lung)	SF268 (astrocytoma)
Spiroscytalin	20.5	17.6	21.9
Leporin B	0.2	0.1	0.2
Purpactin A	11.4	17.3	19.3
Camptothecin	0.07	0.01	0.03

Leporin B, isolated from MSX68345, has shown good cytotoxicity against human breast carcinoma, human large cell lung carcinoma, and human astrocytoma, as shown above. In this context, and in order to increase the supply of this metabolite, a large scale fermentation (150 g of rice) of the strain was conducted. After the fermentation process, the organic extract was obtained using an established protocol. The organic extract was dereplicated to determine the existence of leporin B and assess potential for new compounds. Dereplication provided three compounds including: spirobotrin, alternariol monomethyl ether, penicillide, and vermixocin A. However, leporin B was not detected. However, the extract was further fractionated by flash chromatography. This procedure gave four fractions. A small aliquot of each organic extract and fractions were evaluated

for cytotoxicity in three different cancer cell lines, including: melanoma, breast and ovarian cancer cells. Cytotoxicity testing test a sample against three cancer cell lines using two concentrations of 20 μg/mL and 2 μg/mL of the sample. Boxes that show a number less than 50, means that less than half of the cancer cell survived with the addition of the sample. The cytotoxicity results showed very little activity in the large scale extract and the four flash chromatography fractions, as seen in table 2 where none of the boxes show a number less than 50, or less than 50% survival of those cancer cell lines. This data can be compared to the original extract cytotoxicity data that was tested against breast lung and astrocytoma cancer cell lines. The cancer cell lines vary, however, the original extract showed activity in three different cancer cell lines, therefore, it would be likely that the compound should show some activity in the other cancer cell lines as well, even if they are different types of cancer cells. Therefore, this was another sign that leporin B was not present in a significant amount or at all in the sample, due to the lack of activity in the new extract. For further confirmation, mass data on the organic extract and fractions was collected multiple times in search for leporin B, looking for the specific mass of leporin (352.19003 amu), but the mass was not found. Mass defect filtering was also performed in the hopes that it may just be in small supply or analogues may be present, but nothing was found. Looking back at the chromatogram for the small scale flash chromatography analysis (Fig. 7), there is a peak in between fraction 49-53. Based on the data that led to the original isolation of leporin B, that is the peak that should have contained leporin B. Looking at the flash chromatography for the large scale, the peaks in the beginning and end of the chromatogram relatively match up, but the peak for leporin

B, that should be around 22 minutes based on the chromatogram shown from the previous isolation, is missing (15). Therefore, it was determined that leporin B was not in that specific fermentations of Mycosythetix fungal strain 68345.

Figure 6. Structures of Spirobotrin, Alternariol Monomethyl Ether, and Penicillide; Vermixocin A, Respectively

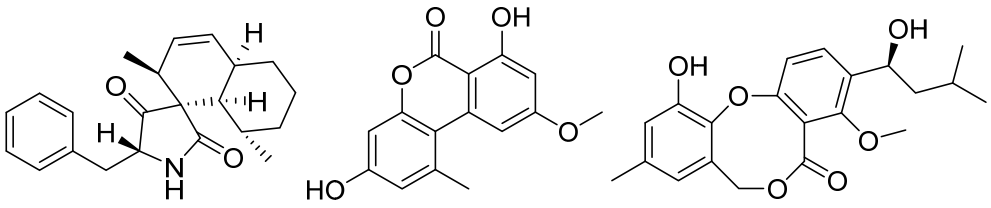
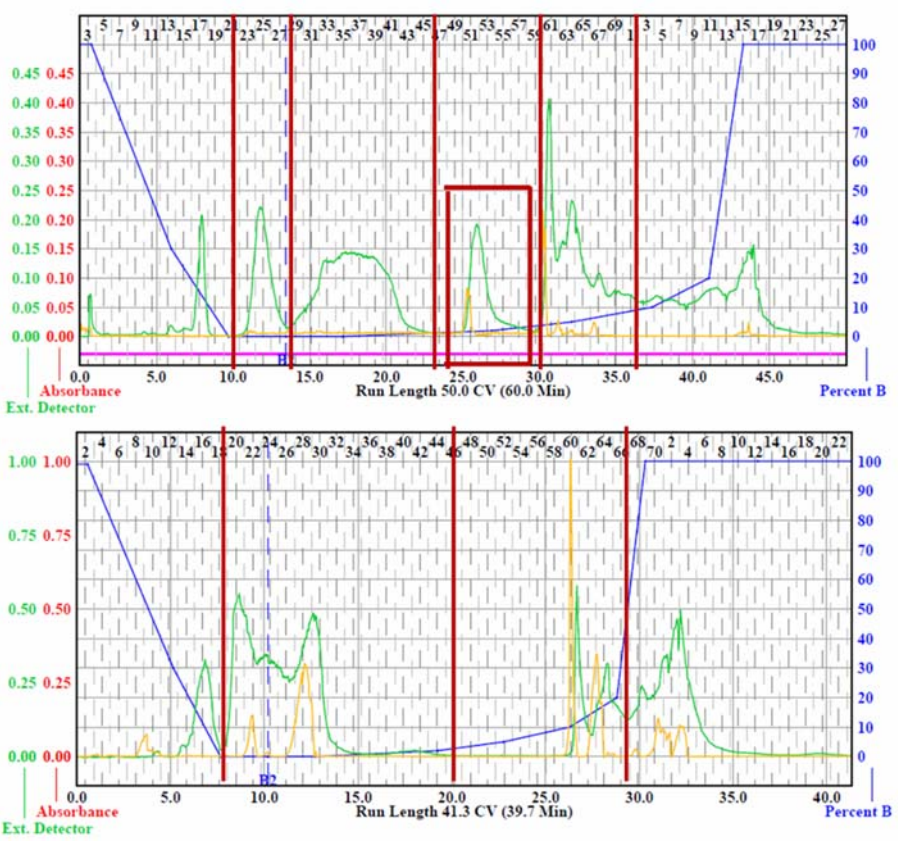


Table 2. Cytotoxicity Data for the Original Organic Fraction Isolation of Leporin B and Current Cytotoxicity Data for Fractions Isolated from the Same Fungus

	MCF-7-WTK (breast)		H460 (lung)		SF268 (astrocytoma)	
	20 µg/mL	2 µg/mL	20 µg/mL	2 µg/mL	20µg/mL	2 µg/mL
MSX68345	36	97	4	102	17	100

	MDA-MB-435 (melanoma)		MDA-MB-231 (breast)		OVCAR3 (ovarian)	
Fraction	20 µg/mL	2 µg/mL	20 µg/mL	2 µg/mL	20µg/mL	2 µg/mL
SM	66	68	77	77	79	85
1	100	100	91	100	100	100
2	100	100	100	100	100	100
3	98	97	66	84	100	95
4	96	100	98	100	100	95

Figure 7. Flash Chromatography of Original Leporin B Isolation and Recent Isolation of Leporin B



The next step in the search for leporin B was to obtain a slant growth of MSX68345 and another large scale. The four small scale slants and the large scale slants were extracted using the methods presented above. In order to save time and determine which of the five organic fractions to work with, each organic extract was analyzed through high resolution mass spectrometry, and the relative mass for leporin B was searched for. The data was processed using the software expressed above for mass defect filtering. The data showed the presence of the leporin B mass of 351.19003 in four of the five organic fractions,

including the new large scale extract and three of the four small scale slants. Due to the presence of a nitrogen in the ring of leporin B, even numbered $[M+H]^+$ peaks were recorded. Mass defect analysis provides data for potential analogue masses of the compound that is being searched for, therefore the even masses that were recorded were potential analogue matches. The next step was to analyze the masses through a molecular formula calculator. Molecular formulas, index of hydrogen deficiency values and parts per million values were all recorded. A number of molecular formulas were taken out of the list of potential analogues, as the changes were too drastic in relation to the core structure of leporin B. It was determined at this juncture to proceed with the large scale organic extract, due to the presence of the leporin B mass, potential for analogues, and more potential for increased mass of pure compounds.

Figure 8. Purification Fractions for MSX68345

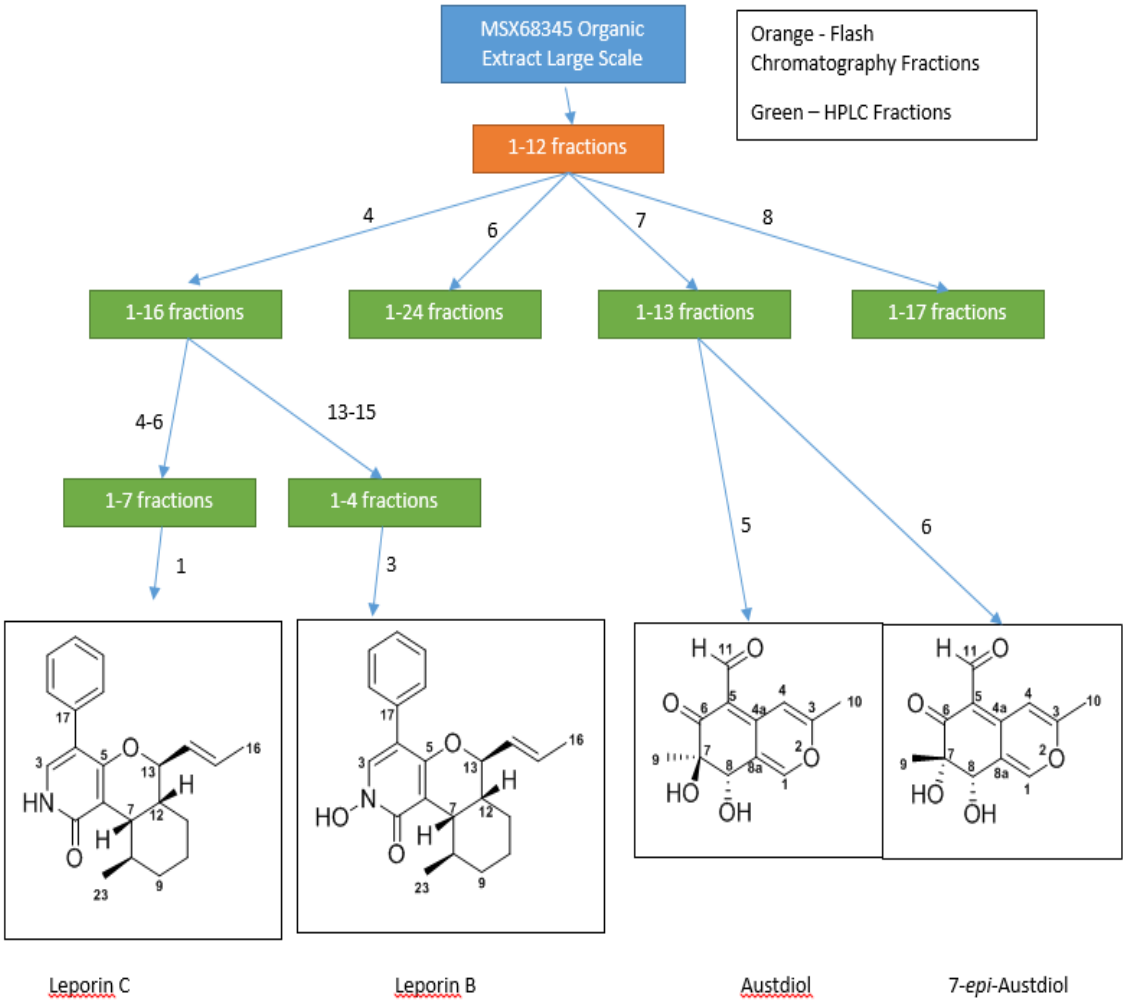
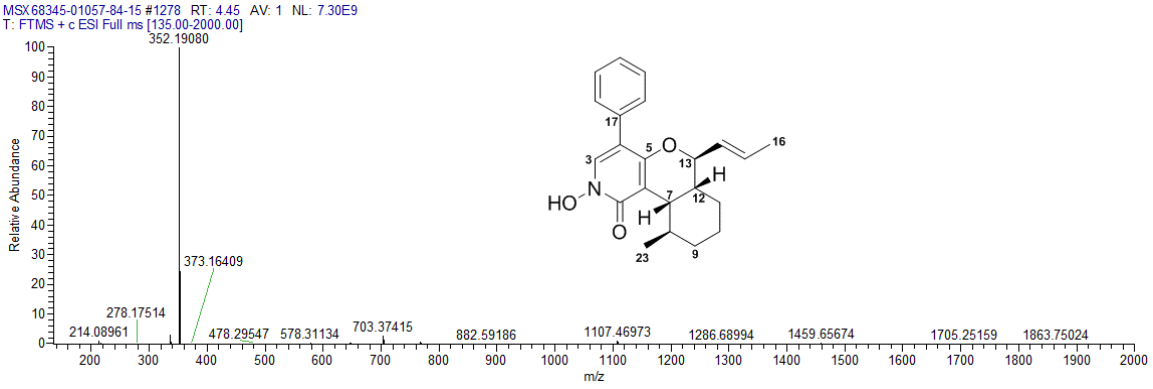


Figure 9. Mass Spectrometry Data for Re-isolation of Leporin B



It can be seen that fraction 15 from (Fig. 2) showed the presence of the leporin B $[M+H]^+$ mass of 352.1908 amu. This mass can be confirmed by finding the $[2M+1]^+$, which is 703.38, a peak that can be seen in the mass spectrum above. To confirm the existence of leporin B, a proton NMR was performed and compared to the previous isolation proton NMR of leporin B. Therefore, fractions that showed the same proton NMR data were combined and further purified to obtain more pure sample of each compound. After further purification, additional NMR experimentation was done including: carbon and proton data. The carbon and proton data was matched with the previous isolation data to confirm the identity of leporin B. The fraction also contained 7.02 mg of sample, which can be combined with the previously isolated 2.49 mg to make 9.51 mg. The importance of the mass increase means that leporin B can be tested *in vivo* to determine if the compound acts in the same manner as it does with in vitro studies.

Leporin B

White powder; $[\alpha]_D^{25}$-41.7 acetonitrile; ^1H, ^{13}C NMR data, see (Tables 3 and 4); HRESIMS m/z 352.1908 for $[M+H]^+$, calcd for $C_{22}H_{26}NO_3$, 352.1907.

The confirmation of the re-isolation of leporin B by NMR can be seen in (Fig. 9). The NMR experiments for leporin B were analyzed in deuterated chloroform on the 400 MHz NMR. The NMR data between the isolated sample and the original sample are compared in (Tables 3 and 4).

Figure 10. Proton and Carbon NMR Data for Leporin B Both Past and Present Isolation

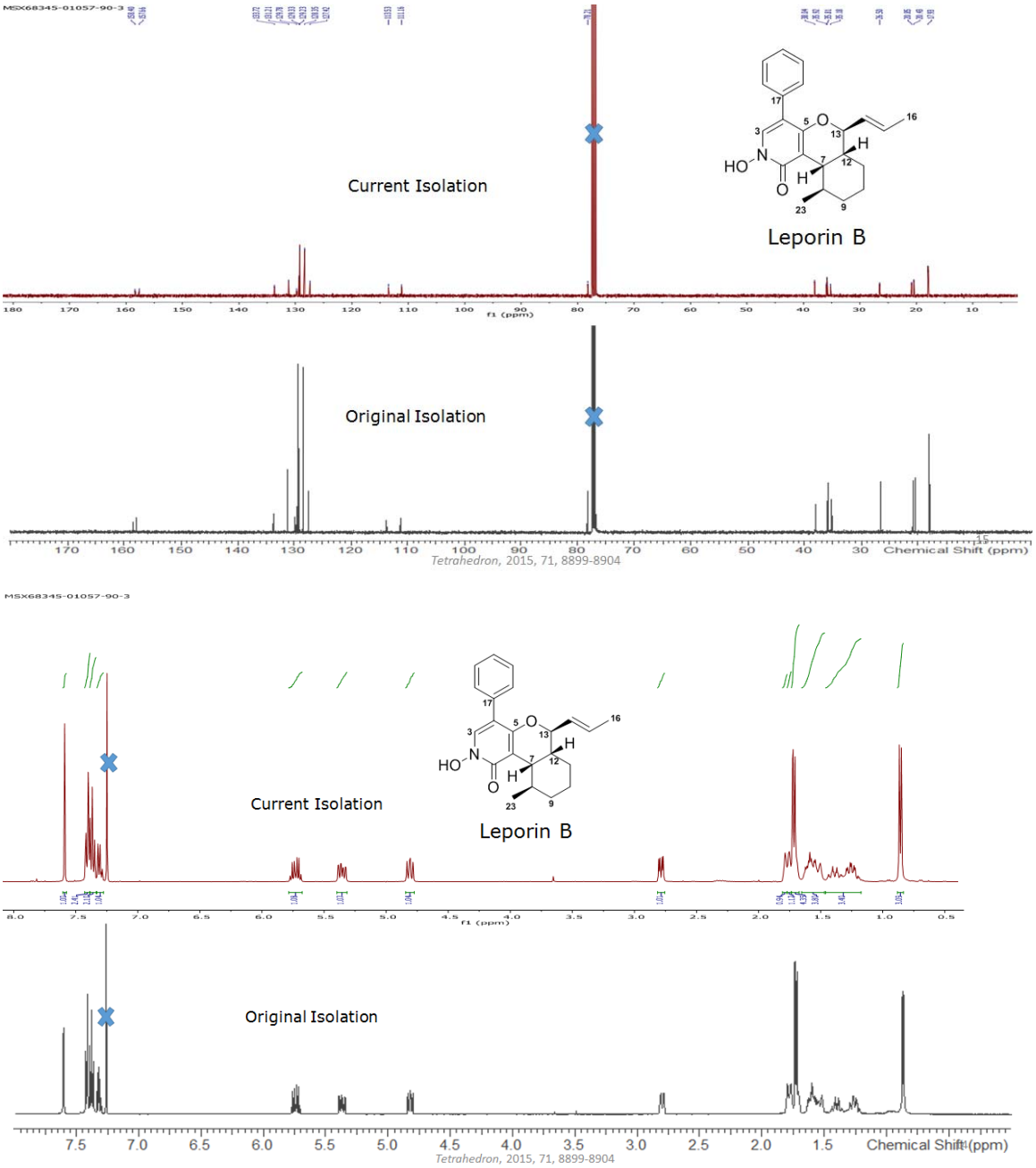


Table 3. Tabulated Proton NMR Data for Leporin B

leporin B	Literature			Experimental		
Position	Proton shift	Multiplicity	J-value	Proton Shift	Multiplicity	J-value
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	7.60	s	-	7.59	s	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	2.79	dd	10,3.5	2.79	dd	10.85, 3.73
8	1.77	m	-	1.79	m	-
9a,b	1.60	m	-	1.60	m	-
10a	1.51	m	-	1.51	m	-
10b	1.39	tq	3.5,13	1.39	qt	12,4
11a	1.76	m	-	1.75	m	-
11b	1.60	m	-	1.60	m	-
12	1.72	m	-	1.75	m	-
13	4.82	dd	11,9	4.81	dd	11.22, 8.23
14	5.37	ddq	15,8.6,2	5.36	ddq	15.20, 8.21,1.56
15	5.73	dq	16,7	5.73	dq	15.31, 6.55
16	1.71	dd	7,2	1.72	dd	6.51,1.64
17	-	-	-	-	-	-
18	7.41	m	-	7.41	m	-
19	7.37	m	-	7.36	m	-
20	7.29	m	-	7.30	m	-
21	7.37	m	-	7.37	m	-
22	7.41	m	-	7.41	m	-
23	0.86	d	6	0.86	d	6.53

Table 4. Tabulated Carbon NMR Data for Leporin B

leporin B	Literature	Experimental
Position	Carbon shift	Carbon Shift
1	158.2	158.40
2	-	-
3	129.2	129.33
4	111.2	111.16
5	157.6	157.66
6	113.7	113.53
7	38.1	38.04
8	35.9	35.92
9a,b	35.8	35.81
10a	20.9	20.85
10b	20.9	20.85
11a	26.5	26.50
11b	26.5	26.50
12	35.2	35.18
13	78.2	78.21
14	129.8	129.78
15	131.2	131.21
16	17.9	17.93
17	133.7	133.72
18	129.1	129.23
19	128.3	128.35
20	127.5	127.42
21	128.3	128.35
22	129.1	129.23
23	20.4	20.43

Analogue

Continuing from fraction four of the large scale flash chromatography, fraction six of the HPLC purification showed the presence of a 336.19565 amu peak. This mass can be confirmed by the $[2M+H]^+$ peak at 671.38422. The interesting aspect of this mass is the absence of an oxygen from the leporin B molecular formula. Therefore, the fraction was analyzed by proton and carbon NMR.

Figure 11. Mass Spectrum Leporin C

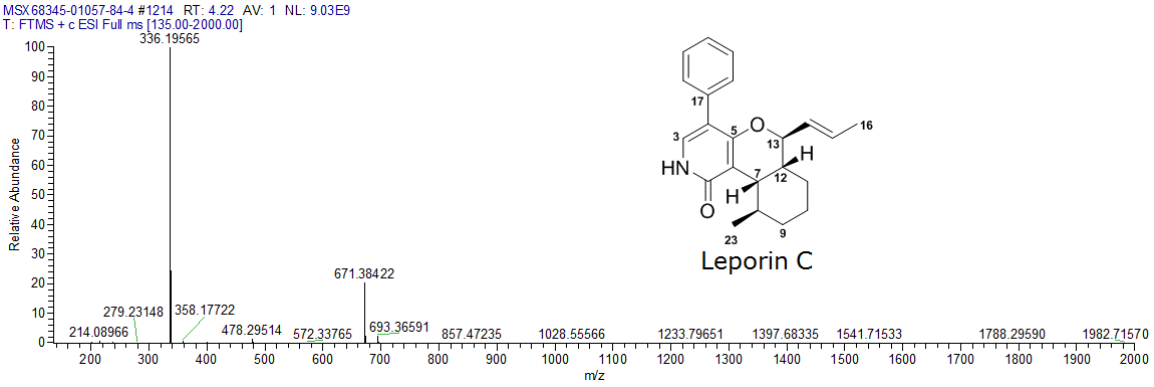


Figure 12. Proton NMR Leporin C in Comparison to Proton NMR of Leporin B

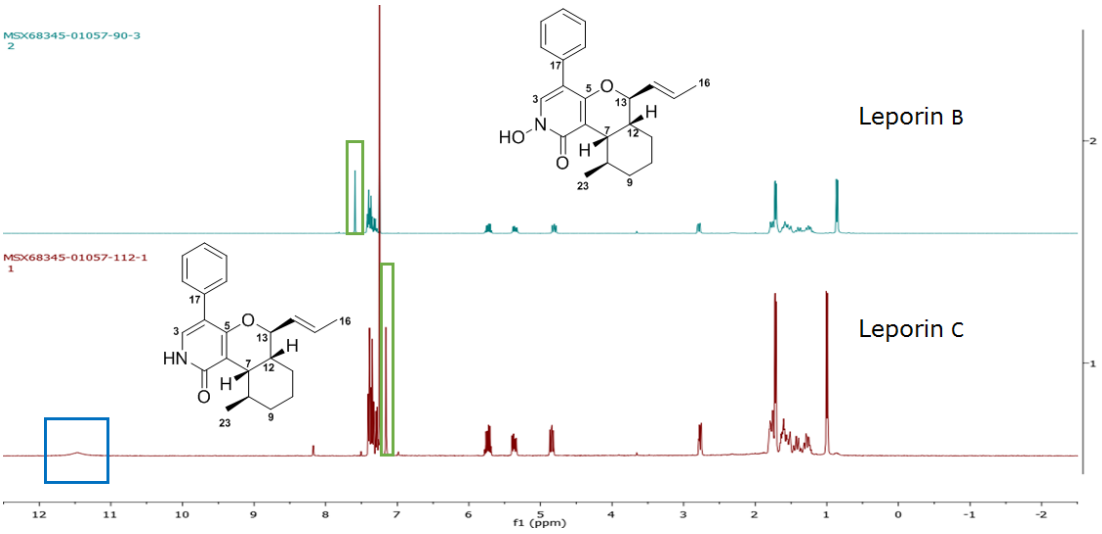


Table 5. Tabulated Proton NMR Data for Leporin C

leporin C	Literature			Experimental		
Position	Proton shift	Multiplicity	J-value	Proton shift	Multiplicity	J-value
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	7.21	s	-	7.16	s	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	2.79	dd	10.9,3.7	2.77	dd	10.87,3.77
8	1.72	dd	6.5,1.5	1.80	m	-
9a,b	1.83-1.26	m	-	1.58	m	-
10a	1.83-1.26	m	-	1.41	m	-
10b	1.83-1.26	m	-	1.27	m	-
11a	1.83-1.26	m	-	1.76	m	-
11b	1.83-1.26	m	-	1.58	m	-
12	1.83-1.26	m	-	1.71	m	-
13	4.58	dd	11.3,8.1	4.84	dd	11.40,8.24
14	5.39	ddq	15.2,8.1,1.5	5.37	ddq	15.17,8.26,1.83
15	5.76	dq	15.2,6.5	5.73	dq	15.43,6.49
16	1.83-1.26	m	-	1.71	dd	6.58,1.62
17	-		-	-	-	-
18	7.43-7.28	m	-	7.40	m	-
19	7.43-7.28	m	-	7.35	m	-
20	7.43-7.28	m	-	7.28	m	-
21	7.43-7.28	m	-	7.35	m	-
22	7.43-7.28	m	-	7.40	m	-
23	1.01	d	6.5	1.00	d	6.58

Table 6. Tabulated Carbon NMR Data for Leporin C

leporin C	Literature	Experimental
Position	Carbon shift	Carbon shift
1	165.2	164.70
2	-	-
3	129.7	129.51
4	111.7	111.62
5	160.5	160.51
6	115.3	115.44
7	37.3	37.09
8	36.2	36.05
9a,b	36.1	35.90
10a	21.1	20.98
10b	21.1	20.98
11a	26.7	26.58
11b	26.7	26.58
12	35.4	35.22
13	78.3	78.25
14	131.0	131.01
15	131.8	131.29
16	18.0	17.94
17	134.7	134.43
18	129.3	129.14
19	128.3	128.23
20	127.2	127.15
21	128.3	128.23
22	129.3	129.14
23	20.7	20.60

Leporin C was compared by NMR, as shown in (Fig. 11) and (Tables 5 and 6).

Additional Compounds

Leporin B was re-isolated from MSX68345, however, there were a number of other fractions that could be purified from the fungus that showed promise in that the fractions were somewhat pure and there was enough material to work with. A particular set of fractions showed interesting HPLC chromatograms with UV data. The UV data

was interesting due to the fact that it was showing absorbance's at three different wavelengths as opposed to the typical two. Therefore, those fractions were further purified. During the purification process, fractions five and six crystalized. The crystals from fraction five were good enough to send for x-ray crystallography. The data was analyzed and the compound was found to be the known compound, austdiol, as seen in (Fig. 2). To confirm this structure, proton NMR was performed on the structure and confirmed the austdiol structure. The fraction 6 also crystalized, however the crystals were too small for x-ray crystallography. Therefore, a proton NMR was performed on the sample. As seen in (Fig. 12), the proton NMR data matches up with the exception of the aromatic peaks around 8 ppm. The mass data was also the same for both fractions, showing a mass of 237.07 amu and an index of hydrogen deficiency of seven. The interesting aspect about this process was a color change that occurred when the sample was dissolved in chloroform. The sample went from a yellowish color to a blue color. Fraction six also changed to a green color. The fractions did not dissolve well with chloroform, so DMSO was needed to dissolve the samples. Figure 13 shows the initial crystals in the vial and the fractions transformation to various colors when further dissolved in DMSO. The following picture shows the sample in the NMR tubes and the final picture shows the samples after leaving them in the NMR tubes overnight. In each case, the color seemed to transform to darker and darker colors as time passed. A possible explanation for this could be the degradation of the compound, which would affect the number of conjugated pi electrons in the structure. Loss of the aldehyde would be a

potential point of degradation that would affect the conjugated π electrons, which would affect the light that is absorbed, causing a color change.

Figure 13. Proton NMR Data Compared for Austdiol and 7-*epi*-Austdiol

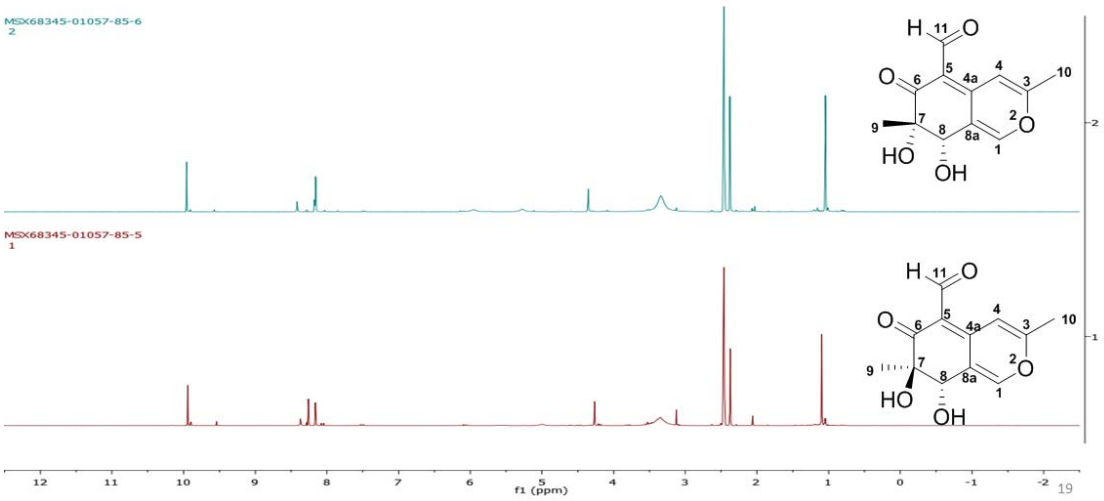


Figure 14. Photos of Austdiol and 7-*epi*-Austdiol in Crystal and Color Altered Forms

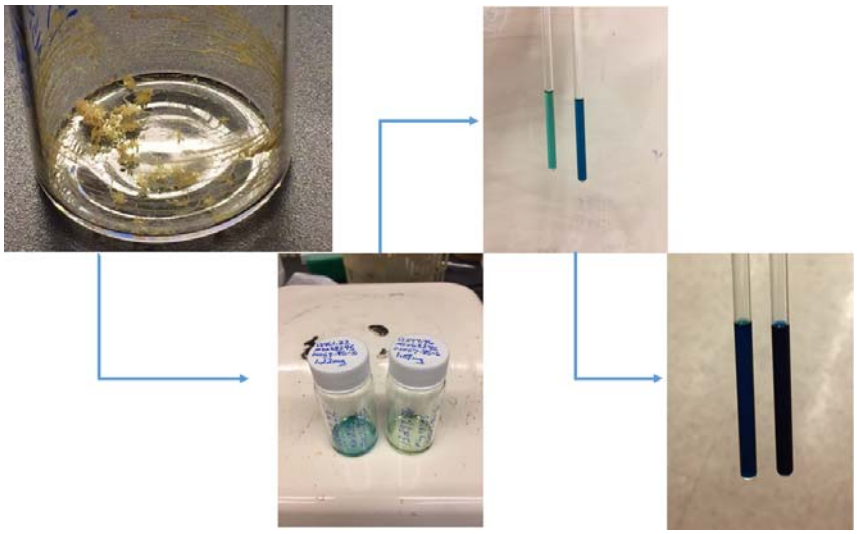
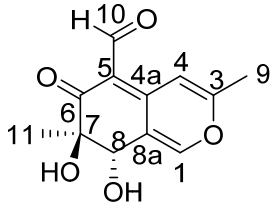


Table 7. Tabulated Proton NMR Data for Austdiol

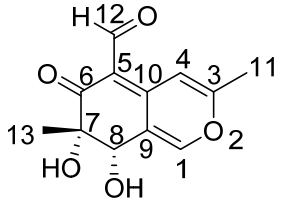


austdiol	Literature		Experimental	
Position	Proton shift	Multiplicity	Proton shift	Multiplicity
1	8.19	-	8.26	s
3	-	-	-	-
4	8.19	-	8.16	s
4a	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
7-OH	5.25	S	4.99	s
8	4.39	S	4.27	s
8a	-	-	-	-
8-OH	5.92	S	5.57	s
9	2.21	S	2.37	s
10	9.99	-	9.94	s
11	1.08	S	1.09	s

Austdiol

Yellow crystals; $[\alpha]_D^{28}$68.9 DMSO; ^1H, ^{13}C NMR data, see (Table 7); HRESIMS m/z 237.0756 for $[M+H]^+$, calcd for $C_{12}H_{13}O_5$, 237.0757.

Table 8. Tabulated Proton NMR Data for 7-*epi*-Austdiol



7-*epi*-austdiol	Literature			Experimental	
Position	Proton shift	Multiplicity	J-value	Proton shift	Multiplicity
1	8.28	S	-	8.17	s
3	-	-	-	-	-
4	8.19	S	-	8.16	s
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
7-OH	4.94	S	-	5.27	s
8	4.31	D	4.0	4.36	s
8-OH	5.50	D	4.0	5.95	s
9	-	-	-	-	-
10	-	-	-	-	-
11	2.41	S	-	2.37	s
12	9.98	S	-	9.96	s
13	1.14	S	-	1.04	s

7-*epi*-austdiol

Yellow crystals; $[\alpha]_D^{28}$202.8 DMSO; ^1H, ^{13}C NMR data, see (Table 8); HRESIMS m/z 237.0755 for $[M+H]^+$, calcd for $C_{12}H_{13}O_5$, 237.0757.

CHAPTER V

BENZOISOQUINOLINE

Fungus MSX68345 was the specific fungus that led to the re-isolation of leporin B, however there are other Mycosynthetix fungi that can be studied for secondary metabolites that are active against varying cancer cells. One fungus, MSX74367, was one of the fungi that produced good activity against melanoma, breast, and ovarian cancer cell lines. Table 9, shows the activity of the sample material and the three fractions created after flash chromatography. Active fractions will show less than 50% survival of the different cancer cell lines. Therefore, fraction three is highlighted in red due to its good activity, less than 50% survival, in the 20 µg/mL concentration for all three cancer cell lines. Fraction three was further purified to determine the active compound by using flash chromatography as seen in (Fig. 3). Further purification by HPLC of fraction three showed the presence of a known compound, 9-*O*-methylbostrycoidin.

Figure 15. Structure of 9-*O*-Methylbostrycoidin

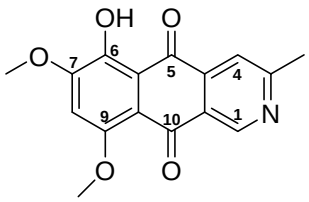
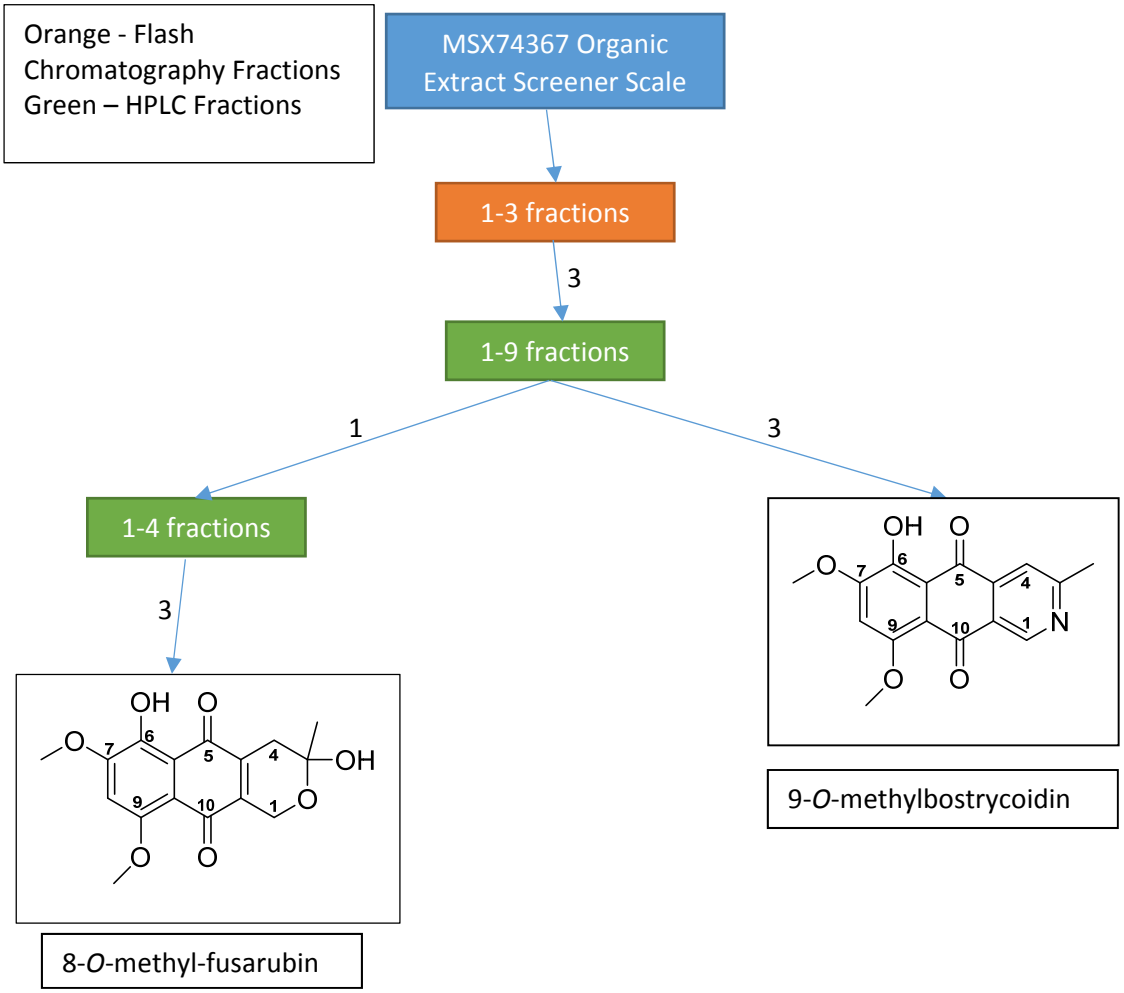


Table 9. Cytotoxicity Data for MSX74367

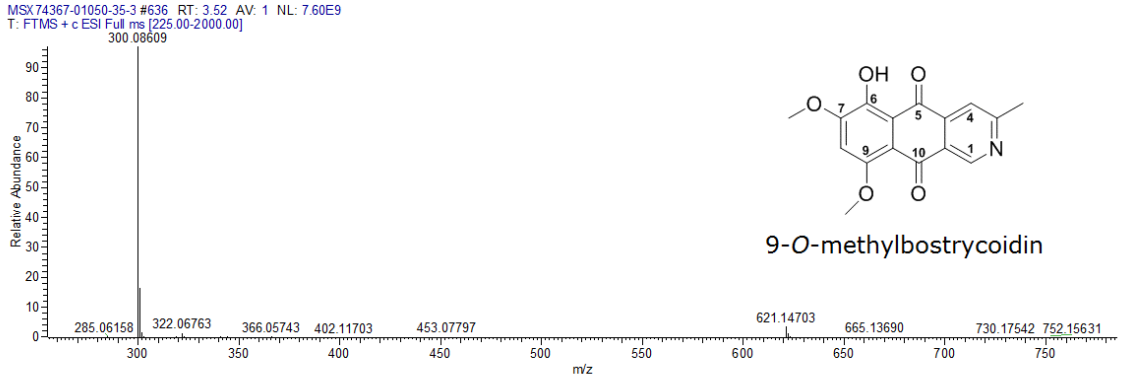
MSX74367	MDA-MB-435 (melanoma)		MDA-MB-231 (breast)		OVCAR3 (ovarian)	
	20 µg/mL	2 µg/mL	20 µg/mL	2 µg/mL	20 µg/mL	2 µg/mL
SM	39	72	100	100	53	100
1	93	100	100	100	100	100
2	89	100	100	100	100	100
3	17	100	32	100	24	100

Figure 16. Purification Fractions for MSX74367



The mass spectrum for the purified fraction showed a mass of 300.08609 amu. This mass can be confirmed by the 322.06763 amu mass, which represents the addition of sodium. This mass is also within 5 ppm of the calculated mass for the compound and shows a degree of unsaturation of 11. The proton NMR data was matched with previously isolated 9-*O*-methylbostrycoidin (Fig. 15 and Table 10).

Figure 17. Mass Spectrometry and Proton NMR Data for 9-*O*-Methylbostrycoidin



[M+H] 300.0861, Calculated for $C_{16}H_{14}NO_5$ (300.0865), 1.8ppm, IHD 11

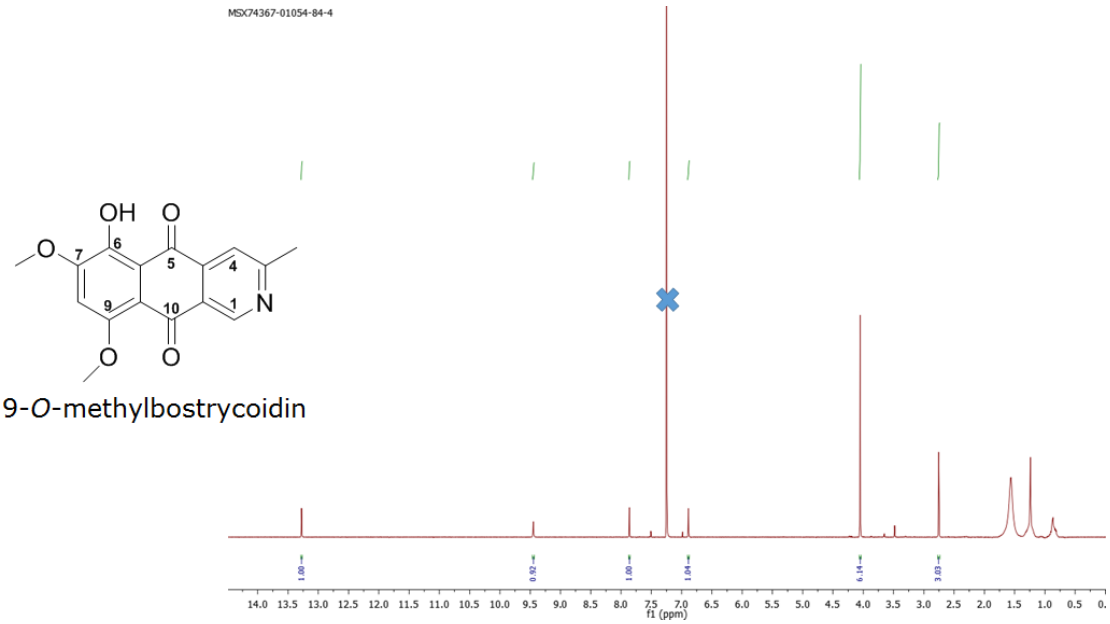
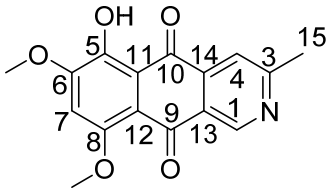


Table 10. Tabulated Proton NMR Data for 9-*O*-Methylbostrycoidin

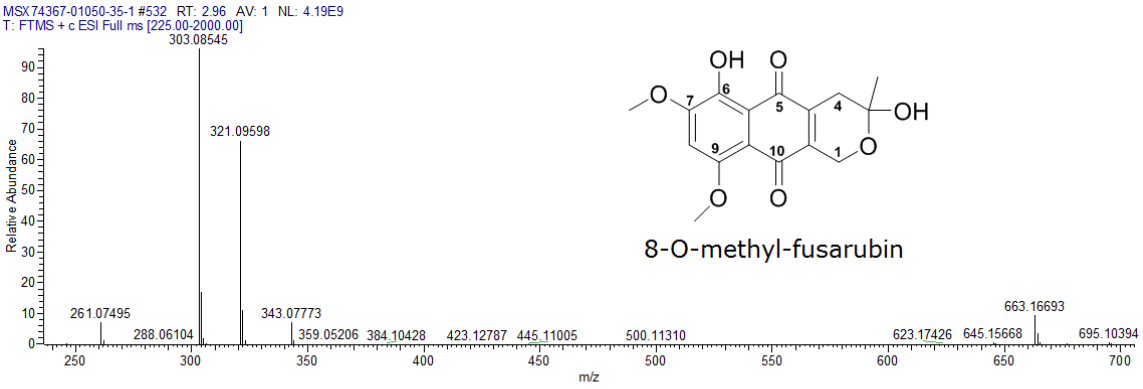


9-*O*-methylbostrycoidin	Literature		Experimental	
Position	Proton shift	Multiplicity	Proton shift	Multiplicity
1	9.44	S	9.45	s
3	-	-	-	-
4	7.85	S	7.86	s
5	-	-	-	-
6	-	-	-	-
7	6.86	S	6.89	s
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	2.76	S	2.75	s
6-OCH_3	4.05	S	4.05	s
8- OCH_3	4.05	S	4.05	s
OH	13.19	S	13.28	s

Another fraction was purified from the same fungus to isolate the compound 8-*O*-methyl-fusarubin. The mass spectrum shows a mass of 321.09598 amu. This mass was confirmed by the peak showing the loss of water, (303.08545 amu peak). The compound mass is shown to be within 5 ppm of the calculated mass, with an index of hydrogen deficiency of 9. Proton NMR data was obtained for the compound and matched through

dictionary of natural products with the known compound 8-*O*-methyl-fusarubin (Fig. 16 and Table 11).

Figure 18. Mass Spectrometry and Proton NMR Data for 8-*O*-Methyl-Fusarubin



[M+H] 321.0959, Calculated for $C_{16}H_{16}O_7$ (321.09688), 3ppm, IHD 9

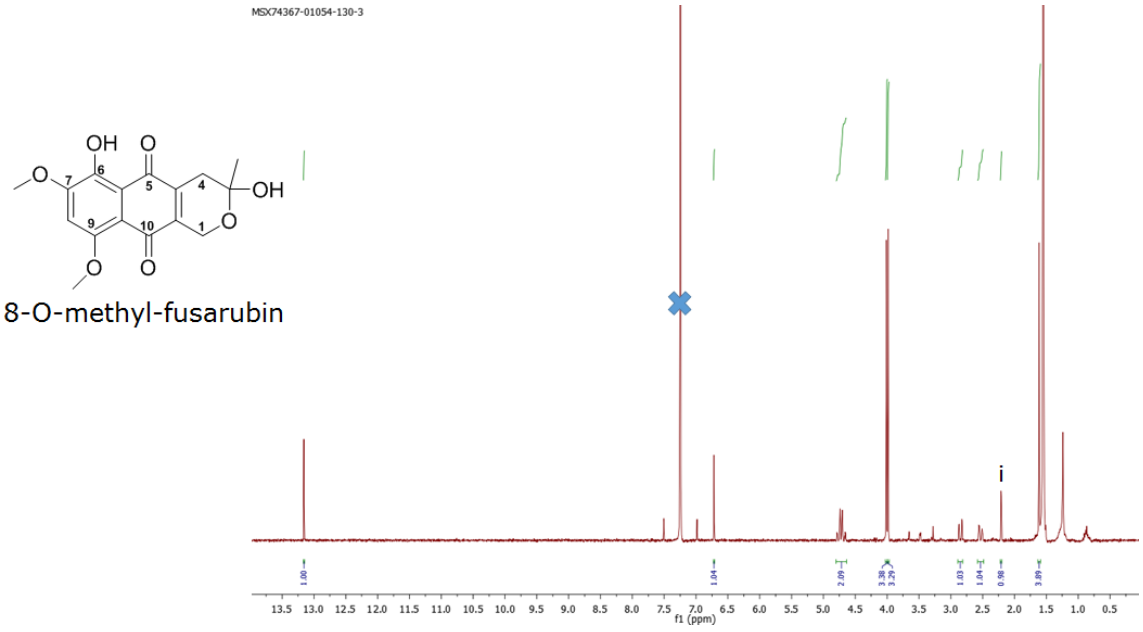
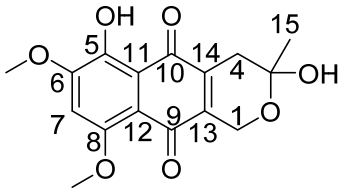


Table 11. Tabulated Proton NMR Data for 8-*O*-Methyl-Fusarubin



8-*O*-methyl-fusarubin	Literature			Experimental		
Position	Proton shift	Multiplicity	J-value	Proton shift	Multiplicity	J-value
1	4.75	dd	18,2.8	4.72	m	-
3	-	-	-	-	-	-
4ax	2.53	d	18	2.53	dd	20.16, 4.18
4eq	2.84	d	18	2.85	dd	18.58, 2.45
5-OH	13.16	s	-	13.16	s	-
6	-	-	-	-	-	-
6-OMe	4.03	s	-	3.98	s	-
7	6.73	s	-	6.72	s	-
8	-	-	-	-	-	-
8-OMe	4.00	s	-	4.01	s	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	1.55	s	-	2.21	d	2.13

A large scale growth of MSX74367 was obtained. The new goal was to specify the purification process towards obtaining benzoisoquinoline containing compounds and nitrogen containing compounds. In order to do this, mass defect filtering was performed. Masses were chosen based on even $[M+H]^+$ values, which are representative of nitrogen

containing compounds. The list of masses was narrowed down to masses that could represent the addition or loss of substituents on the outside of the core structure of 9-*O*-methylbostrycoidin. This list is shown in (Table 12), where green numbers represent the addition of substituents, red numbers show the subtraction of substituents, and black numbers show the same mass as 9-*O*-methylbostrycoidin. The black numbers could represent analogues of the compound, or they could represent the compound bleeding through the varying fractions. The list of masses obtained through mass defect filtering were correlated with high resolution mass spectrometry of each stage of purification of the large scale growth. This process allowed for guided fractionation of compounds found in the large scale. The advantage of this process was the re-isolation of 9-*O*-methylbostrycoidin, however the disadvantage was that other nitrogen containing analogues were not obtained. Based on this analysis, it may have been better to perform a cytotoxicity guided fractionation. This would have been more time consuming, however it may have resulted in more interesting compounds from this fungus.

Figure 19. Flow Chart for Benzoisoquinoline Mass Guided Fractionation

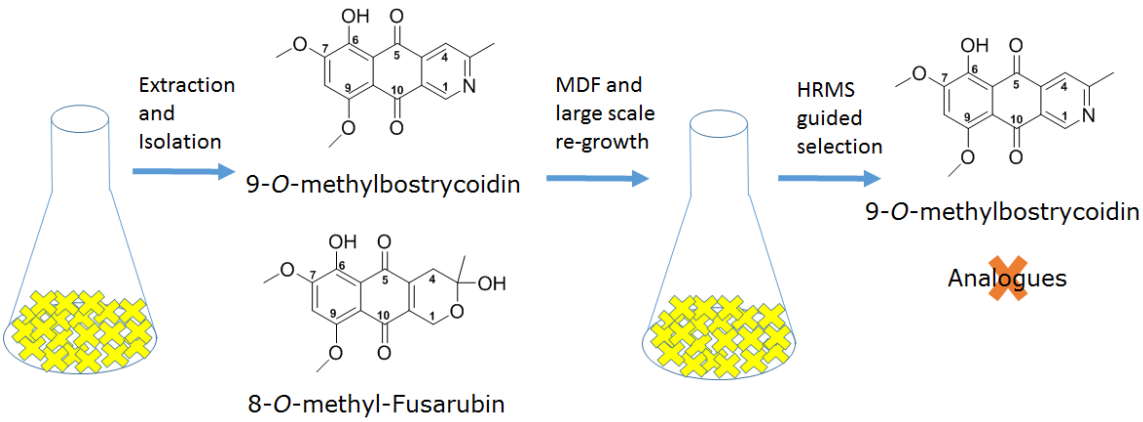
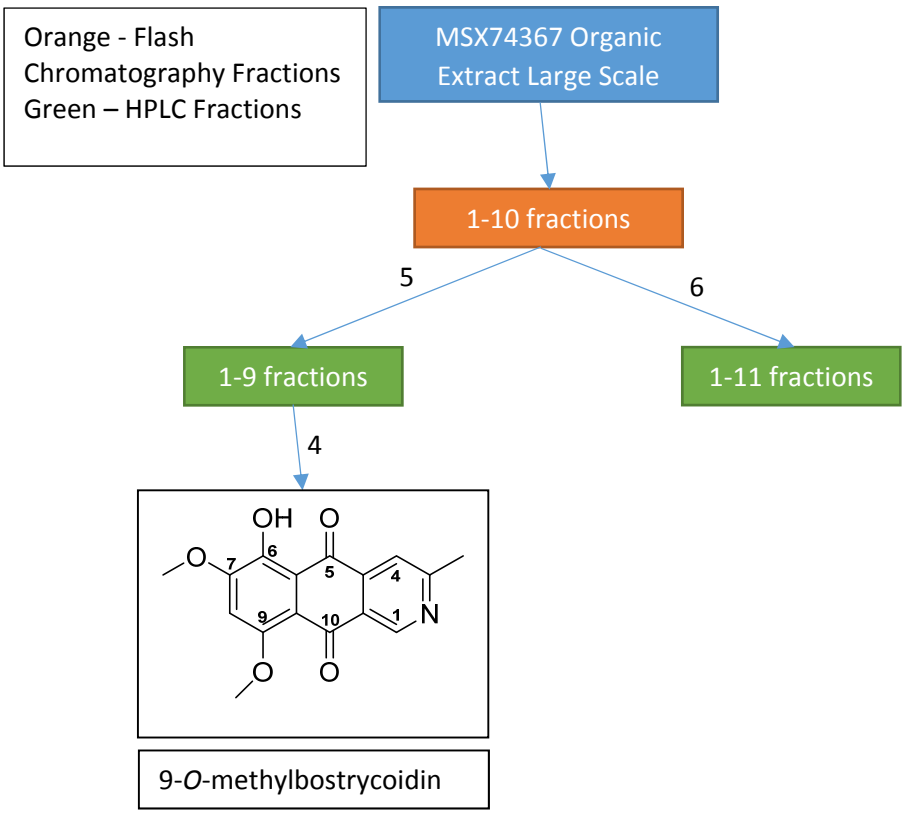


Table 12. MDF Data Collected from Screener Scale Fractions

Fraction	M+H Mass	Calculated For M+H
1	286.07040	$C_{15}H_{12}NO_5$ (2.1ppm)(IHD-11)
2	314.10175 330.09671	$C_{17}H_{16}NO_5$ (1.7ppm)(IHD-11) $C_{17}H_{16}NO_6$ (1.5ppm)(IHD-11)
3	286.07047 330.09647	$C_{15}H_{12}NO_5$ (1.8ppm)(IHD-11) $C_{17}H_{16}NO_6$ (2.3ppm)(IHD-11)
4	300.08621	$C_{16}H_{14}NO_5$ (1.5ppm)(IHD-11)
5	300.08609	$C_{16}H_{14}NO_5$ (1.9ppm)(IHD-11)
6	300.08636	$C_{16}H_{14}NO_5$ (1.0ppm)(IHD-11)
7	300.08633	$C_{16}H_{14}NO_5$ (1.1ppm)(IHD-11)
9	284.09119 300.08612 322.09149 332.07584	$C_{16}H_{14}NO_4$ (1.9ppm)(IHD-11) $C_{16}H_{14}NO_5$ (1.8ppm)(IHD-11) $C_{15}H_{16}NO_7$ (2.0ppm)(IHD-9) $C_{16}H_{14}NO_7$ (1.9ppm)(IHD-11)

Figure 20. Purification Fractions for MSX74367



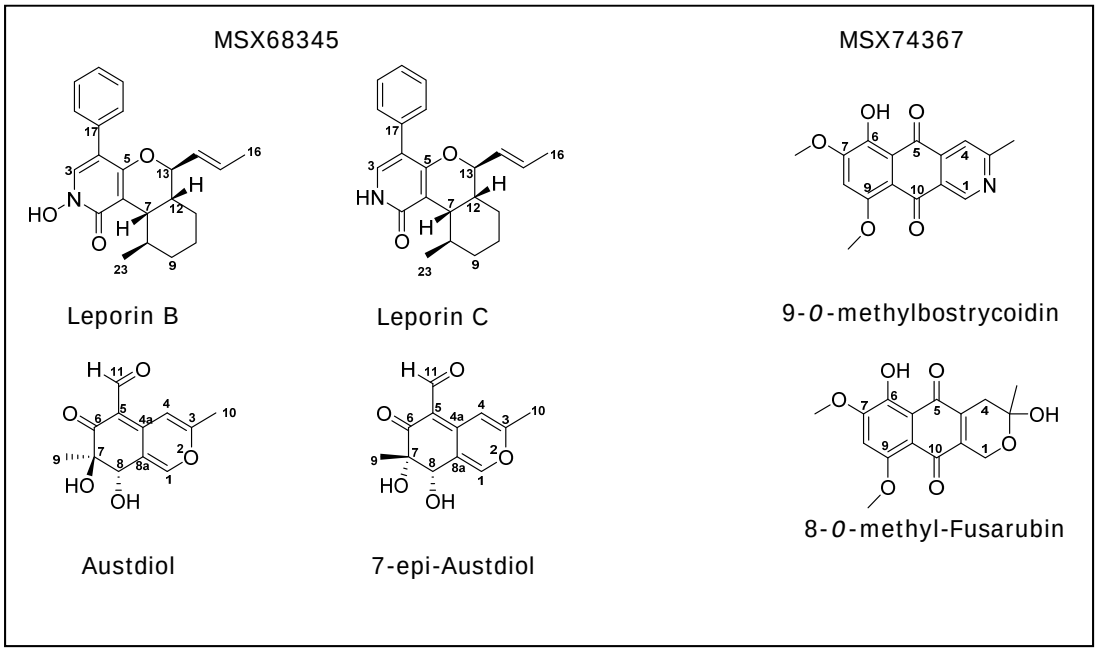
CHAPTER VI

CONCLUSIONS

Two fungal species, MSX68345 and MSX74367, were studied. The study of secondary metabolites from fungal species MSX68345 led to the re-isolation of leporin B and the increase of the in house supply. A new natural product was also isolated, leporin C, which is just the absence of the oxygen group in position two. Other compounds isolated from *Scytalidium cuboideum* were austdiol and 7-epi-austdiol. Re-isolation of leporin B was the main goal due to its activity against breast, lung, and astrocytoma cancer cell lines. With the increased supply of leporin B from less than three mg to approximately 10 mg, the compound can now be used for further testing in vivo. Leporin C was also found from the same fungus. It is a known compound from the synthesis of leporin A, however, it is new to natural product isolation (13). Leporin C can be added to the in house database of over 300 compounds. Finding this compound and leporin B could mean that other analogues are present in *Scytalidium cuboideum*. Austdiol and 7-epi-austdiol were also isolated from MSX68345. Fungal species MSX74367 was another fungal species that was

interesting to study due to it containing benzoisoquinoline structures, something that is lacking with the in house database of over 300 compounds. The fungus also produced the compound 8-*O*-methylfusarubin, which has the same core structure as 9-*O*-methylbostrycoidin with the loss of the nitrogen.

Figure 21. All Characterized Structures



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