FAYE, BOUKAR KOUMBA SENE, M.S. Precursor Directed Biosynthesis for the Discovery of 'Non-Natural' Natural Products in Fungi. (2020)
Directed by Dr. Nicholas H. Oberlies. 37 pp.

Verticillins are a class of dimeric epipolythiodioxopiperazine alkaloids that are known to have IC_{50} values of 10 nM or less in various human cancer cell lines This biological activity makes them potential candidates in the search of new anticancer drugs. However, further studies are hindered by limited supply when isolating verticillin class secondary metabolites from fungal sources. Thus, the search for fungal strains that produce these secondary metabolites at equal or greater abundances than the fungal strain currently being used in our lab, MSX59553, is necessary. This study revealed MSX59549 as a fungal strain of interest due to its production of verticillin A and verticillin H in higher abundances than MSX59553. Three additional fungal strains, MSX39480, MSX43578 and MSX60138, were also found to produce more verticillin H than MSX59553. These results lead to optimizing the isolation of verticillins for use in future chemical and pharmacological studies.

The generation of fluorinated verticillin derivatives has been achieved using precursor directed biosynthesis to incorporate 5-fluorotryptophan into the verticillin structures while retaining the potent biological activity. Further precursor directed biosynthesis experiments using 5-hydroxytryptophan, 5-chlorotryptophan and 5-bromotryptophan lead to the production of 9-hydroxyverticillin A and 9-chloroverticllin A. Further studies involving the isolation of these verticillin derivatives and

semisynthetic modification via cross coupling reactions may improve pharmacological properties within the verticillin class.

PRECURSOR DIRECTED BIOSYNTHESIS FOR THE DISCOVERY OF 'NON-NATURAL' NATURAL PRODUCTS IN FUNGI

by

Boukar Koumba Sene Faye

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
2020

Approved by

____
Committee Chair

APPROVAL PAGE

This thesis written by Boukar Koumba Sene Faye 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 ________________________
Kimberly S. Petersen

____
Daniel A. Todd

____
Date of Acceptance by Committee

____
Date of Final Oral Examination

TABLE OF CONTENTS

Page

LIST OF TABLES iv

LIST OF FIGURES v

CHAPTER

I. INTRODUCTION 1

1.1 Verticillin Class Compounds 1
1.2 Verticillin Supply 4
1.3 Verticillin Modification 4
1.4 Cross-Coupling Reactions 7

II. OPTIMIZING ISOLATION OF VERTICILLIN SECONDARY METABOLITES 9

2.1 Experimental 9
2.1.1 Fungal Fermentation 9
2.1.2 Extraction and Isolation 9
2.1.3 Identification of Verticillin Class Secondary Metabolites 10
2.1.4 Preparation of Fungal Extracts 11
2.1.5 Preparation of Verticillin A and Verticillin H Standards 11
2.1.6 LC-MS Analysis 12
2.2 Results 12
2.3 Conclusion 15

III. PRECURSOR DIRECTED BIOSYNTHESIS OF VERTICILLINS 16

3.1 Experimental 16
3.1.1 Fungal Culture for Precursor Directed Biosynthesis of Verticillin Derivatives 16
3.1.2 Extraction of Fungal Cultures 17
3.1.3 Identification of Modified Verticillin Derivatives 18
3.2 Results 20
3.3 Conclusion 35

REFERENCES 36

LIST OF TABLES

Page

Table 1. Cytotoxicities of Verticillin A, Verticillin H
and Fluorinated Analogues. 6

Table 2. Observed (+)-ESI Mass Spectral Data of Naturally
Occurring Verticillin Compounds..23

Table 3. The Predicted Mass Spectral Data for 9-Cl-Verticillin A
via (+)-ESI HMRS. 32

Table 4. The Predicted Mass Spectral Data for Verticillin B and
9-OH-Verticillin A via (+)-ESI HMRS. 33

Table 5. The Predicted Mass Spectral Data for Gliocladicillin C and
9-OH-Verticillin H via (+)-ESI HMRS. 34

LIST OF FIGURES

Page

Figure 1. Structures of Verticillin A and Related Analogues. 3

Figure 2. The Structures of Verticillin A, Verticillin H and the
Fluorinated Analogues. 5

Figure 3. Cross-Coupling Reactions. 8

Figure 4. Standard Curves for Verticillin A (A) and Verticillin H (B).............................14

Figure 5. The Percentage of Secondary Metabolites of Interest per
Milligram of Extract for Each Fungal Strain Grown on
Oatmeal. 15

Figure 6. Chemical Structures of the Modified Tryptophans. 17

Figure 7. Expected Secondary Metabolites from Precursor
Directed Biosynthesis. 19

Figure 8. (+)-ESI Base Peak Chromatogram and Total Scan
PDA of Verticillin B. 20

Figure 9. (+)-ESI Mass Spectrum of Verticillin B...21

Figure 10. (+)-ESI Tandem Mass Spectrum (MS/MS) of Verticillin B. 22

Figure 11. The (+)-ESI-HRMS Analysis of MSX59553 Control.....................................25

Figure 12. The (+)-ESI-HRMS Analysis of MSX59553 Grown with
5-OH-DL-Trp..27

Figure 13. Observed and Expected Isotopic Pattern of
9-Hydroxyverticillin A via (+)-ESI HMRS. 28

Figure 14. The (+)-ESI-HRMS Analysis of MSX59553
Grown with 5-Cl-DL-Trp. 30

CHAPTER I

INTRODUCTION

1.1 Verticillin Class Compounds

Fungi are an abundant source of structurally diverse and biologically active secondary metabolites.[1-3] Fungal secondary metabolites have been utilized as antibacterial and antifungal drugs, such as, penicillin and griseofulvin, respectively. In continuing the search for anticancer secondary metabolites from filamentous fungi,[4, 5] epipolythiodioxopiperazine (ETP) alkaloids have become a promising group due to their unique structure and activity. Verticillins, as an important member of ETP alkaloids, are a lead class of dimeric structures that exemplify potent antitumor activity,[6-10] along with antibacterial,[11-14] nematicidal[15] and immune induction properties.[16]

Verticillin A was first isolated in 1970 from what was first reported to be a *Veriticillium* sp., at which point they were only able to characterize the disulfide-bridged diketopiperazine system using ^{1}H NMR, IR and mass spectrometry.[17] It was not until one year later that the complete structure and absolute configuration was elucidated using chemical and physicochemical experiments.[12] In 2011 it was determined that the *Veriticillium* sp. was unlikely to produce verticillin A due to the absence of ETP gene clusters. It was suggested that the fungal culture was contaminated with *Clonostachys*

rosea, formerly known as *Gliocladium roseum*, which could have been responsible for the biosynthesis of verticillin A.[18]

Analogues of verticillin A were first discovered in 1973 and the first total synthesis of (+)-11, 11'-dideoxyverticillin A was considered to be a big step forward due to the complexity of the verticillin structural core.[19, 20] Verticillin H, the most recent analogue, was isolated from a *Clonostachys* sp. (Bionectriaceae) and tested for cytotoxicity in various cancer cell lines. These tests showed potent activity in human breast carcinoma, large cell lung carcinoma and astrocytoma at the same order of magnitude as the positive control drug, camptothecin.[4] Verticillins are an interesting class of ETP alkaloids that are characterized by their dimeric core and the disulfide bridge that is responsible for their potency.[21] The combination of all of these characteristics make verticillins desirable for further pharmacological use.

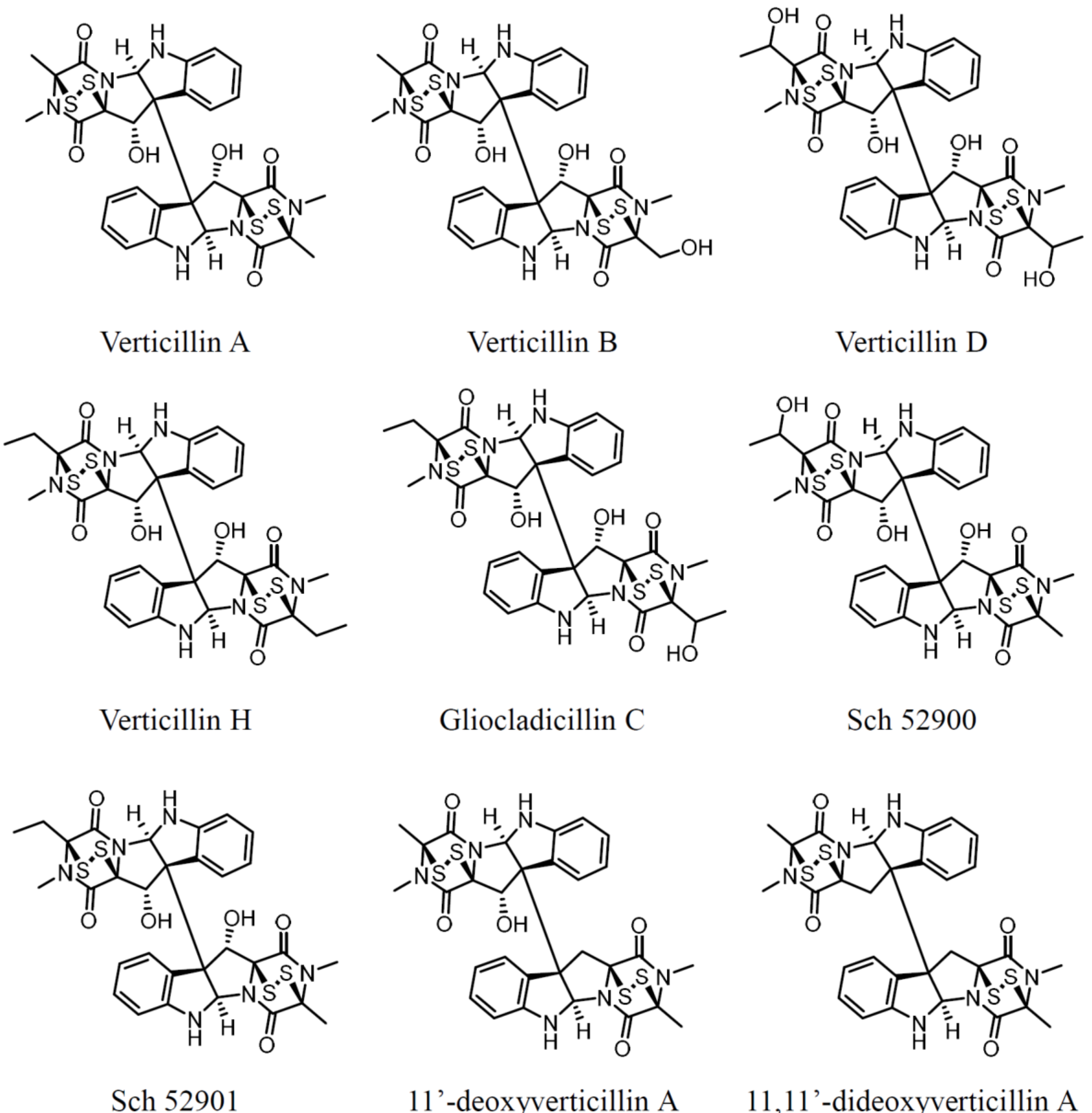

Figure 1. Structures of Verticillin A and Related Analogues.

1.2 Verticillin Supply

A common problem pursuing these compounds of interest is their low abundance, which in turn limits supply for pharmacological studies. In past studies, various fungal strains and growth conditions have been compared to optimize verticillin isolation. The results indicated that *Clonostachys rogersoniana* (MSX59553) produced more verticillin related compounds when grown on oatmeal media.[22] Twelve fungal strains, provided by Mycosynthetix, Inc., were analysed in this study to further optimize the isolation of verticillins and verticillin analogues.

1.3 Verticillin Modification

Approximately 20% of all pharmaceutical drugs on the market today contain fluorine because of its ability to improve pharmacological properties.[23-28] Verticillins are derived from multiple amino acids and believed to be biosynthesized via non-ribosomal peptide synthases (NRPSs).[29] Previous studies to incorporate fluorine into verticillin structures have applied this hypothesis by providing 5-fluoro-*DL*-tryptophan (5-F-*DL*-tryptophan) to fungal cultures. This experiment resulted in the isolation and characterization of 9-fluoroverticillin A, 9,9'-difluoroverticillin A, 9-fluoroverticillin H and 9,9'-difluoroverticillin H (Figure 2).[30] When these modified verticillins were tested against human breast, ovarian cancer and melanoma cell lines; the cytotoxicity results showed retention of biological activity (Table 1).

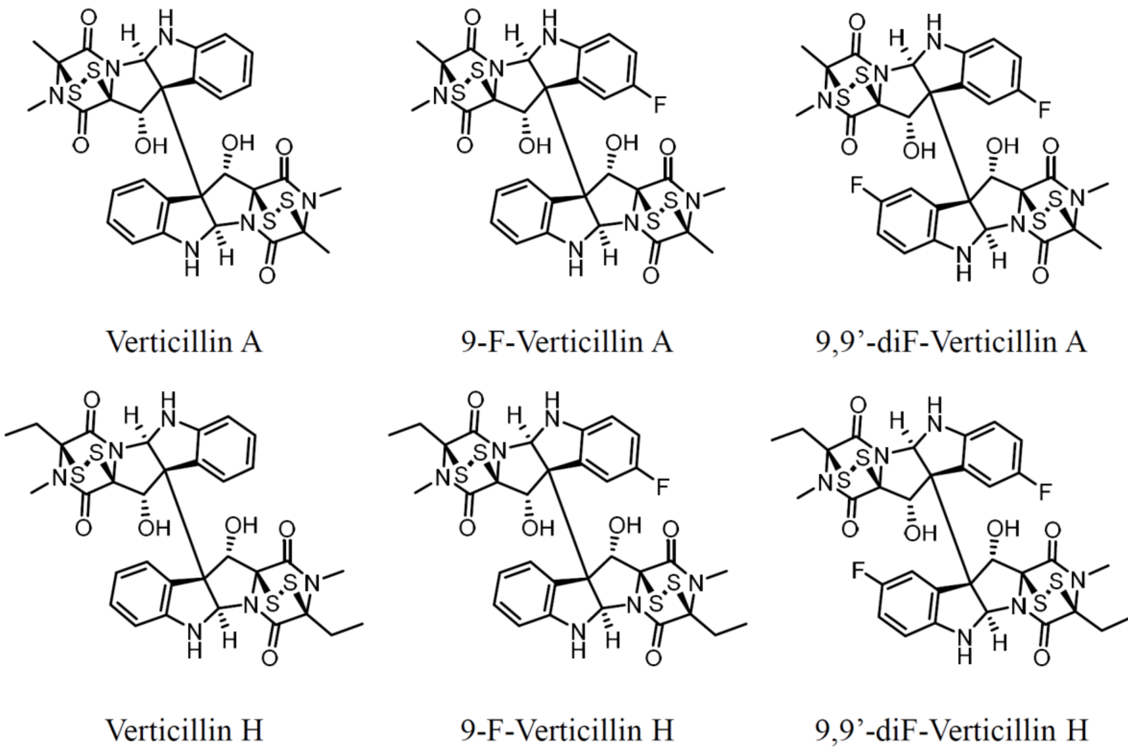

Figure 2. The Structures of Verticillin A, Verticillin H and the Fluorinated Analogues.

This biosynthetic approach for modification helps maintain the integrity of the core structure and thus activity. With a variety of modified tryptophan commercially available, we can pursue additional precursor directed biosynthesis experiments for the diversification of verticillin compounds.

Table 1. Cytotoxicities of Verticillin A, Verticillin H and Fluorinated Analogues.

compound	IC_{50} (nM)		
	MDA-MB-231 breast cancer	OVCAR3 ovarian cancer	MDA-MB-435 melanoma
Verticillin A	43	37	37
Verticillin H	71	100	88
9-F-Verticillin A	230	180	240
9-F-Verticillin H	46	39	49
9,9'-diF-Verticillin A	59	75	65
9,9'-diF-Verticillin H	840	920	830
Taxol (control)	170	5	0.4

1.4 Cross-Coupling Reactions

Natural product biological activity can be improved upon via semisynthetic reactions. The targeted verticillin modifications will enhance reactivity for semisynthetic reactions, specifically cross-coupling reactions. The Suzuki-Miyaura reaction has been used for various synthetic experiments due to its flexibility and mild conditions.[31, 32] The use of this reaction in natural product research has led to the engineering of natural product derivatives with improved biological activities.[33, 34] Targeting the addition of halides and hydroxy groups to the tryptophan moiety of the verticillin structure establishes potential reactive centers for Suzuki-Miyaura and phenol cross-coupling reactions, respectively (Figure 3).[35, 36] Modifications of natural products can not only enhance biological activity, but also allow for patenting, which is considered to be significant when trying to commercialize a drug.[37] The modification of verticillin class compounds via precursor directed biosynthesis followed by semisynthetic reactions can lead to improved biological activity as well as open the door for patentability.

A

X = Cl, Br

R_1 = aryl vinyl alkyl

B

R_2 = sulfonates, esters, carbamates, ethers, salts

Figure 3. Cross-Coupling Reactions. The verticillin A structure was simplified to focus on the tryptophan moiety. The proposed Suzuki-Miyaura reaction for the chlorinated and brominated verticillin analogues (A). The proposed cross-coupling reactions for the hydroxylated verticillin analogues (B).[31, 36]

CHAPTER II

OPTIMIZING ISOLATION OF VERTICILLIN SECONDARY METABOLITES

2.1 Experimental

2.1.1 Fungal Fermentation

Each fungal strain is maintained on oatmeal agar in Petri dishes and transferred once a month to a new plate. In order to scale up culturing, a fungus is grown on solid media. A mycelium agar plug from the Petri dish is transferred to Yeast Extract Soy Peptone Dextrose (YESD) broth to yield a seed culture. The seed culture is then incubated for three days at room temperature with agitation (~100 rpm). A 250 mL Erlenmeyer flask containing 10 g of oatmeal (Quaker) and 17 mL of deionized water is autoclaved. A seed culture is transferred to the sterilized flask and left to grow at room temperature for 7 to 10 days.

2.1.2 Extraction and Isolation

The fungal culture growth on solid media (oatmeal) is ceased using acetone, which is then chopped and shaken (~100 rpm) overnight at room temperature. The culture is then vacuum filtered, and the remaining residue is washed with acetone. The filtrate is then evaporated under vacuum until only water and a precipitate remained. To this, a mixture of 2:1 ethyl acetate:water is added, stirred and transferred to a separatory funnel. The mixture was left to separate overnight, then the ethyl acetate layer containing

the verticillin compounds was collected. The desugared extract is then evaporated to dryness under vacuum and defatted with 1:1:2 methanol:acetonitrile:hexane. The defatted, 1:1 methanol:acetonitrile layer is collected and evaporated to dryness under vacuum. The extract is then subjected to chromatographic separation via normal phase flash chromatography and normal phase high performance liquid chromatography (HPLC) in order to isolate the secondary metabolites of interest. The flash chromatographic separation was performed on a Teledyne ISCO Combiflash Rf system connected to an evaporative light scattering detector (ELSD) and a photodiode array detector (PDA) with UV detection set at 200-400 nm with a specific wavelength set at 300 nm. The HPLC separation was completed using a Varian ProStar HPLC system connect to a ProStar 355 PDA with UV detection set at 240 and 300 nm. The preparative normal-phase HPLC purification was achieved using a silica (5 µm; 250 x 21.2 mm) column with a mobile phase consisting of ethyl acetate and hexanes.

2.1.3 Identification of Verticillin Class Secondary Metabolites

The isolated metabolites are characterized and identified using a series of spectrometric and spectroscopic techniques. The HRMS data is collected on either a Thermo LTQ Orbitrap XL mass spectrometer or a Thermo Q Exactive Plus (Thermo Fisher Scientific); both equipped with an electrospray ionization source and a Waters Acquity UPLC (Waters Corp.) using a BEH C18 column (1.7 µm; 50 mm × 2.1 mm) set to a temperature and flow rate of 40ºC and 0.3 mL/min, respectively. The mobile phase consists of acetonitrile and water (both acidified with 0.1% formic acid), starting at 15% acetonitrile and increasing linearly to 100% over 8 minutes, with a 1.5-minute hold

before returning to the starting conditions. The scan range was narrowed (m/z 600 to m/z 800) in order to optimize detection of secondary metabolites in the verticillin class. The NMR data is collected using a JEOL ECS-400 spectrometer equipped with a JEOL normal geometry broadband Royal probe and 24-slot autosampler, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C as well as a JEOL ECA-500 spectrometer, operating at 500 MHz for ^{1}H and 125 MHZ for ^{13}C (Both from JEOL USA, Inc.). MS data and NMR data are analyzed using Xcalibur software and MestReNova software, respectively. All data is compared to values within the in-house dereplication database, ensuring that the isolates are verticillin class compounds. In the case that a secondary metabolite does not match a known compound, the structure is elucidated using the spectrometric and spectroscopic data gathered.

2.1.4 Preparation of Fungal Extracts

The fungal extracts were prepared at a 50 µg/mL concentration in 1.5 mL Eppendorf and 200 µL of the solutions were transferred to an Ansi 96 well – 1 mL plate.

2.1.5 Preparation of Verticillin A and Verticillin H Standards

Aliquots of each metabolite were weighed and prepared with 1,1,1,3,3,3-hexafluoropropanol at a concentration of 1.05 mg/mL, then 0.50 mL of each solution were combined in a 1.5 mL Eppendorf tube to yield a solution containing 0.525 mg/mL of each metabolite. Using this solution, serial dilutions were conducted to produce concentrations ranging from 1 ng/mL to 262144 ng/mL.

2.1.6 LC-MS Analysis

To quantify verticillin A and verticillin H within the fungal extracts, an LC-MS analysis was conducted in positive ion mode. The mass spectrometer scanned across a mass range of *m/z* 600 to 800 and a spray voltage of 4 kV. This was coupled with an Acquity UPLC system (Waters Corp.), which had a flow rate of 0.3 mL/min and utilized a BEH C_{18} column (2.1 mm x 50 mm, 1.7 μm) that was operated at 40°C. The mobile phase consisted of Fisher Optima LC-MS grade acetonitrile:water (both with 0.1% formic acid). The gradient began at 15% acetonitrile and linearly increased to 100% acetonitrile over 8 min. It was then held at 100% for 1.5 min before returning to starting conditions to re-equilibrate. All data was analyzed using Thermo Xcalibur Qual Browser and Thermo Xcalibur Quan Browser.

2.2 Results

The standard solutions containing verticillin A and verticillin H were ran in triplicate to create calibration curves for the concentration of each metabolite. The quantitation range for verticillin A and verticillin H were 16 ng/mL to 2048 ng/mL and 32 ng/mL to 4096 ng/mL, respectively. These results enable quantification of secondary metabolite presence in mixtures such as fungal extracts (Figure 4).

Mycosynthetix, Inc. provided twelve fungal strains suspected of biosynthesizing verticillin compounds which were grown in this experiment to compare the amount of verticillin A and verticillin H produced. The fungal strains that produced the most verticillin A were MSX59549 and MSX59553 with MSX59549 producing 1.85 % verticillin A by mass of extract and MSX59553 producing 1.71 %. The most abundant

amounts of verticillin H were produced by MSX39480, MSX43578, MSX59549 and MSX60138 at 1.11 % ,1.19 % , 1.23 % and 1.04 % per milligram of extract, respectively. Of the twelve fungal strains analysed, four fungal strains (MSX60030, MSX64561, MSX70918 and MSX71844) did not biosynthesize detectable amounts of verticillin A and verticillin H. MSX64546 was found to produce quantifiable amounts of verticillin A, however the amount of verticillin H fell below the quantitation range limit (Figure 5).

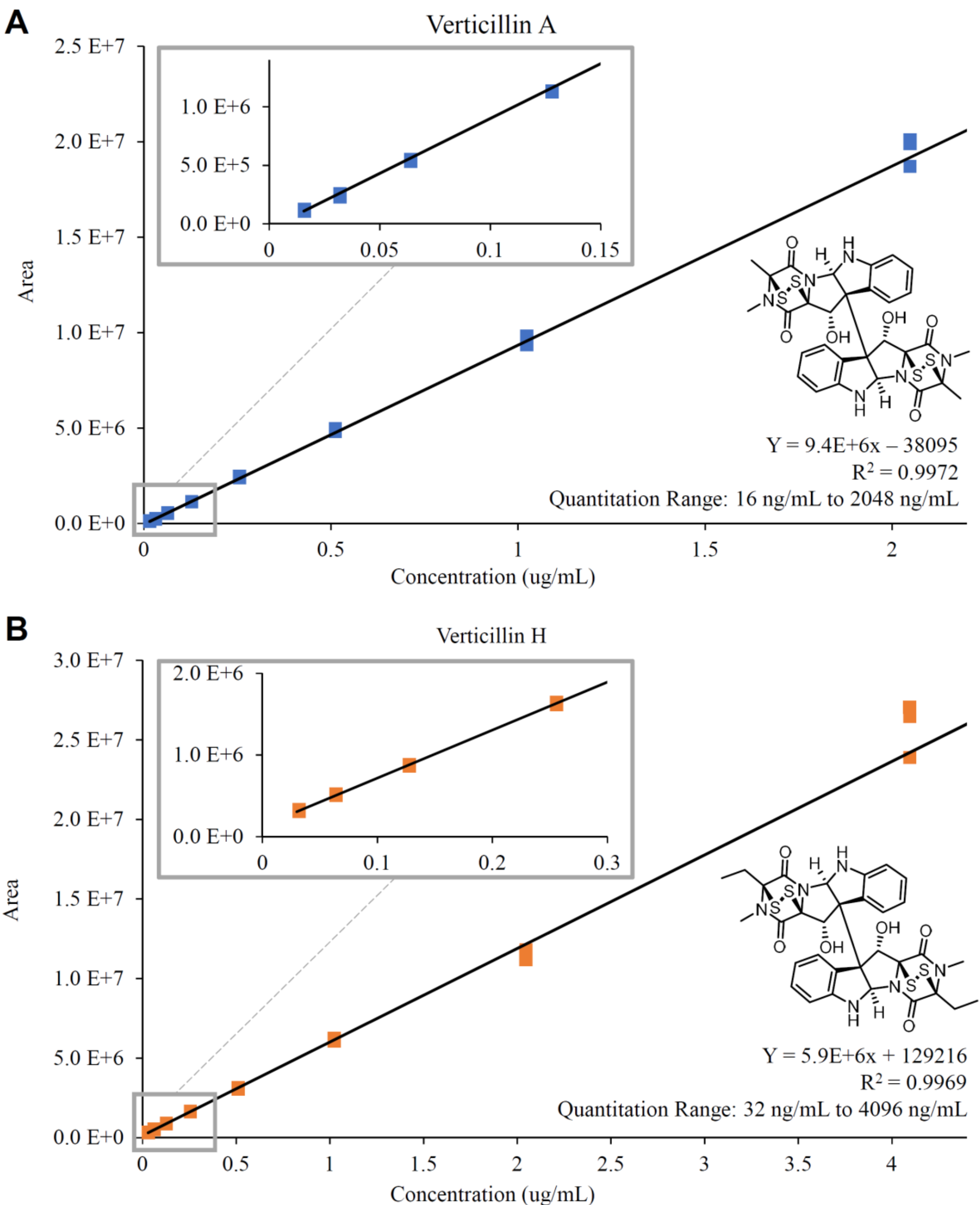

Figure 4. Standard Curves for Verticillin A (A) and Verticillin H (B).

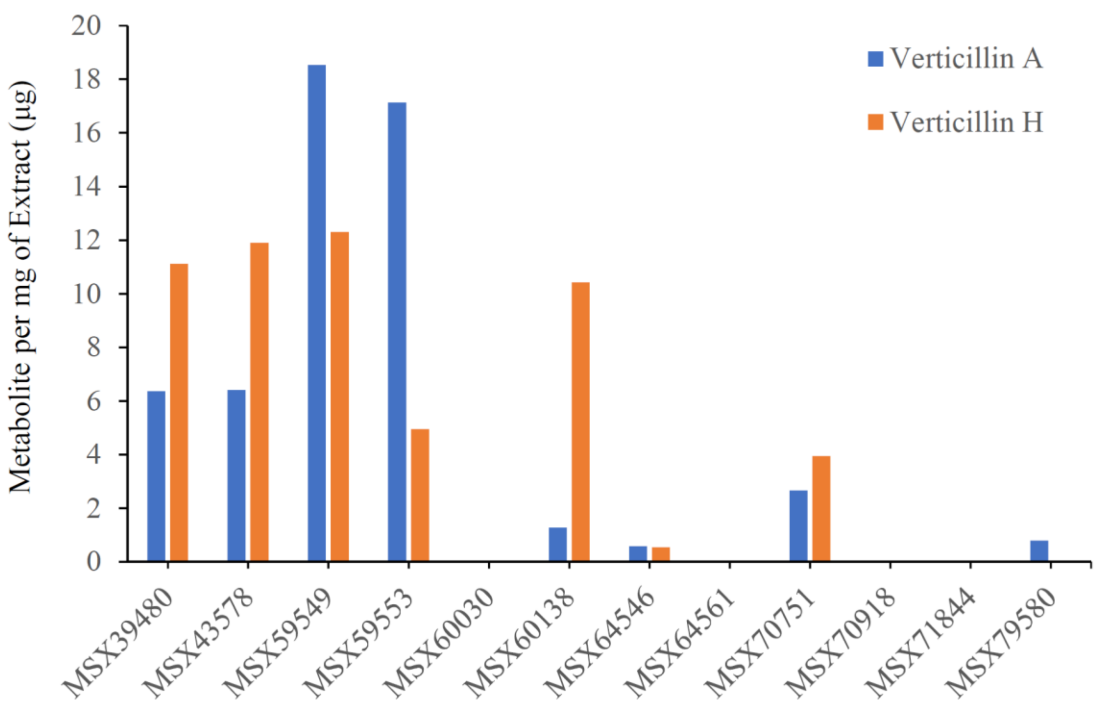

Figure 5. The Percentage of Secondary Metabolites of Interest per Milligram of Extract for Each Fungal Strain Grown on Oatmeal.

2.3 Conclusion

Based on the results from comparing various fungal strains, MSX59549 and MSX59553 were used to pursue the isolation of verticillin A and verticillin H. Prior to beginning this there was about 20 mg of verticillin A and 10 mg of verticillin H in storage. With the use of both MSX59553 and MSX59549, an additional 140 mg of verticillin A and 50 mg of verticillin H was isolated thereby decreasing the limitation of supply for future studies with these compounds. Continuing efforts to isolate more material will advance pharmacological research on verticillin compounds in hopes of improving anticancer activity.

CHAPTER III

PRECURSOR DIRECTED BIOSYNTHESIS OF VERTICILLINS

3.1 Experimental

3.1.1 Fungal Culture for Precursor Directed Biosynthesis of Verticillin Derivatives

Two types of fungal cultures were prepared for this experiment: Petri dish and solid media. A 1.125 g sample of 5-hydroxy-*DL*-tryptophan (5-OH-*DL*-Trp) powder, purchased from Chem-Impex Int'l. Inc., was added to 150 mL of sterilized 50 °C water to make a 7500 ppm amino acid stock solution of the modified amino acid. The solution is then stirred for 20 minutes to homogenize it, then filtered using a sterile Nalgene™, 0.45μm filter. For the Petri dish fungal culture, an amino acid oatmeal agar solution was prepared by combining 20 mL of the latter solution and 200 mL of oatmeal agar solution. An agar plug was then transferred to the amino acid oatmeal agar plate and left to grow at room temperature for 7 - 10 days. This procedure was also used to make Petri dishes containing 5-chloro-*DL*-tryptophan (5-Cl-*DL*-Trp) and 5-bromo-*DL*-tryptophan (5-Br-*DL*-Trp) (Figure 6).

5-OH-*DL*-tryptophan 5-Cl-*DL*-tryptophan 5-Br-*DL*-tryptophan

Figure 6. Chemical Structures of the Modified Tryptophans.

To prepare the solid media fungal culture, a seed culture was started by combining 1 mL of 7500 ppm amino acid stock solution and 10 mL of YESD broth in a falcon tube. This was then inoculated with an agar plug taken from a fungal culture grown on oatmeal agar. The seed culture was shaken (~100 rpm) at room temperature for 5 days. A 250 mL Erlenmeyer flask containing 10 g of oatmeal (Quaker) and 15 mL of deionized water is autoclaved. The seed culture along with 2 mL of 7500 ppm amino acid stock solution was transferred to the sterilized flask, then left to grow at room temperature for 7 to 10 days.

3.1.2 Extraction of Fungal Cultures

In this study, two extraction methods are used: Petri dish (oatmeal agar) and solid media (oatmeal). For the former, the culture is chopped using a spatula and transferred to a scintillation vial. The vial is then filled with acetone and left to sit for 4 hours before being vortexed and filtered. The extract is then dried under nitrogen before being subjected to further analysis. For the latter, the fungal culture growth is ceased using acetone, and then it is chopped and shaken (~100 rpm) overnight at room temperature. The culture is then vacuum filtered, and the remaining residue is washed with acetone.

The filtrate is then evaporated under vacuum until only water and a precipitate remained. To this, a mixture of 2:1 ethyl acetate:water is added, stirred and transferred to a separatory funnel. The ethyl acetate layer contains the verticillins, while the water removes unnecessary sugars. The desugared extract is then evaporated to dryness under vacuum and defatted with 1:1:2 methanol:acetonitrile:hexane. The defatted, 1:1 methanol:acetonitrile layer is collected and evaporated to dryness under vacuum.

3.1.3 Identification of Modified Verticillin Derivatives

The HRMS data is collected on either a Thermo LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization source and a Waters Acquity UPLC (Waters Corp.) using a BEH C18 column (1.7 µm; 50 mm × 2.1 mm) set to a temperature and flow rate of 40ºC and 0.3 mL/min, respectively. The mobile phase consists of acetonitrile and water (both with 0.1% formic acid), starting at 15% acetonitrile and increasing linearly to 100% over 8 minutes, with a 1.5-minute hold before returning to the starting conditions. The scan range was narrowed (m/z 600 to m/z 800) in order to optimize detection of secondary metabolites of interest. Similar to the results obtained during the incorporation of fluorine into verticillin structures, it is expected that the modification can appear at the 9 and 9' positions of the verticillin core structure. For verticillin A and verticillin H, it is expected that each compound will have two analogues per modified tryptophan (Figure 7).

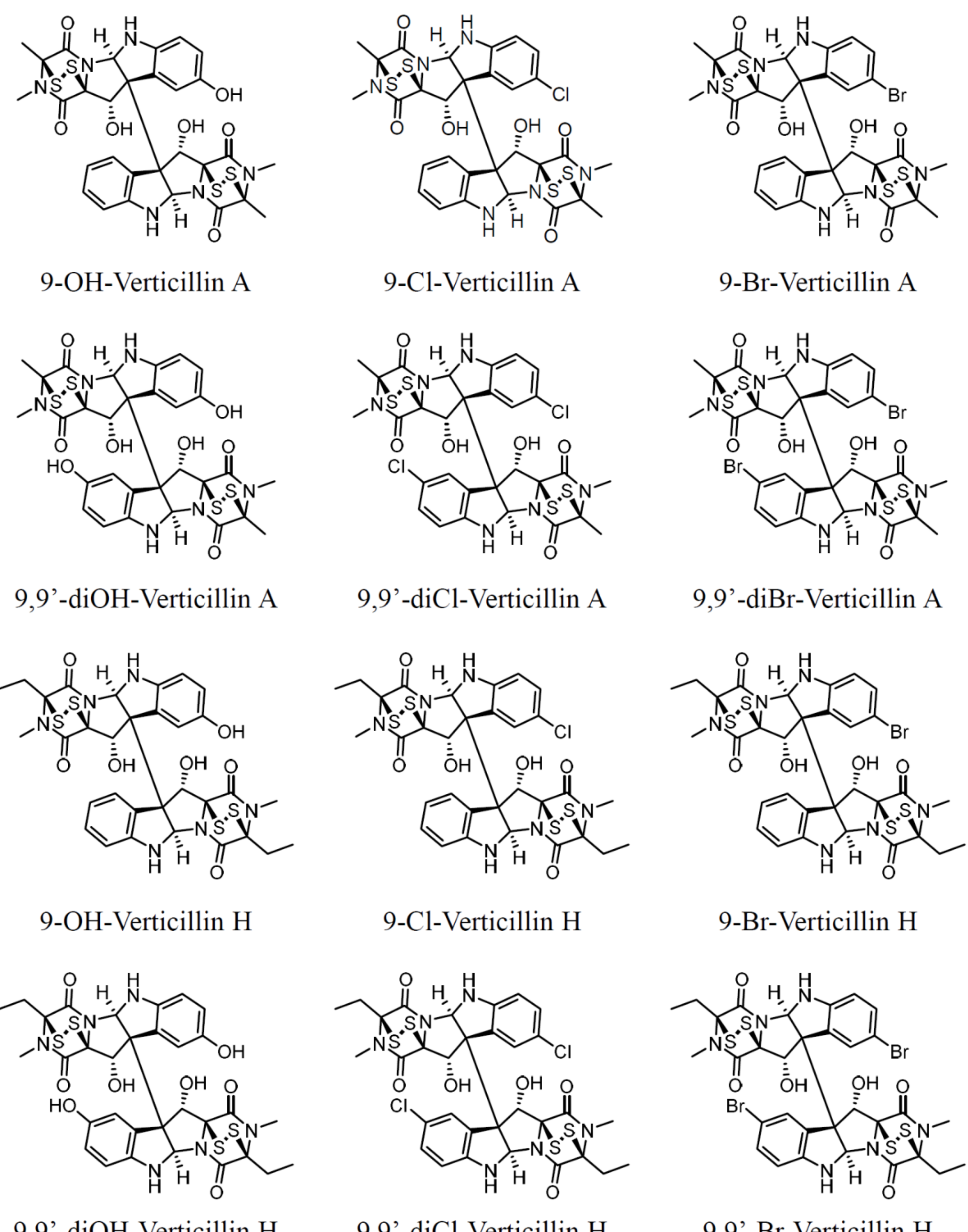

Figure 7. Expected Secondary Metabolites from Precursor Directed Biosynthesis.

3.2 Results

A series of verticillin secondary metabolites naturally metabolised by *Clonostachys* spp. were extracted and isolated for analysis via UPLC-MS. The identification and purity of each standard was supported using NMR and MS analysis (Figure 8 and 9).

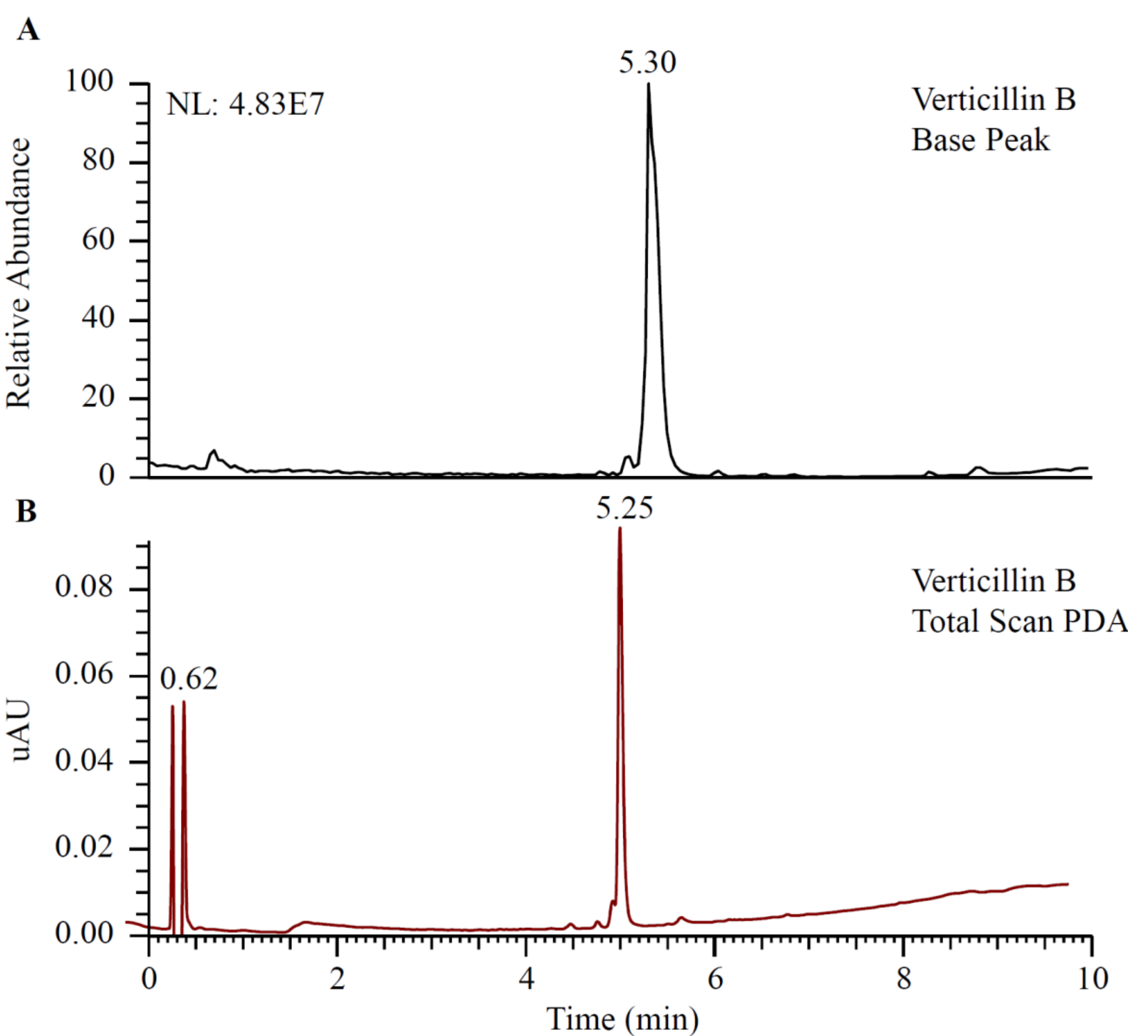

Figure 8. (+)-ESI Base Peak Chromatogram and Total Scan PDA of Verticillin B.

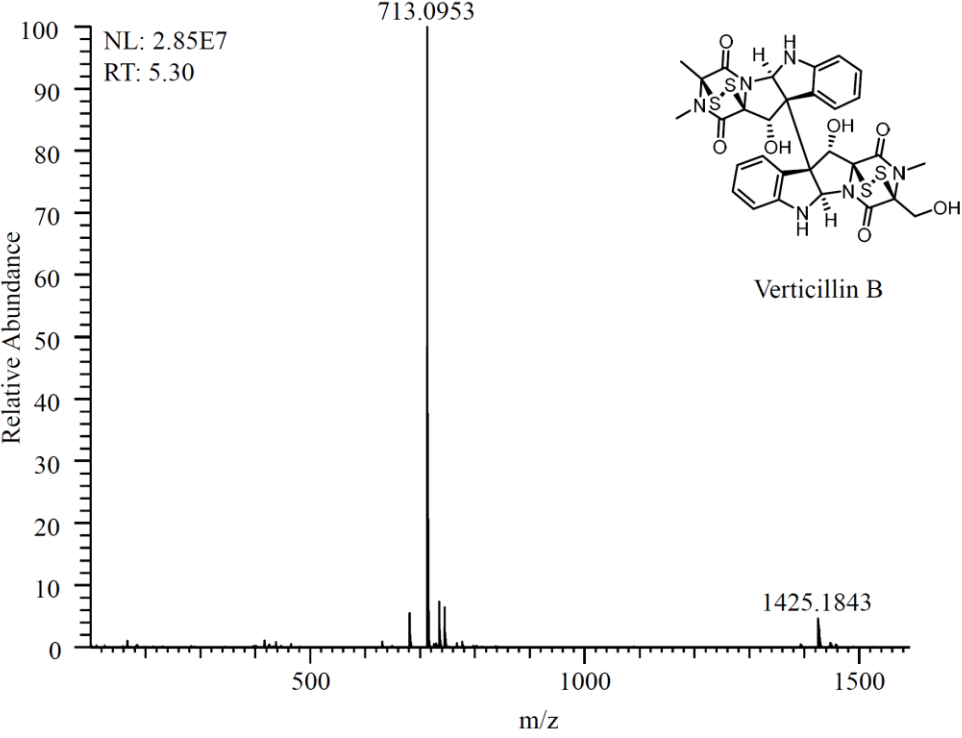

Figure 9. (+)-ESI Mass Spectrum of Verticillin B.

Each standard was then subjected to tandem mass spectrometry (MS/MS) experiments to generate fragmentation patterns for each metabolite (Figure 10). The observed retention times and ten most abundant MS/MS fragments were recorded and referenced for identification of secondary metabolites (Table 2).

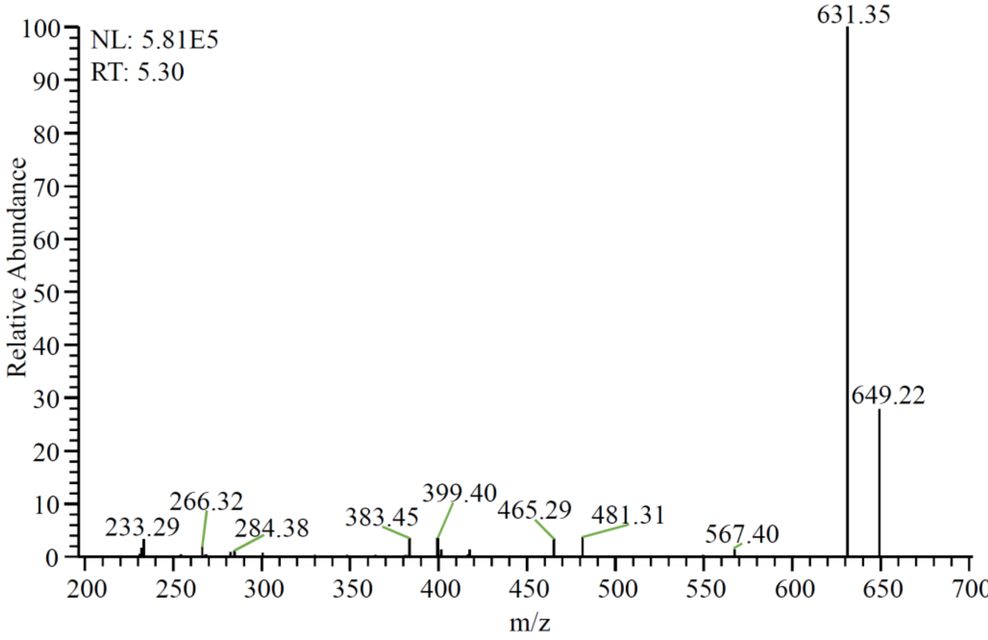

Figure 10. (+)-ESI Tandem Mass Spectrum (MS/MS) of Verticillin B.

Table 2. Observed (+)-ESI Mass Spectral Data of Naturally Occurring Verticillin Compounds.

Compound	Retention Time (min)	Calculated $[M+H]^+$	MS/MS Fragments*	
Verticillin A	5.42	697.1031	633.01	632.28
			615.16	551.25
			465.23	401.22
			383.18	284.13
			233.09	232.11
Verticillin B	5.30	713.0980	631.34	649.22
			481.33	465.29
			399.40	383.44
			233.29	266.31
			284.39	567.51
Verticillin H	6.02	725.1345	643.09	661.02
			397.26	415.10
			479.22	579.36
			233.05	232.12
			298.20	478.50
Gliocladicillin C	5.54	741.1293	659.16	677.04
			413.26	280.16
			495.12	676.40
			397.31	296.16
			479.00	233.06

*Ten most abundant MS/MS fragments.

A control flask of MSX59553 was grown on oatmeal and extracted for UPLC-MS analysis. A search for the exact masses of each expected modified verticillin was performed to determine if MSX59553 was producing metabolites with similar masses. As seen in Figure 11, the extracted ion chromatograms (XICs) produced several peaks that can be considered to be within the baseline because they had normalisation levels 4 orders of magnitude lower than that of the extract. An XIC for the mass of 741.1293 amu produced a peak at the retention time that is associated with the presence of gliocladicillin C, indicating that MSX59553 naturally metabolites this verticillin compound.

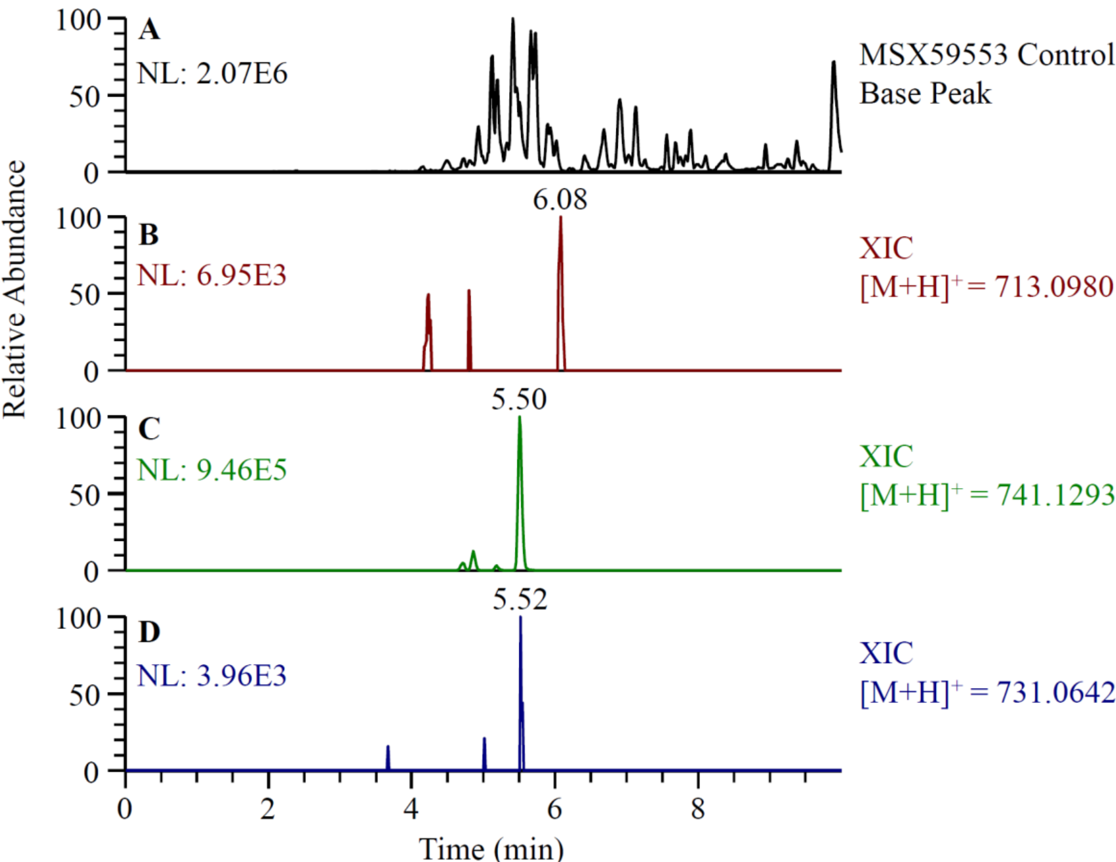

Figure 11. The (+)-ESI-HRMS Analysis of MSX59553 Control. The base peak chromatogram of MSX59553 grown on Oatmeal (A). The extracted-ion chromatogram (XIC) of 9-hydroxyverticillin A (B), gliocladicillin C/9-hydroxyverticillin (C), and 9-chloroverticillin A (D).

The extract of MSX59553 grown on solid media with 5-OH-DL-Trp was analysed via UPLC-HMRS in pursuit of modified verticillin A and verticillin H. The expected analogues were 9-hydroxyverticllin A, 9,9'-dihyrdroxyverticillin A, 9-hydroxyverticillin H, and 9,9'-dihydroxyverticillin H (Figure 7). The production of secondary metabolites was first assessed using extracted-ion chromatograms (XICs) by searching for their respective mass-to-charge ratios with an accurate mass tolerance of 5 ppm. In addition to HRMS data, retention times and UV data were also used as mutually supportive data. Further analysis in search of *m/z* 713.0980, the calculated $[M+H]^+$ of 9-hydroxyverticillin H and verticillin B, produced a peak at 4.97 min (Figure 12).

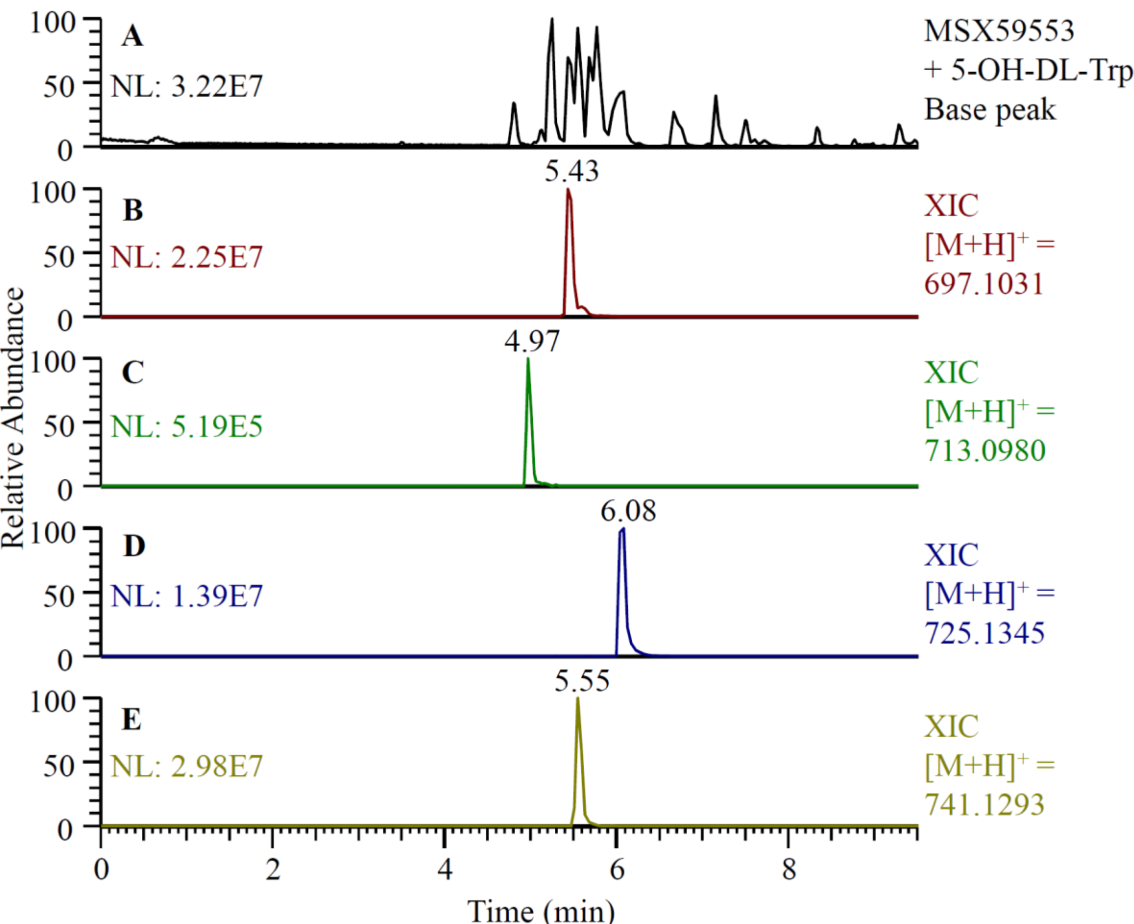

Figure 12. The (+)-ESI-HRMS Analysis of MSX59553 Grown with 5-OH-DL-Trp. The base peak chromatogram of MSX59553 grown with 5-OH-DL-Trp (A). The extracted-ion chromatogram (XIC) of verticillin A (B), 9-hydroxyverticillin A (C), verticillin H (D) and gliocladicillin C/9-hydroxyverticillin H (E).

When pure verticillin B was analyzed using the same solvents, mass spectrometer, and method; it was observed at a retention time of 5.30 min which led to the conclusion that the peak at 4.97 min was 9-hydroxyverticillin A. The isotopic pattern of the ion found within the peak at 4.97 min was compared to that of a simulated isotopic pattern

for 9-hydroxyverticillin A. The comparison yielded very similar results, indicating that 9-hydroxyverticllin A was responsible for the peak observed at 4.97 min (Figure 13).

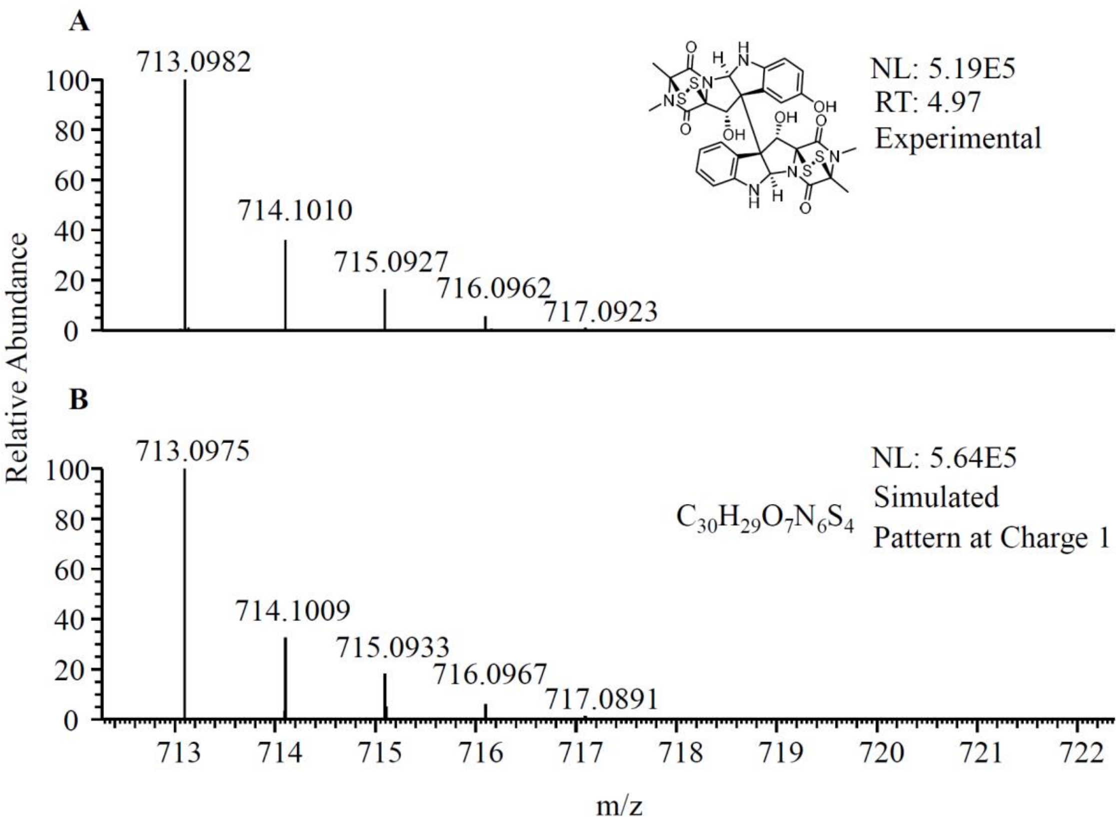

Figure 13. Observed and Expected Isotopic Pattern of 9-Hydroxyverticillin A via (+)-ESI HMRS.

Ratios of the naturally occurring secondary metabolites to the modified metabolites were estimated using the normalisation levels obtained in HRMS analysis. The ratios were estimated to be about 1:40 for Verticillin A to 9-hydroxyverticillin A (Figure 6). When searching for the presence of 9-hydroxyverticillin H, a peak at 5.55 min was observed. However, this peak cannot be identified at the modified compound

because the mass and retention time match that of gliocladicillin C. Therefore, it is unclear whether 9-hydroxyverticillin H was produced. Similar UPLC-HRMS analyses of the Petri dish extracts of MSX59553 with 5-Cl-DL-Trp indicated that the fungus is able to produce 9-chloroverticillin A at an estimated ratio of about 1:240 (verticillin to 9-chloroverticillin A). However, 9-chloroverticillin H was not observed. Extracts of the fungus grown with 5-Br-DL-Trp did not indicate the presence of any modified verticillin compounds and the peak at 1.52 min was identified as single scan event from an ion that appeared near the base of the chromatogram (Figure 14).

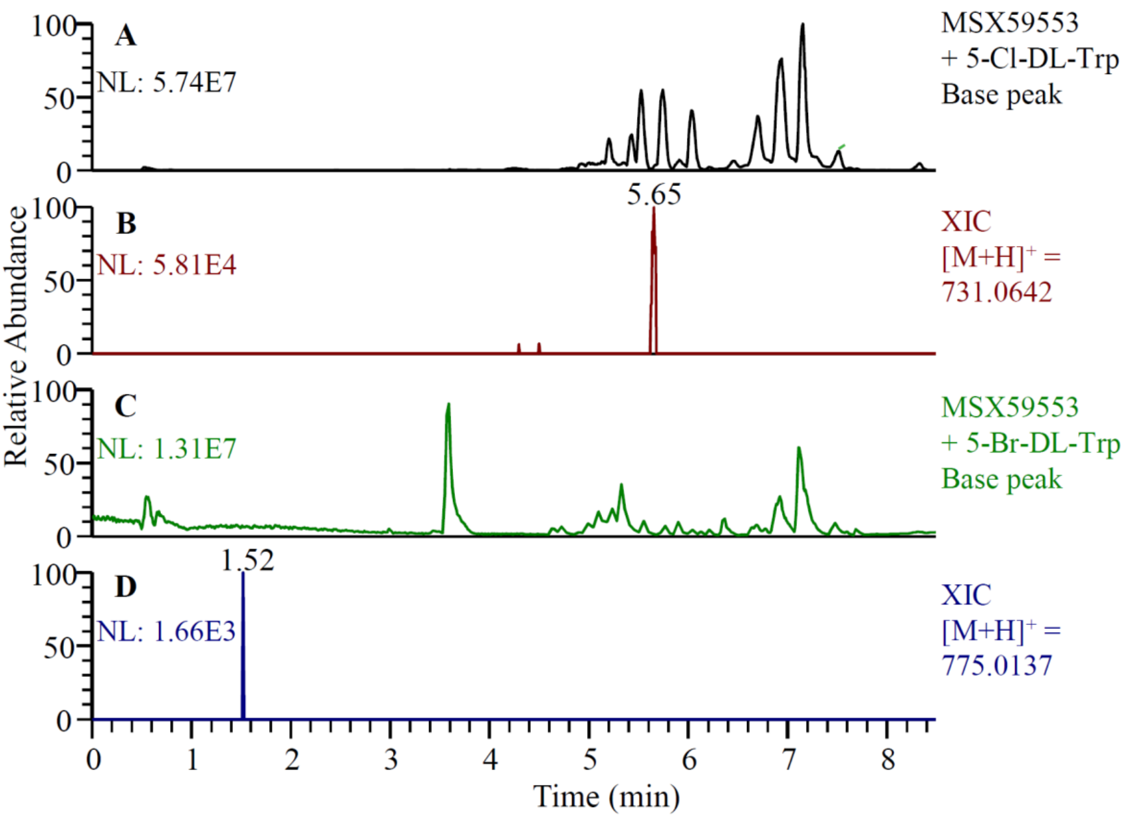

Figure 14. The (+)-ESI-HRMS Analysis of MSX59553 Grown with 5-Cl-DL-Trp. The base peak chromatogram of MSX59553 grown with 5-Cl-DL-Trp (A) and the extracted-ion chromatogram (XIC) of 9-chloroverticillin A (B). The (+)-ESI-base peak chromatogram of MSX59553 grown with 5-Br-DL-Trp (C) and the extracted-ion chromatogram (XIC) of 9-bromoverticillin A (D).

Considering that NRPS biosynthesis may be responsible for the incorporation of the tryptophan moiety within verticillin structures,[29, 30, 38] the success of precursor directed biosynthesis in MSX59553 might be limited by the size of the atom or molecule being added. The successful incorporation of 5-F-DL-Trp, done previously by Amrine, C.S. *et al*, and 5-OH-DL-Trp to produce isolable amounts of modified verticillins

compounds has likely been due to their smaller atomic sizes in comparison 5-Cl-DL-Trp and 5-Br-DL-Trp[30].

Tandem mass spectrometry (MS/MS) can be utilized to further confirm the production of the modified verticillin analogues due to the potential of unique fragmentations. ACD/Labs software was used to predict the expected MS/MS fragmentation of 9-chloroverticillin A, verticillin B, 9-hydroxyverticillin A, gliocladicillin C and 9-hydroxyverticillin H. Most of the MS/MS fragments of 9-chloroverticillin A would be unique in comparison to the naturally biosynthesized secondary metabolites (Table 3). The retention times of verticillin B and 9-hydroxyverticillin A were observed to be different and to further confirm the identity of each peak, the unique MS/MS fragments of each molecule can be used to indicate their presence (Table 4). The MS/MS fragments of gliocladicillin C and 9-hydroxyverticllin H can be analysed to distinguish between their identical accurate masses (Table 5).

Table 3. The Predicted Mass Spectral Data for 9-Cl-Verticillin A via (+)-ESI HMRS.

Compound	Calculated $[M+H]^+$	Predicted MS/MS Fragments		
9-Cl-Verticillin A	731.0642	730.0563	712.0458	703.0454
		702.0614	701.0298	699.0141
		696.0686	694.0797	694.0352
		684.0509	676.0691	676.0458
		673.0349	666.1122	664.0966
		655.0243	654.0250	648.1016
		645.0036	642.0581	636.0145
		620.0640	610.0860	602.0534
		528.0693	526.0536	498.0587
		496.0431	480.0481	383.0165
		381.0009	365.0059	362.9903
		349.0555	347.0398	331.0449
		329.0293	234.0133	231.9976
		204.0027	201.9871	

Table 4. The Predicted Mass Spectral Data for Verticillin B and 9-OH-Verticillin A via (+)-ESI HMRS.

Compound	Calculated $[M+H]^+$	Predicted MS/MS Fragments			
Verticillin B	713.0980	712.0902	**695.0637**	694.0797	685.0793
		684.0953	683.0637	**682.0797**	681.048
		678.1025	676.0691	666.0847	**664.0691**
		658.0797	655.0688	648.1461	**648.0919**
		646.1304	**642.0847**	637.0582	636.0589
		630.1335	627.0375	624.0919	619.0324
		618.0484	592.1199	**576.125**	510.1032
		508.0875	**492.0926**	480.0926	478.0769
		464.0977	462.082	**446.0871**	365.0504
		363.0347	349.0555	347.0398	345.0242
		332.0289	331.0449	329.0293	**250.0082**
		247.9925	234.0133	231.9976	**229.982**
		219.9976	204.0027	201.9871	
9-OH-Verticillin A	713.0980	712.0902	694.0797	685.0793	684.0953
		683.0637	681.048	678.1025	676.0691
		666.0847	658.0797	655.0688	648.1461
		646.1304	637.0582	636.0589	630.1335
		627.0375	624.0919	**620.064**	618.0484
		602.0534	592.1199	510.1032	508.0875
		480.0926	478.0769	462.082	365.0504
		363.0347	349.0555	347.0398	345.0242
		331.0449	329.0293	234.0133	231.9976
		204.0027	201.9871		

Table 5. The Predicted Mass Spectral Data for Gliocladicillin C and 9-OH-Verticillin H via (+)-ESI HMRS.

Compound	Calculated $[M+H]^+$	Predicted MS/MS Fragments
Gliocladicillin C	741.1293	740.1215 722.1110 713.1106 712.1266 712.0902 711.0950 709.0793 706.1338 704.1004 696.0953 694.1160 694.0797 683.1001 678.1025 **678.0847** 676.1774 674.1617 672.0953 665.0895 664.0902 662.1076 658.1668 **656.1004** 646.0797 640.1232 606.1355 **590.1406** 579.1246 522.1032 **506.1082** 494.1082 **478.1133** 476.0977 **460.1028** 379.0660 377.0504 363.0711 361.0555 359.0398 345.0606 343.0449 **264.0238** **262.0082** 248.0289 246.0133 **243.9976** **234.0133** 218.0184
9-OH-Verticillin H	741.1293	740.1215 722.1110 713.1106 712.1266 712.0902 711.0950 709.0793 706.1338 704.1004 **698.1110** **694.1338** 694.1160 694.0797 683.1001 678.1025 676.1774 674.1617 672.0953 665.0895 664.0902 662.1076 658.1668 **648.0953** 646.0797 640.1232 **630.0847** 606.1355 579.1246 522.1032 494.1082 476.0977 379.0660 377.0504 363.0711 361.0555 359.0398 345.0606 343.0449 248.0289 246.0133 218.0184

3.3 Conclusion

Precursor directed biosynthesis of modified verticillin compounds by the addition of 5-OH-DL-Trp to solid media has shown to increase the chemical diversity found within MSX59553. Scale up of these growths would yield isolable amounts of 9-hydroxyverticillin A and help determine if the modified tryptophan was also used to biosynthesize 9-hydroxyverticlllin H. In addition to the patentable nature of these 'non-natural' natural products, the modifications allow for the possibility for semi-synthetic experiments, specifically at the modified position of the tryptophan moiety.

REFERENCES

1. El-Elimat, T.; Zhang, X.; Jarjoura, D.; Moy, F.J.; Orjala, J.; Kinghorn, A.D.; Pearce, C.J.; Oberlies, N.H. *ACS Med. Chem. Lett.* **2012**, *3*, 645-649.
2. González-Medina, M.; Prieto-Martínez, F.D.; Naveja, J.J.; Méndez-Lucio, O.; El-Elimat, T.; Pearce, C.J.; Oberlies, N.H.; Figueroa, M.; Medina-Franco, J.L. *Future Med. Chem.* **2016**, *8*, 1399-1412.
3. González-Medina, M.; Owen, J.R.; El-Elimat, T.; Pearce, C.J.; Oberlies, N.H.; Figueroa, M.; Medina-Franco, J.L. *Front. Pharmacol.* **2017**, *8*, 1-12.
4. Figueroa, M.; Graf, T.N.; Ayers, S.; Adcock, A.F.; Kroll, D.J.; Yang, J.; Swanson, S.M.; Munoz-Acuna, U.; Carcache de Blanco, E.J.; Agrawal, R.; Wani, M.C.; Darveaux, B.A.; Pearce, C.J.; Oberlies, N.H. *J. Antibiot.* **2012**, *65*, 559-564.
5. 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. *Anticancer Res.* **2016**, *36*, 5623-5637.
6. Chen, Y.; Guo, H.; Du, Z.; Liu, X.Z.; Che, Y.; Ye, X. *Cell Prolif.* **2009**, *42*, 838-847.
7. Chen, Y.; Zhang, Y.-X.; Li, M.-H.; Zhao, W.-M.; Shi, Y.-H.; Miao, Z.-H.; Zhang, X.-W.; Lin, L.-P.; Ding, J. *Biochem. Biophys. Res. Commun.* **2005**, *329*, 1334-1342.
8. Jordan, T.W.; Cordiner, S.J. *Trends Pharmacol. Sci.* **1987**, *8*, 144-149.
9. Son, B.W.; Jensen, P.R.; Kauffman, C.A.; Fenical, W. *Nat. Prod. Lett.* **1999**, *13*, 213-222.
10. Zhang, Y.-X.; Chen, Y.; Guo, X.-N.; Zhang, X.-W.; Zhao, W.-M.; Zhong, L.; Zhou, J.; Xi, Y.; Lin, L.-P.; Ding, J. *Anti-Cancer Drugs* **2005**, *16*, 515-524.
11. Joshi, B.K.; Gloer, J.B.; Wicklow, D.T. *J. Nat. Prod.* **1999**, *62*, 730-733.
12. Minato, H.; Matsumoto, M.; Katayama, T. *J. Chem. Soc. D* **1971**, 44-45.
13. Zheng, C.-J.; Kim, C.-J.; Bae, K.S.; Kim, Y.-H.; Kim, W.-G. *J. Nat. Prod.* **2006**, *69*, 1816-1819.
14. Zheng, C.-J.; Park, S.-H.; Koshino, H.; Kim, Y.-H.; Kim, W.-G. *J. Antibiot.* **2007**, *60*, 61-64.
15. Dong, J.-Y.; He, H.-P.; Shen, Y.-M.; Zhang, K.-Q. *J. Nat. Prod.* **2005**, *68*, 1510-1513.
16. Erkel, G.; Gehrt, A.; Anke, T.; Sterner, O. *Z. Naturforsch. C* **2002**, *57*, 759-768.
17. Katagiri, K.; Sato, K.; Hayakawa, S.; Matsushima, T.; Minato, H. *J. Antibiot.* **1970**, *23*, 420-422.
18. Schenke, D.; Böttcher, C.; Lee, J.; Scheel, D. *J. Antibiot.* **2011**, *64*, 523-524.
19. Minato, H.; Matsumoto, M.; Katayama, T. *J. Chem. Soc., Perkin Trans. 1* **1973**, 1819.
20. Kim, J.; Ashenhurst, J.A.; Movassaghi, M. *Science* **2009**, *324*, 238-241.
21. Li, E.; Hou, B.; Gao, Q.; Xu, Y.; Zhang, C.; Liu, X.; Jiang, X.; Che, Y. *J. Nat. Prod.* **2020**, *83*, 601-609.

22. Amrine, C.S.M.; Raja, H.A.; Darveaux, B.A.; Pearce, C.J.; Oberlies, N.H. *J. Ind. Microbiol. Biotechnol.* **2018**, *45*, 1053-1065.
23. Yamazaki, T.; Taguchi, T.; Ojima, I. *Fluorine in Medicinal Chemistry and Chemical Biology*; Ojima, I., Ed.; John Wiley & Sons: Wiltshire, Great Britain, 2009; pp 3-46.
24. Gillis, E.P.; Eastman, K.J.; Hill, M.D.; Donnelly, D.J.; Meanwell, N.A. *J. Med. Chem.* **2015**, *58*, 8315-8359.
25. Moore, P.; Swallow, S.; Gouverneur, V. *Chem. Soc. Rev.* **2008**, *37*, 320-330.
26. Müller, K.; Faeh, C.; Diederich, F. *Science* **2007**, *317*, 1881-1886.
27. Meanwell, N.A. *J. Med. Chem.* **2018**, *61*, 5822-5880.
28. Wang, J.; Sánchez-Roselló, M.; Aceña, J.L.; del Pozo, C.; Sorochinsky, A.E.; Fustero, S.; Soloshonok, V.A.; Liu, H. *Chem. Rev.* **2014**, *114*, 2432-2506.
29. Fox, E.M.; Howlett, B.J. *Mycol. Res.* **2008**, *112*, 162-169.
30. Amrine, C.S.M.; Long, J.L.; Raja, H.A.; Kurina, S.J.; Burdette, J.E.; Pearce, C.J.; Oberlies, N.H. *J. Nat. Prod.* **2019**, *82*, 3104-3110.
31. Littke, A.F.; Dai, C.; Fu, G.C. *J. Am. Chem. Soc.* **2000**, *122*, 4020-4028.
32. Kirchhoff, J.H.; Netherton, M.R.; Hills, I.D.; Fu, G.C. *J. Am. Chem. Soc.* **2002**, *124*, 13662-13663.
33. Deng, Y.; Su, M.; Kang, D.; Liu, X.; Wen, Z.; Li, Y.; Qiu, L.; Shen, B.; Duan, Y.; Huang, Y. *J. Med. Chem.* **2018**, *61*, 11341-11348.
34. Nakama, Y.; Yoshida, O.; Yoda, M.; Araki, K.; Sawada, Y.; Nakamura, J.; Xu, S.; Miura, K.; Maki, H.; Arimoto, H. *J. Med. Chem.* **2010**, *53*, 2528-2533.
35. More, N.Y.; Jeganmohan, M. *Eur. J. Org. Chem.* **2017**, *2017*, 4305-4312.
36. Zeng, H.; Qiu, Z.; Domínguez-Huerta, A.; Hearne, Z.; Chen, Z.; Li, C.-J. *ACS Catal.* **2017**, *7*, 510-519.
37. Harrison, C. *Nat. Biotechnol.* **2014**, *32*, 403-404.
38. Süssmuth, R.D.; Mainz, A. *Angew. Chem. Int. Ed.* **2017**, *56*, 3770-3821.