1-1-2013

# Screening of chemical libraries in search of inhibitors of aflatoxin biosynthesis

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# Screening of Chemical Libraries in Search of Inhibitors of Aflatoxin Biosynthesis

**A Thesis Submitted to the**

**University Honors Program**

**In Partial Fulfillment of the**

**Requirements of the Baccalaureate Degree**

**With Upper Division Honors**

**Department Of**

Biological Sciences

**By**

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December 2013

University Honors Program

Capstone Approval Page

Capstone Title (print or type)

Screening of Chemical Libraries in Search of Inhibitors of Aflatoxin Biosynthesis

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Department of (print or type) Biological Sciences

Date of Approval (print or type) December 3, 2013

# HONORS THESIS ABSTRACT
# THESIS SUBMISSION FORM

AUTHOR: Justin Durancik

THESIS TITLE: Screening of Chemical Libraries in Search of Inhibitors of Aflatoxin Biosynthesis

ADVISOR: Dr. Ana Calvo

ADVISOR'S DEPARTMENT: Biological Sciences

DISCIPLINE: Microbiology YEAR: 2013

PAGE LENGTH: 7 pages

BIBLIOGRAPHY: See Attached

ILLUSTRATED: Yes

PUBLISHED (YES OR NO): No

LIST PUBLICATION:

COPIES AVAILABLE (HARD COPY, MICROFILM, DISKETTE): Hard Copy

ABSTRACT (100-200 WORDS): See Attached

# Screening of Chemical Libraries in Search of Inhibitors of Aflatoxin Biosynthesis

Abstract:

*Aspergillus nidulans* is a filamentous fungus and model organism for other *Aspergillus* species, such as *A. flavus*. *A. flavus* and some other *Aspergillus* species produce a toxin known as aflatoxin. This compound is a carcinogenic secondary metabolite produced by these organisms. *A. nidulans* is often used to study this pathway because it presents a conserved biosyntheticpathway that leads to the production of the related mycotoxin sterigmatocystin (ST). For my project, I have searched for chemicals that disrupt the activation of the biosynthetic pathway responsible for producing aflatoxin. Specifically, I screened for chemicals that inhibit the nuclear VeA-LaeA protein-protein interaction. This protein interaction has been previously shown to affect production of secondary metabolites, including aflatoxin, in *Aspergillus* species. The experiment was done by performing a yeast 2 hybrid strain, so that growth of the yeast is dependent upon the interaction of these two proteins. After performing the high throughput screening, I found one chemical of interest. However, application of this chemical on *A. nidulans* cultures did not prevent toxin production, fungal growth, or morphogenesis. It is possible that the compound is not incorporated in *A. nidulans* cells and reach the nucleus.

Introduction:

Fungi are important organisms in agriculture, industry, and the medical field. Numerous fungal species are capable of producing beneficial products as well as harmful products. One important fungal genus is *Aspergillus*. This is a diverse group that includes species that are pathogenic as well as species that are able to synthesize beneficial compounds, such as antibiotics (Wortman et al., 2009). These fungi are especially detrimental to patients with compromised immune systems, such as those with AIDS or going through chemotherapy. A model organism within this group, *Aspergillus nidulans,* has been used to study eukaryotic genetics for over 60 years (Wortman et al., 2009). Using *A. nidulans* as a model system allows us to better understand fungal biology at a faster pace. This knowledge sets the bases to develop strategies to enhance a desired trait or inhibit a harmful one.

Chemicals generated by fungi that are not necessary for the survival of the organism in pure culture are denominated secondary metabolites. These secondary metabolites are postulated to play an ecological role for the organism in competition with other organisms. Aflatoxin, a secondary metabolite produced by some *Aspergillus* species including *Aspergillus flavus* and *A. parasiticus*, is especially harmful to human. It is not only toxic at high concentrations but also carcinogenic at lower concentrations. These opportunistic pathogens are commonly found contaminating oil seed crops of importance such as corn or peanuts. Contamination from *A. flavus* in the U.S. corn industry alone causes approximately $200 million in damages annually (Duran et al., 2009). In order to prevent the devastating effects of aflatoxin contamination, research efforts are focused on understanding the genetic mechanisms that regulate the synthesis of this detrimental fungal compound. The model fungus *Aspergillus nidulans* produces a precursor of aflatoxin called sterigmatocystin through the same conserved biosynthetic pathway. For this reason, *A. nidulans* has also been used as a model system to study mycotoxin production (Dhingra and Calvo, 2011).

One particularly interesting regulator of aflatoxin production is the protein known as Velvet A, or VeA. VeA is a global genetic regulator that is unique to fungal species (Calvo

2008). This protein has been shown in *A. nidulans* to interact in the nucleus with the protein LaeA, under dark conditions. This interaction leads to morphological changes and an increased production of secondary metabolites, including toxin (Bayram et al. 2008). For this reason, the interaction of these two proteins was the focus of the research I performed. In particular, I did a high throughput chemical screening using a visual yeast two-hybrid screening previously developed in Dr. Calvo's lab to find chemicals inhibiting the interaction between VeA and LaeA. I was able to test over 12,000 chemicals and found 5 that appeared to inhibit the interaction, with one of them having the strongest effect. The effect of the selected compound on VeA-LaeA interaction was then tests by split-YFP method in *A. nidulans*. Additionally, I have characterized the effects this compound on growth and toxin production in *Aspergillus* cultures.

Materials and Methods:

Fluorescent Microscopy:

A strain of *A. nidulans* containing a split yellow fluorescent protein attached to VeA and LaeA was grown in wash minimum medium containing a final concentration of 25μM of the chemical of interest. Mycelia from these plates were then viewed using fluorescent microscopy to further analyze possible protein complex disruption in the presence of the antifungal compound.

Vegetative Growth:

Fungal growth rate was evaluated as colony diameter in *A. nidulans* point-inoculated solid GMM cultures with 25μM of the chemical of interest. Cultures were grown under dark conditions for 72 hours. All experiments were done using three replicates in 6-well microtitle plates.

Mycotoxin Analysis:

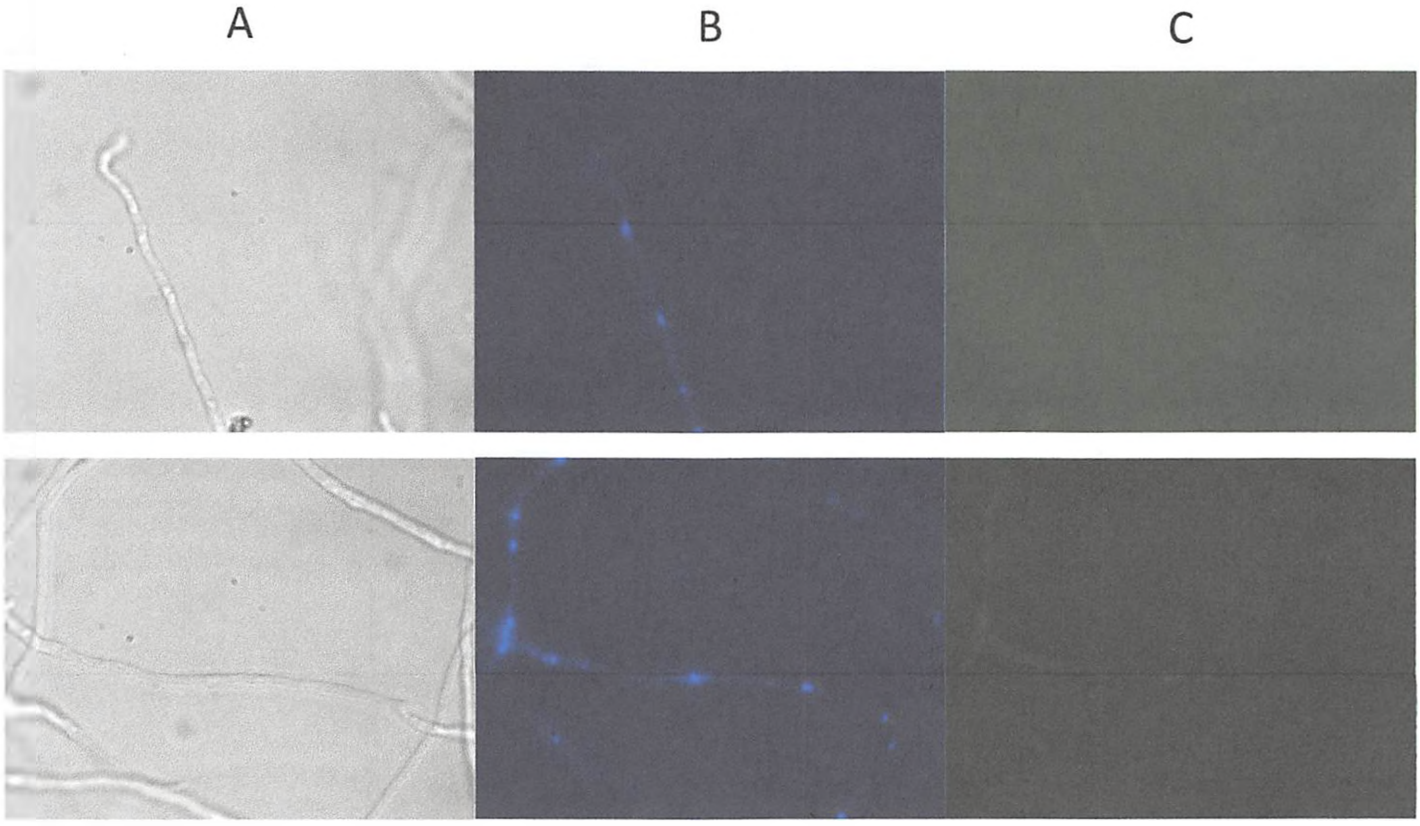
Plates containing solid glucose minimum medium with 25μM to 50μM of the chemical of interest were inoculated with top agar containing fungal spores. Cores from each replicate plate were extracted with $CHCl_3$. Samples were then fractionated by thin-layer chromatography using benzene and glacial acetic acid [95:5 (v/v)] as a solvent system. The plates were sprayed with aluminum chloride and baked at 80°C. Toxin bands on TLC plates were viewed by exposing the plates under UV light (375-nm).

Results:

Fluorescent Microscopy:

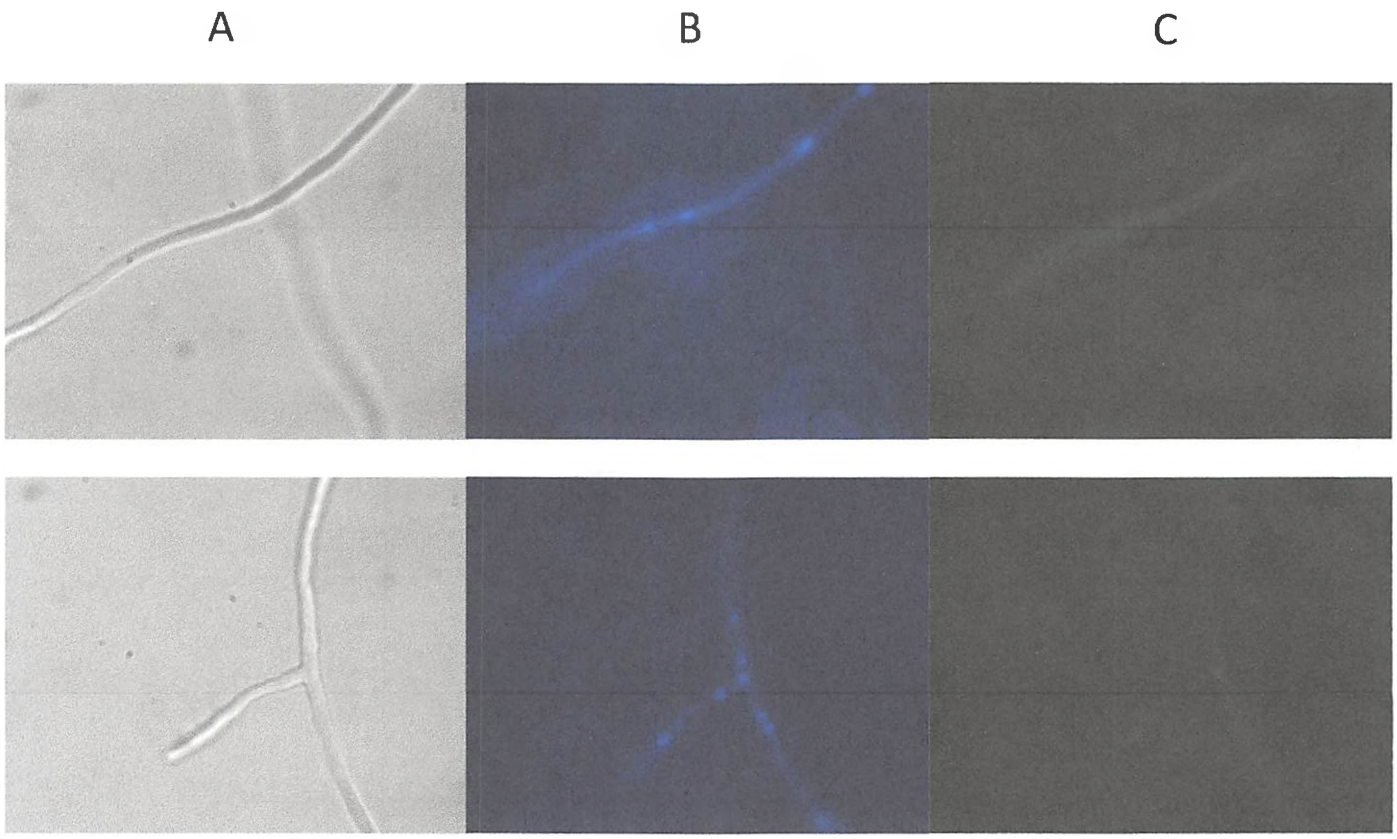
Fluorescent microscopy was performed as described above. Since VeA and LaeA have been shown to interact in the nucleus, a nuclear localization of fluorescence was expected from the VeA-LaeA split yfp strain when the proteins interact. In Figure 1, the fungus was grown without the addition of the compound. The images of the hyphae stained with dapi (column B) shows the location of the nucleus. The yfp images (column C) show the localization of the Vea-LaeA interaction. This interaction appears to overlap with the fluorescence of the dapi images, suggesting nuclear localization as expected. Figure 2 shows images of hyphae from the VeA-LaeA split yfp strain grown in 25μM of the compound. Column B is the hyphae stained with dapi to show the location of the nucleus. Column C shows the yfp image with fluorescence showing any interaction between VeA and LaeA.

# Figure 1




Column A shows the brightfield image of the VeA-LaeA split yfp strain with no compound added. Column B shows the image of the same hyphae stained with dapi to show nuclear localization. Column C shows the yfp image of the hyphae showing the interaction of VeA and LaeA where fluorescence appears.

# Figure 2

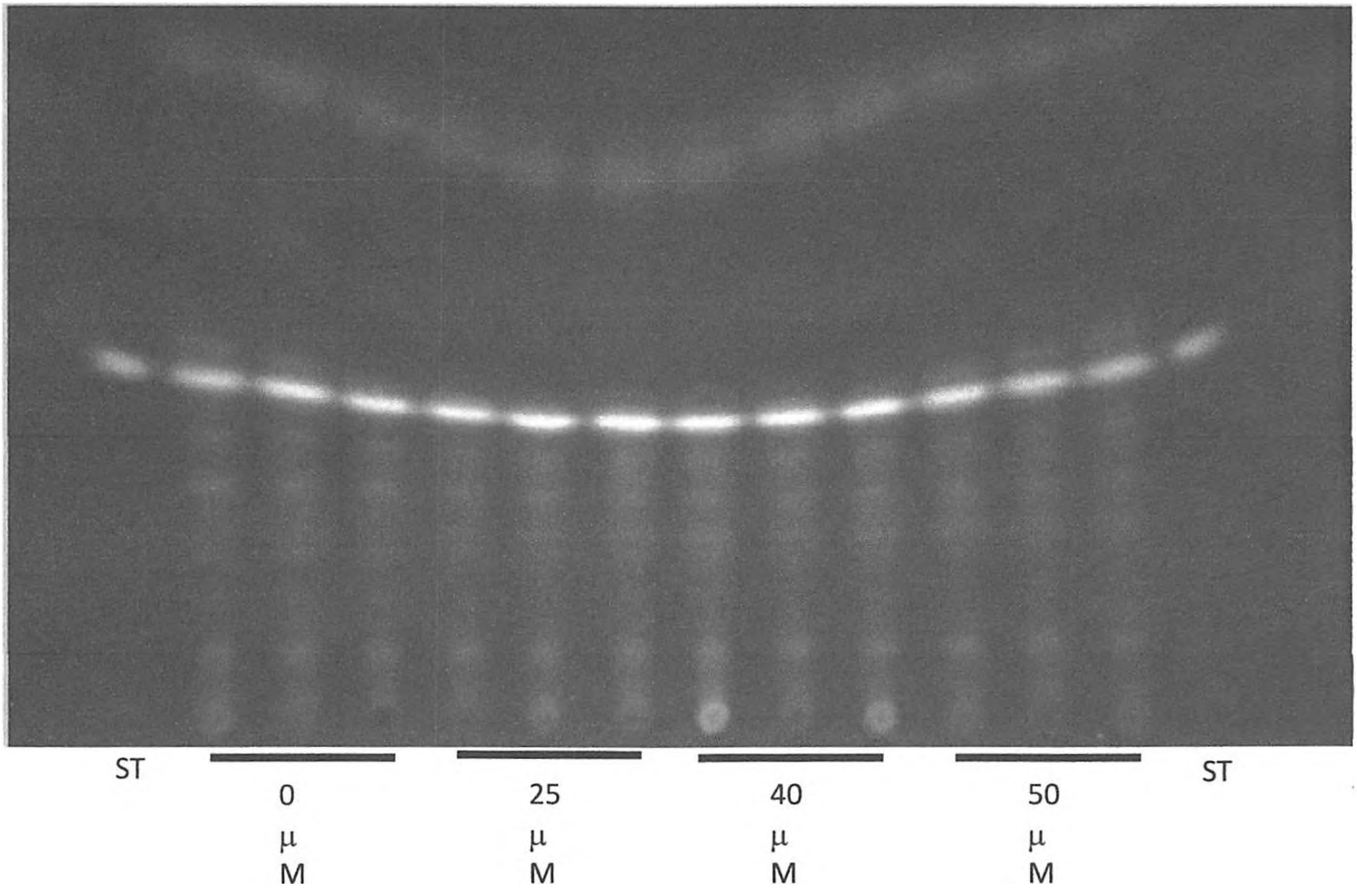



Column A shows the brightfield image of the VeA-LaeA split yfp strain with 25μM compound added. Column B shows the image of the same hyphae stained with dapi to show nuclear localization. Column C shows the yfp image of the hyphae showing the interaction of VeA and LaeA where fluorescence appears.

Mycotoxin Analysis:

To analyze the production of sterigmatocystin a thin layer chromatography was performed as mentioned above. Samples were extracted from cultures grown with no compound, 25μM compound, 40μM compound, and 50μM compound. Figure 3 shows the TLC with sterigmatocystin standard on both the right and left sides. This marker is used to determine the band corresponding to sterigmatocystin in the fractionalized samples. The intensity of the band is used to determine the relative amount of sterigmatocystin in the sample. The band intensity does not appear to change with the different concentrations of compound added.

## Figure 3

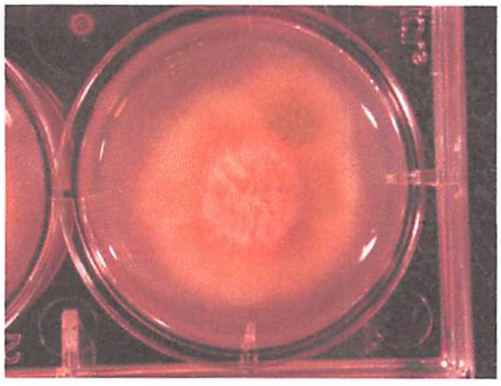


Growth Rate:

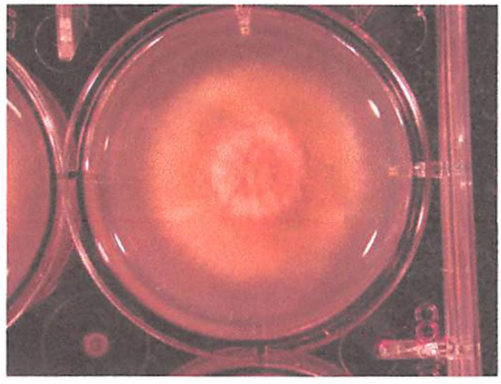
The growth rate was analyzed by performing point inoculations and measuring colony diameter (in mm). Figure 4 shows images of the fungus grown without compound added (A), with 25mM compound added (B), and a table of the colony diameters (C) after three days of growth.

## Figure 4

A No Compound Added



B 25μM Compound Added



C

| Colony Diameter (in mm) | | |
|---|---|---|
| Colony # | No Compound | 25μM Compound |
| 1 | 26 | 26 |
| 2 | 27 | 25 |
| 3 | 26 | 26 |
| Average | 26.3 | 25.7 |

Discussion:

In this study, I analyzed the effects of a compound previously found to disrupt the interaction between the proteins Vea and LaeA, an interaction known to effect growth and mycotoxin production. More specifically, I analyzed if this interaction was disrupted *in vivo*, the effect on mycotoxin production, and growth rate. The fluorescent microscopy images taken to determine the effect on the VeA-LaeA interaction *in vivo* did not conclusively show a disruption of the interaction. By analyzing the mycotoxin production shown in Figure 3, it is clear that this compound did not affect the production of sterigmatocystin. The growth rate also appears to be unaffected, shown in Figure 4.

The lack of effect on toxin production and growth rate along with inconclusive fluorescent microscopy images leads me to the hypothesis that the compound is unable to be incorporated in the hyphae of *A. nidulans*. Perhaps the cell wall structure found in *Aspergillus* fungi presents variations compared to that of *Saccharomyces cerevisiae*, the organism initially used in the study finding the compound to disrupt the interaction. This hypothesis can be tested by inoculating germlings on media containing the compound. These germlings do not contain a fully developed cell wall. If the given hypothesis is true, the compound would be able to enter the fungus and disrupt the VeA-LaeA interaction, reducing the growth rate and mycotoxin production. It could be possible that derived compounds of similar structures and with the same activity could be developed to overcome the cell wall barrier.

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