

College of Saint Benedict and Saint John's University

DigitalCommons@CSB/SJU

---

Honors Theses, 1963-2015

Honors Program

---

1999

## An Endophytic Fungus as a Source of New Antifungal Compounds

Lisa Marie Jungbauer

*College of Saint Benedict/Saint John's University*

Follow this and additional works at: [https://digitalcommons.csbsju.edu/honors\\_theses](https://digitalcommons.csbsju.edu/honors_theses)

 Part of the [Chemistry Commons](#), and the [Fungi Commons](#)

---

### Recommended Citation

Jungbauer, Lisa Marie, "An Endophytic Fungus as a Source of New Antifungal Compounds" (1999).

*Honors Theses, 1963-2015*. 719.

[https://digitalcommons.csbsju.edu/honors\\_theses/719](https://digitalcommons.csbsju.edu/honors_theses/719)

Available by permission of the author. Reproduction or retransmission of this material in any form is prohibited without expressed written permission of the author.

**AN ENDOPHYTIC FUNGUS AS A SOURCE OF NEW ANTIFUNGAL COMPOUNDS**

**A THESIS**

**The Honors Program**

**The College of St. Benedict/St. John's University**

**in Partial Fulfillment**

**of the Requirements for the Distinction "All College Honors"**

**and the Degree Bachelor of Arts**

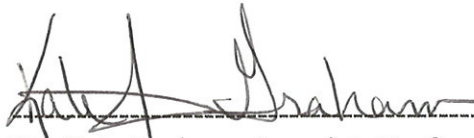
**in the Department of Chemistry**

**by Lisa M. Jungbauer**

**May 1999**

**PROJECT TITLE: AN ENDOPHYTIC FUNGUS AS A SOURCE OF NEW  
ANTIFUNGAL COMPOUNDS**

**Approved by:**


  
----- (advisor)  
Dr. Kate Graham, Associate Professor of Chemistry

  
-----  
Dr. Ellen Jensen, Associate Professor of Biology

  
-----  
Fr. John Klassen, Associate Professor of Chemistry

  
-----  
Dr. Brian Johnson, Chair, Chemistry Department

  
-----  
Margaret Cook, Director, Honors Thesis Program

  
-----  
Charles Bobertz, Director, Honors Program

## ABSTRACT

Medical advances in society such as organ transplants, prolonged chemotherapy, and those that lengthen the lives of AIDS patients and the elderly increase the number of immunocompromised individuals.<sup>1</sup> When the immune system is compromised, opportunistic fungi can flourish and become fatal. Current antifungal treatments are limited and often toxic.<sup>2,3</sup> In addition, strains of fungi resistant to available antifungals are emerging.<sup>4,5</sup>

Fungi were selected as the source of potential new antifungal agents because fungal antagonism has been reported in most fungal ecosystems.<sup>6</sup> Endophytic fungi, which inhabit the spaces between plant cells, are known producers of natural products and that assist plants in fending off plant fungal pathogens.<sup>7</sup> Therefore, endophytic fungi should produce compounds with antifungal activity. The endophytic fungus, KG146A, a basidiomycete found in the wild rosemary tree/shrub *Ceratiola ericoides*, demonstrated antifungal activity in plug assays against *Candida*, a human pathogen. KG146A was cultured in liquid Sabouraud's Dextrose broth and extracted with ethyl acetate. The organic extracts (~5mg/ diffusion disk) revealed antifungal activity in disc diffusion assays against *Candida albicans* 406, *Candida albicans* wisconsin, *Candida albicans* MEN, and *Saccharomyces cerevisiae*. Purification of the active component involving LH-20 gel chromatography followed by reverse-phase HPLC was developed. Final purification will be completed through the use of HPLC and structure elucidation will be achieved through NMR spectroscopy, IR spectroscopy, and mass spectrometry.

## INTRODUCTION

In recent years, a continued increase both in the frequency and severity of fungal infections has been noted.<sup>8, 9, 10</sup> Paradoxically, one major factor contributing to this growth is the medical advances that prolong the lives of many patients.<sup>11</sup> Advances in medicine such as improvements in organ transplantation, intensive care, chemotherapy, and antibiotics<sup>11</sup> continue to create a growing population with substantially suppressed immune function.<sup>12</sup> Immunocompromised individuals have increased susceptibility to serious fungal infections.<sup>1</sup> This at-risk population includes increasing numbers of AIDS patients, longer-lived elderly, premature infants, and chemotherapy patients. Also included are burn victims, patients with prosthetic devices, and those receiving prolonged antibiotic therapy. Hodgkin's disease and lymphomas also leaves patients severely immunocompromised,<sup>13</sup> and patients with diabetes, especially when not controlled are also at high risk for fungal infection.<sup>9-18</sup>

A variety of genera and species of fungi are known to cause human fungal infection (Table 1). There are at least 300,000 known species of fungi, of which only 200 are known to be parasites.<sup>19</sup> *Candida* are a family of fungi well known for human pathogenicity. The *Candida* family alone comprise about 200 different species.<sup>14</sup> Of these *Candida* species, fewer than ten are known to be human pathogens (Table 1). Although these species share the ability to infect humans, there is great intraspecies diversity with respect to virulence and pathogenic mechanisms.<sup>14</sup>

**Table 1: Genera and Species of Fungi known to cause Infection in Human Hosts<sup>20</sup>**

Genus	Species
<i>Candida</i>	<i>albicans, glabrata, tropicalis, parapsilosis, krusei, guilliermondii, kefir</i>
<i>Aspergillus</i>	<i>fumigatus, flavus, terreur, ustus, nidulans</i>
<i>Cryptococcus</i>	<i>neoformans</i>
<i>Histoplasma</i>	<i>capsulatum</i>
<i>Coccidioides</i>	<i>immitis</i>
<i>Blastomyces</i>	<i>dermatitidis</i>
<i>Trichosporon</i>	<i>beigelii</i>
<i>Penicillium</i>	<i>marneffeii</i>
<i>Fusarium</i>	<i>solani, oxysporum, moniliforme, chlamydosporum</i>
<i>Sporothrix</i>	<i>schenckii</i>

*Candida* is a dimorphic yeast (single-celled fungus) normally found growing on humans. *Candida* is responsible for the majority of opportunistic fungal infections.<sup>14</sup> The most common species causing infection is *Candida albicans*. However several other species such as *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. glabrata*, and *C. kefir* have been found to cause Candidiasis.<sup>21</sup> Candidiasis most frequently infects the mucous membranes of the oral cavity, and vagina, but can also cause infection of the skin, the esophagus, and the gastrointestinal tract, (the locations in which it normally resides as a commensal). In the immunocompromised host, lesions can occur in the kidneys, gastrointestinal tract, lungs, central nervous system, or endocardium.<sup>22</sup>

In a study by the National Nosocomial Infection Surveillance System (NNIS) *Candida* were documented to cause 85% of all nosocomial (hospital acquired) infections.<sup>23</sup> Of these infections *Candida albicans* were causative agents of 60%.<sup>23</sup> However, non-*albicans Candida* species are more frequently being seen as pathogens in the hospital. The major problem surrounding the non-*albicans* species is that they are significantly less susceptible to azole antifungal agents,<sup>9</sup> and they cause more complications and higher mortality.<sup>17</sup>

In addition to *Candida*, there are two other types of pathogenic yeasts known to infect humans, especially with incompetent immune function. *Cryptococcus* causes cryptococcosis, which can occur as pneumonia, meningitis, or disseminated infection.<sup>24</sup> *Trichosporon* are fungi that are uncommon, but usually cause fatal infection in immunocompromised patients.<sup>24</sup> Fungal infection can also be due to inhalation of the spores of dimorphic fungi found in soil, (*Histoplasma*, *Coccidioides*, *Blastomyces*, *Penicillium*) which have the ability to become pathogenic when introduced into the human host. These mycoses (histoplasmosis, blastomycosis, coccidioidomycosis, and penicilliosis) are normally endemic and often seen as pulmonary infection.<sup>24</sup>

Not only does a compromised immune system provide increased opportunity for pathogenic fungi to invade and colonize the host, but these patients are also susceptible to opportunistic fungi.<sup>25</sup> Opportunistic fungi are not harmful in their normal habitat.<sup>26</sup> However, when presented with the opportunity—a host with compromised immune defenses, or sites for open entry through the skin or mucous membranes—they can become pathogenic.<sup>26</sup> *Candida* are the most frequently encountered opportunists, however, there are four other families of filamentous fungi currently known to cause opportunistic infection in humans: *Aspergillus*, *Zygomycetes*, *Fusarium*, and *Pseudallescheria*.<sup>24</sup> Aspergillosis is an opportunistic infection caused by airborne spores of a fungus that exists widespread in the environment (in soil and decaying vegetation). Zygomycosis (a.k.a. mucormycosis) is not susceptible to current antifungal treatments<sup>5</sup> and pseudallescheriasis (which is almost indistinguishable from Aspergillosis) does not respond to any available antifungal therapy.<sup>24</sup> Although much less frequently encountered than *Candida*, these infections can be fatal to the immunocompromised host.<sup>21</sup>

The most common fungi responsible for human infections are: *Candida*, *Aspergillus*, *Cryptococcus*, *Trichosporon*, and *Mucor*.<sup>19</sup> However, further complexity is added to the situation because immunocompromised patients are also susceptible to serious and unusual infection by unexpected fungi.<sup>18, 25, 27</sup> Recently, saprophytic fungi that normally exist in the environment with no previous documentation of human pathogenicity such as *Rhizopus*, *Absidia*, and *Scopulariopsis* have been found to cause fungal infection in the immunocompromised.<sup>25, 27</sup>

Systemic opportunistic mycoses occur in up to 80% of immuno-suppressed patients.<sup>19</sup> Fungal infections in immuno-compromised patients often continue to persist even with antifungal therapy.<sup>28</sup> In hospitals with more than 500 beds, infections due to the fungus *Candida* have increased by almost 500% from 1980-1990 and by 75% in hospitals with 200 or fewer beds.<sup>29</sup> In the last decade, *Candida* caused as many nosocomial infections (those acquired in the hospital setting) as *E. coli*,<sup>29</sup> a bacteria which until recently was responsible for 60% of nosocomial infections.<sup>26</sup> Fungal overgrowth in persons lacking a functioning immune system have a 20-40% chance of leading to a fatal infection.<sup>30</sup> In some cases, fungal infections in these immuno-compromised patients have higher mortality rates: in 1996, the mortality rates for *Aspergillus* infection were greater than 90%, and for *Fusarium* (a fungus that normally causes toe-nail onychomycosis) and *Trichosporon* the mortality rates were 100%.<sup>29</sup> Even *Candida* infections (the most common fungal pathogen) continue to have a mortality rate of 50%.<sup>29</sup>

Not only does immune suppression provide an open door for opportunistic fungi to invade, it causes numerous other complications to fungal infection. An incompetent immune system can result in asymptomatic fungal infection due to such things as nonfunctioning agents responsible for inflammatory response.<sup>13</sup> Additionally, the severity of the fungal infection is magnified, posing difficulties in safe and effective treatment. For example, Cryptococcosis in



the patient with normal immune defenses consists of a localized pulmonary lesion. However, without immune defenses intact the infection can spread to the CNS, lungs, lymph nodes, skin, adrenal glands, kidney, and prostate.<sup>13</sup> Immediate, intense and highly monitored treatment of fungal infection in the immunocompromised patient is crucial to prevent the spread of infection. Once treated for the original infection, the immunocompromised patient is susceptible to relapse. Therefore, the utilization of some form of constant, life-long therapy to prevent resurgence of the infection seems necessary.<sup>13</sup> However, due to the risks involved, life-long therapy to prevent resurgence of the infection is debatable.<sup>31</sup> The prolonged antifungal therapy would prevent relapse, but would also create an opportunity for resistant forms of the pathogens to emerge.<sup>31</sup>

Furthermore, different types of immunosuppression have specific fungal infections associated with them.<sup>13</sup> For example, the opportunistic fungus, *Pneumocystis carinii*, commonly infects hosts immunocompromised by AIDS, but not those whose source of immune suppression is rigorous corticosteroid therapy.<sup>13</sup> Also, the mechanism of protection offered by the immune cells depends on the infecting organism. The interference is often not obvious, and sometimes questionable. Different responses are generated by pathogenic fungi (*Histoplasma*, *Coccidioides*, *Cryptococcus*) than opportunistic fungi (*Candida*, *Aspergillus*).

Fungal infections of humans can be placed into three classes based on the location and severity of the infection.

1. Superficial infections of the skin, hair, and other cutaneous appendages, comprise the *superficial/cutaneous*, or *dermatophytic mycoses*. Superficial mycoses are rarely life-threatening, but a source of discomfort and inconvenience to the host, that respond well to treatment with topical (sometimes oral) antifungal agents.<sup>10</sup> However, even these surface infections can pose more serious and recurring problems in the immunocompromised host.

2. Mycoses involving trauma to the skin or subcutaneous tissues are known as the *subcutaneous mycoses*. These are typically localized in the gastrointestinal, genitourinary, and respiratory tracts.<sup>10</sup>

3. The *systemic mycoses* are fungal infections that have disseminated into the host, usually affecting internal organs. These pose the most serious therapeutic problem because systemic fungal infections are the most severe, occur mainly in immunocompromised patients, and are the most difficult to treat.<sup>10</sup>

Because pathogenic fungi and the diseases they cause are changing with time and increasing in frequency, medical workers need to discover a consistent approach to combat fungal infections. Medical students are not well-trained, or not trained at all, in medical mycology<sup>32</sup>; hence, it is not rare for physicians to be unfamiliar with fungal disease.<sup>33</sup> The treatment process of fungal infections has been slowed by the lack of universal standards for the treatment of fungal infections. When invasive fungal diseases were first recognized, they were extremely difficult to diagnose and treat.<sup>35</sup> Decades later, the same treatment and clinical diagnostic problems exist, but are slowly being corrected by advances in the fields of mycology, molecular biology, and medicine.<sup>35</sup>

Another major problem is that the pathogenic fungi are not a uniform group of organisms.<sup>27</sup> Mycologists and others studying fungal infections and their treatment document hundreds of studies of the pathogenic yeasts and treatments. However, each group has their own methodology of defining patient response to therapy and, therefore, effectiveness of the antifungal drug. Each group also uses different criteria to diagnose the patient. Systemic infections often develop without showing specific symptoms or indicators of the disease.<sup>19</sup> This makes the crucial early diagnosis and initiation of treatment difficult because of uncertainty in

knowing which specimens to select for laboratory testing, and inconclusive test results.<sup>19</sup> As a result, treatment with a broad-spectrum antifungal is often begun without confirmation of the type of fungal infection or the identity of the species responsible.<sup>19</sup> Rather than withhold treatment from a patient whose infection is in advanced stage (thus having high rates of morbidity and mortality) while waiting to confirm the identity of the infection, therapy is often commenced at the first suspicion of fungal infection.<sup>19</sup> Early diagnosis is especially important in assessing for dissemination of the infection and for decreasing the chance of mortality by earlier intervention.<sup>36</sup> Despite newer, less toxic antifungals, Amphotericin B remains the most effective against a broad range of pathogens.<sup>10,35</sup> However, use of this antifungal agent poses a dilemma: while effective at killing the pathogen, it is toxic also to the patient. Furthermore, the use of lower doses risks ineffective treatment.<sup>19</sup>

Diagnosis of fungal infections needs to be rapid and accurate.<sup>37</sup> Diagnosis is one of the major problem areas resulting in the continued mortality of invasive mycoses. Improvements in diagnosis are particularly needed because of recent formulations of less toxic but more expensive antifungal drugs. To avoid unnecessary expenses, a reliable diagnosis is imperative prior to administration of such costly treatment.<sup>38</sup> Another reason for improved diagnostic methods is that delays in treatment diminish the chance of success, especially if the infection progresses to an advanced stage. Starting therapy based on “clinical indications”<sup>24</sup> attempts to treat without proof of the specific type of pathogen—risking overly potent chemotherapy or ineffective treatment.<sup>24</sup>

New alternatives to culture diagnosis have been developed that reduce the need for blind antifungal therapy by providing rapid, accurate diagnosis. There have been three main non-culture diagnostic techniques: the detection of fungal antibodies, fungal antigens, and fungal

metabolites.<sup>38</sup> Each technique tests for the presence of a feature of the fungus which indicates the invasive fungal infection and, ideally, the species of the causative agent.<sup>38</sup> The first method of antibody detection is no longer relied on for two reasons: the majority of patients prone to fungal infection are immunocompromised and cannot produce antibodies; and second it has been found to have limited diagnostic success.<sup>38</sup> The method of antigen detection is also not used very frequently because of the difficulty in obtaining an antibody specific and selective enough to detect only the fungal antigen. For aspergillosis, antigens are only present late in the infection, when proper treatment based on diagnosis would be too late anyway.<sup>38</sup> The most promising improvement in non-culture diagnostic technique has been the discovery of detecting fungal DNA through polymerase chain reaction (PCR) test systems.<sup>38</sup> However, this technique also needs further improvement and method standardization to yield consistent and accurate specimen collections and function in a highly specific mechanism.<sup>38</sup>

Therefore, an underlying need for more randomized studies to develop standards applicable to most cases of fungal infection, methods of characterizing disease, and universal terminology to describe the type, symptoms, and degree of infection are crucial to advancement. Such studies would be helpful in knowing when to stop therapy and how to assess patient response to therapy, which would lead to the capability to make definitive assignments of which antifungal drugs would be best in each type of infection.<sup>39</sup> The ideal susceptibility test would provide four things: 1) a reliable comparison of the activity of various antifungal agents; 2) results that correlate with *in vivo* activity and can predict the outcome of therapy; 3) a method to detect and observe the acquisition of resistance in a normally susceptible group of pathogens and; 4) the ability to predict the therapeutic potential of novel antifungal agents.<sup>34</sup> An increased

effort to standardize the methods of evaluating antifungal agents must occur concomitantly with the increased focus on locating novel therapeutic agents.<sup>37</sup>

Taking all things involving the development of reliable diagnosis and effective treatment of fungal infections into consideration (the idiosyncratic nature of fungal pathogenicity, the different host responses and abilities to defend against infection, and the specific mechanisms of pathogenicity), mycologists, clinicians, pharmacists and physicians are presented with a very complicated problem. This problem is compounded by the difficulty in selecting safe and effective targets for antifungal therapy because fungal cells and human cells are both eukaryotic, and therefore have very similar structure and function.<sup>20</sup> Structures in common are a defined nucleus, nuclear envelope, and a plasma membrane composed of lipids, glycoproteins, and sterols. This provides an obstacle in development of antifungal compounds because targets must be selected that are specific to the fungi to avoid toxicity to the host.

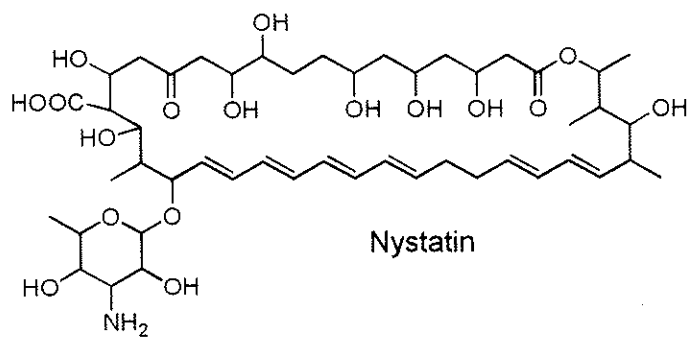
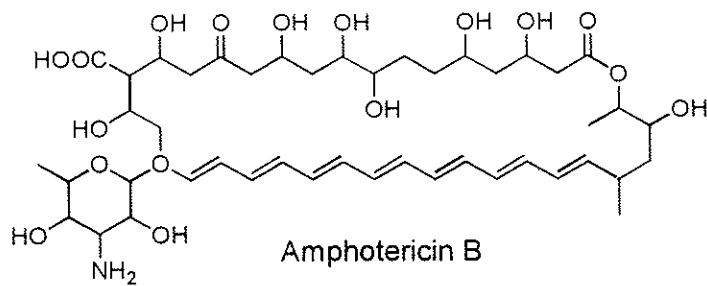
However, some structural differences between the human cell membrane and the fungal cell membrane do exist, and have served as the target for antifungal mechanisms (Mechanims of Action, Table 2a). Many current antifungal drugs target the fungal cell membrane in order to inhibit the proliferation of the fungus in the host by rendering these protective structures ineffective. The cell membrane is important to the fungus because it serves as a matrix for glucan- and chitin synthetases (membrane-bound enzymes involved in formation of polysaccharides that comprise the cell wall), it regulates molecule transport into and out of cells, and it is a barrier between the outside environment and the cytoplasm.<sup>40</sup> The inhibition of sterol biosynthesis and interruption of membrane sterol and phospholipid interaction significantly disrupt the integrity of the cell. Those antifungals that target membrane components more specific to fungal cell membranes, such as 5-FC (Mechanisms of Action, Table 2a) are less toxic

to the patient. On the other hand, antifungals such as Amphotericin B (Table 2a), which complexes with a sterol of similar structure to cholesterol (the major sterol of human cell membranes) cause destruction of the human cells concurrent to fungal cell destruction leading to host toxicity.

The polyene macrolide Amphotericin B (Figure 1a) and the azole Fluconazole (Figure 4e) have been the leading treatments for fungal infections.<sup>3,4</sup> Other antifungal agents currently used include various azoles (Figures 4a-e), the allylamine terbinafine (Figure 3a), Nystatin (Figure 1b), 5-fluorocytosine (Figure 2) (Table 2a). Cell wall synthesis inhibitors such as cilofungin and nikkomycin are still in the experimental stage.<sup>5,6</sup>

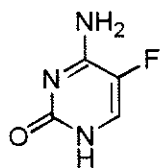
**Figure 1a & 1b:**

**POLYENE MACROLIDES**



**Figure 2:**

**NUCLEOSIDE ANALOGUES**



**Figure 3a & 3b:**

**ALLYLAMINES**

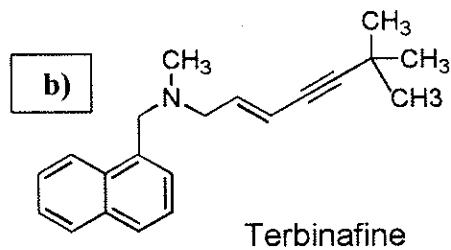
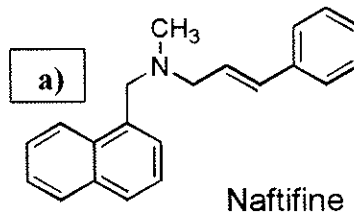
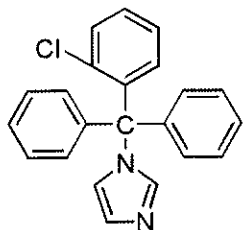
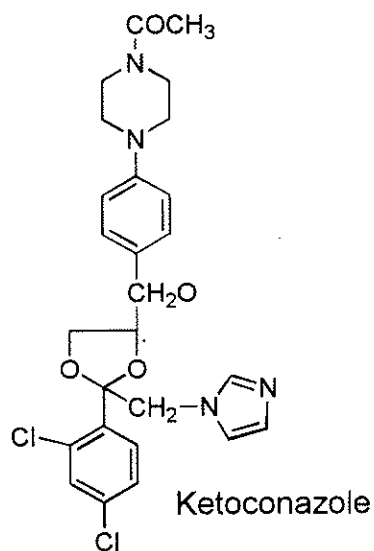


Figure 4a-c

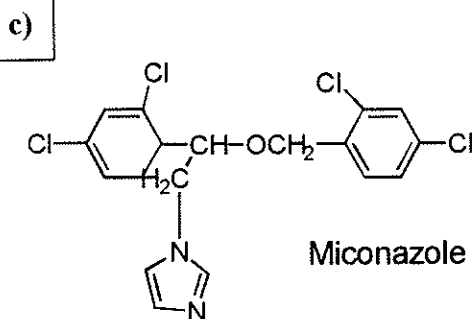
IMIDAZOLES



a) Clotrimazole



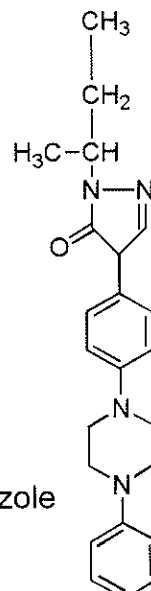
b) Ketoconazole



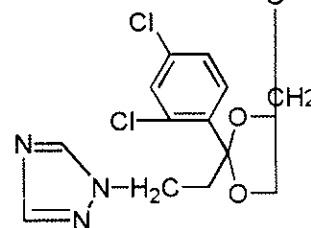
c) Miconazole

Figure 4d-e

TRIAZOLES



d) Itraconazole



e) Fluconazole



**Table 2a: Current Antifungal Drugs**

Drug Name (means of administration)	Class	Mechanism of Action	Type of Fungal Infections
Amphotericin B (Parenteral, Topical)	Polyene Macrolide (isolated from the fungus <i>Streptomyces nodosus</i> ) <sup>35</sup>	Targets membranes with a high ergosterol content, the principal sterol in the cell membrane of fungi, forms a channel through the membrane that promotes the leakage of potassium, sodium, hydrogen ions and other small molecules, preventing the fungus from maintaining its internal environment, leading to cell death <sup>35, 41</sup>	Most deep-seated, invasive, systemic mycoses, candidiasis, aspergillosis, zygomycosis, <i>Cryptococcus neoformans</i> , endemic fungi. <sup>42</sup>
Nystatin (Topical)	Polyene Macrolide (isolated from the fungus <i>Streptomyces noursei</i> ) <sup>35</sup>	Targets membrane barrier function <sup>43</sup>	Superficial (mucosal) <i>Candida</i> infections <sup>35</sup>
Terbinafine (Oral)	Allyl Amine	Inhibits conversion of squalene to lanosterol affecting cell membrane and function. <sup>35</sup>	Dermatophytic mycosis <sup>35</sup>
Flucytosine (Oral)	Nucleoside Analog (Synthetic)	Converted to 5-fluorouracil (5-FU) by cytosine deaminase, which interrupts DNA, RNA, and protein synthesis <sup>33</sup>	Acts against only certain yeasts: <sup>21</sup> Cryptococcal meningitis, cryptococcomas, <i>Candida albicans</i> , candidiasis, aspergillosis <sup>32</sup>
Ketoconazole	(Triazole) Synthetic	Inhibition of a cytochrome P450-dependent enzyme involved in ergosterol synthesis, an important membrane component in fungi, <sup>42</sup> which results in loss of fungi's ability to maintain internal environment due to altered membrane permeability. <sup>35</sup>	Nonmeningeal fungal infections in nonimmunocompromised patients such as paracoccidioidomycosis, blastomycosis, chronic cavitary, disseminated histoplasmosis, mucosal candidiasis <sup>42</sup>
Itraconazole	Triazole (Synthetic)	Inhibits cytochrome P450-dependent enzyme involved in ergosterol synthesis. <sup>35</sup>	Aspergillosis, histoplasmosis, blastomycosis, coccidioidomycosis, sporotrichosis—all disseminated mycoses that fluconazole cannot successfully treat. <sup>10</sup>
Fluconazole	Triazole (Synthetic)	More specific inhibitor of fungal cytochrome P450 sterol C-14 $\alpha$ -demethylation reactions <sup>42</sup> . <sup>10</sup> Infrequent demethylation of host cells—therefore, reduced incidence of severe adverse effects <sup>10</sup>	Systemic mycoses such as <i>Coccidioides</i> meningitis, used in combination with AmB; management of buccal and/or esophageal candidiasis in AIDS patients; stomatitis associated with removable dentures; <sup>42</sup> dermal & vaginal candidiasis, cryptococcal meningitis <sup>10</sup>

**Table 2b: Comparison of Current Antifungal Treatments**

Drug Name	Adverse Effects	Pros	Cons
Amphotericin B	Fever, chills, cardiotoxicity, vomiting, hypotension, renal toxicity (in > 80% patients) <sup>10</sup> , hypokalemia, anemia, hypomagnesemia	Resistance is rare Effective against almost all fungal infections: very broad spectrum of activity. <sup>35</sup>	Severe adverse effects; poor water solubility; instability in solution; Must be administered via IV and in a hospital setting; crosses cell membranes poorly—so it is poorly absorbed from the gut and into the CSF
Nystatin	Possibility of gastrointestinal distress <sup>10</sup>	Effective against oropharyngeal candidiasis <sup>10</sup>	Cannot be parenterally administered due to toxicity; does not get absorbed when taken orally. <sup>35</sup>
Terbinafine	Very minimal side effects—possibility of taste perversion or gastrointestinal distress <sup>44</sup>	Fungicidal for most filamentous fungi <sup>44</sup>	Inhibition is reversible and noncompetitive. <sup>35</sup> Possibly not very active against pathogenic yeasts. <sup>45</sup>
Flucytosine	Bone marrow depression (leukopenia, anemia, thrombocytopenia); gastrointestinal disturbances (nausea, vomiting, diarrhea); CNS toxicity (headache, drowsiness, confusion, vertigo, hallucinations) <sup>10, 35</sup>	Water soluble; rapid and complete absorption upon oral administration, widely distributing throughout the body fluids <sup>10, 35</sup>	Resistance develops rapidly during treatment if not inherently present. Therefore, It cannot be used alone as an antifungal treatment. <sup>10, 35</sup>
Ketoconazole	Nausea, vomiting, possible hepatotoxicity <sup>42</sup> Liver failure (possibly fatal), impotency, gynecomastia <sup>21</sup>	Successfully orally absorbed, broad spectrum of antifungal activity, wide tissue distribution, long half-life. <sup>42</sup>	Adverse effects with prolonged; significant drug interaction, acidic conditions required for its absorption <sup>21</sup> ; fungistatic
Itraconazole	Not common, possible short-lived incidences of gastrointestinal disturbances, dizziness, headache, depressed libido, and leucopenia. Also possible hypertension and hypolakaemia with very high doses, <sup>42</sup> hepatitis <sup>46</sup>	Less toxicity, broader spectrum of activity, well-absorbed orally, fewer and less severe drug interactions. <sup>42</sup> One of most effective available antifungal drugs. <sup>21</sup>	Water insoluble, poor CSF distribution; fungistatic, potential for adverse interactions with other drugs. <sup>44</sup>
Fluconazole	Headache, nausea, abdominal pain, diarrhea, dyspepsia, dizziness, taste perversion <sup>10</sup>	Highly selective, water soluble, <sup>10</sup> high bioavailability after oral administration <sup>47</sup>	Fungistatic mechanism of action, relapse often occurs after completion of treatment <sup>47</sup>

Currently, the four main chemical classes remain inadequate in many ways (Table 2a and 2b). For example, while the new and improved triazole fluconazole seemed safe and effective, it only has fungistatic activity. Consequently, it is fairly ineffective against many disseminated mycoses and it requires continuous use to keep the pathogen in check. It is also fairly prone to the development of resistance.<sup>10</sup>

As summarized by Alice M. Clark,<sup>10</sup> all of the current antifungals have at least one of the following faulty traits:

- Toxicities/side effects
- Fungistatic (rather than fungicidal)
- Possibility for the development of resistance
- Mediocre pharmacokinetics and metabolic profile
- Unattractive means of administration
- Undesirable drug-drug interactions
- Physiochemical problems

Thus it is clear that the current treatments for fungal infections are very limited in comparison to the diversity and frequency of fungal infection. Physicians, mycologists and pharmaceutical companies are all pursuing the same goal: an antifungal agent with the following characteristics.<sup>10</sup>

- Effective in low doses
- Fungicidal (not merely fungistatic)
- No chance of relapse or resistance
- Inexpensive

Clark<sup>10</sup> elaborated on the necessary traits, mentioning the more specific characteristics of

- Stability
- Orally absorbed
- Parenterally effective
- Well distributed
- Metabolically stable
- Water Soluble
- Aesthetically Pleasing

In order to develop effective, selective fungicidal compounds, new cellular targets need to be located.<sup>48</sup> Current antifungals were developed with consideration of a limited range of cellular targets (Mechanism of Action, Table 2a). As a result, they can be toxic, have limited or reduced efficacy, or are ineffective due to natural or induced resistance (Table 2b). Therefore, in the search for an antifungal agent, to avoid the problems of acquired and natural resistance, it is necessary to target components specific to the fungal cell necessary for growth and viability; components that are not susceptible to molecular modification.<sup>48</sup> For example, an essential fungal cell surface enzyme unique to the infecting fungus could lead to the development of drugs with minimal toxicity, since the drug would function without entering the cell.<sup>48</sup> To achieve the development of a highly specific antifungal agent, the combined implication of antifungal screening procedures and rational antifungal design is necessary.

The continued progress of fungal infections without much reduction in mortality or frequency of occurrence, the development of resistance, and the lack of safe and effective treatments are manifestations of the difficulty surrounding the development of an agent suitable for the treatment of fungal infections in the immunocompromised. Due to the variety of fungal pathogens, there is a need for multiple antifungals, not single drugs with broad spectrum of activity, as not all fungi are susceptible to the same drug mechanisms.

Therefore, despite the goal of many researchers to discover an antifungal agent with a broad spectrum of activity, it seems more effective to strive to develop antifungal agents in order to further reduce toxicity, relapse, and resistance. Some strains of pathogenic fungi are inherently resistant to antifungal drugs. For example, *Candida krusei* has no susceptibility to the

antifungal actions of fluconazole.<sup>24</sup> Other fungi can acquire resistance during treatment. These occurrences are highly detrimental to the effectiveness of a broad-spectrum antifungal agent. The development of narrow-spectrum antifungal agents active against a given family of fungi would reveal an immediate treatment regiment upon diagnosis, rather than having to rely on an educated guess of whether or not the broad-spectrum antifungal will be effective. By providing the clinician with a variety of antifungal agents for use against each of the infecting families of fungi, rather than a few broad-spectrum drugs with inconsistent activity against all pathogens, the crucial initial treatment of the host would be much more successful. Moreover, recent efforts focusing on the improvement of rapid diagnostic techniques have allowed for the type of fungal infection to be determined in less than 2 days (contrary to just over 3 days for previous methods)<sup>49</sup> so that a physician could determine the type of infection (i.e. Candidiasis vs. Aspergillosis) and confidently administer an antifungal known to be fungicidal to members of that family of fungi. The odds of fungi in a family being susceptible to the same drug are intuitively much greater than the odds of all the pathogenic fungi being sensitive to powerful broad-spectrum drugs. The available broad-spectrum agents do not address the varying degrees of severity of the mycoses.<sup>50</sup> Expanding the number of available treatments, even if compromising the broad-spectrum capabilities, would provide more options to treat fungal infections occurring with such diversity. Systemic fungal infections are not caused by a mix of pathogens within the host, rather, by one often opportunistic fungus. Therefore, the “ideal” broad-spectrum antifungals are actually not necessary for systemic fungal infections, and a drug targeted to the fungus causing the infection would be better. Directing antifungals at a more selective target improves the safety profile of the drugs.<sup>51</sup> Furthermore, as O. Male<sup>50</sup> points out antifungals with broad therapeutic applications disregard to the fact that as an antifungal drug is made effective against more families of fungi, it

loses potency and tolerability.<sup>50</sup> The fact that the pharmaceutical industry has to this date ignored this inverse correlation, and even discarded potential antifungal therapeutics because of their limited spectrum of activity, is only compounding the problem of fungal infections.

There is a dire need for novel antifungal agents to add to the currently limited pool of available treatments for fungal infections in increasing populations of immunocompromised patients. Looking to nature for clues about species of organisms that would be likely to produce compounds for protection or defense reveals an abundance of resources, creativity, and diversity.<sup>7</sup> Chemical ecology is the study of chemical interactions between species. Examination of chemical interactions between species affords predictions of which organisms are likely to produce useful natural products and/or secondary metabolites.<sup>52, 58</sup>

Secondary metabolites are typically produced in organisms lacking an immune system such as plants and microorganisms to improve chemical defense.<sup>53</sup> A secondary metabolite is a chemical produced by an organism that serves no known metabolic purpose; the organism appears to produce the metabolite solely to increase its survival fitness.<sup>53</sup> Secondary metabolites are usually quite potent and functionally specific, may trigger very specific physiological responses,<sup>53</sup> and have been observed as components of interference competition in nature.<sup>54</sup> Different environmental conditions, or nutrient limitations cause the production of different secondary metabolites.<sup>54</sup> Knowledge of the possible functions of organisms' metabolites can be obtained through chemical ecology based studies.

Chemical ecology to date has had a very impressive success record in drug discovery. In the last decade many drugs developed by Merck Research Laboratories (a major pharmaceutical company) and made available to patients were a result of chemical ecological studies.<sup>52</sup> Another example of chemical ecology leading to the discovery of bioactive compounds occurred with the

shrub *Myrica gale* L. found in nutrient-deficient Scottish wetlands. The shrub was noticed to not be plagued by herbivores or pathogenic fungi. Investigation of the chemistry of the leaves of this shrub, revealed a fairly potent flavenoid antifungal molecule.<sup>55</sup>

Fungal ecology is of particular interest because antagonism (interference competition) has been reported in most fungal ecosystems.<sup>6, 58</sup> Environments inhabited by fungi (dung, soil, water, wood, leaf litter, living plants, and lichens)<sup>7</sup> are constantly changing forcing the fungal inhabitants to be versatile in order to survive. Fungi are known to produce a wide array of secondary metabolites as a primary mechanism of competition with other fungi, and are thought to have a selective advantage over other competitors in nature due to their production of secondary metabolites.<sup>6, 58</sup> There is numerous documentation of compounds with antifungal activity that have been isolated from other fungi.<sup>59, 60</sup> Because of intraspecies and interspecies competition, fungi produce compounds with antibacterial activity, and bacteria produce compounds with antifungal activity. Novel antifungal agents (substituted nine-membered dilactones) have been isolated from the fungus-like filamentous bacteria *Streptomyces* sp. 517-02 at Osaka City University in Japan.<sup>56</sup> Novel pradimicin antibiotic derivatives were isolated from an actinomycete fungus and demonstrated activity against many strains of *C. albicans*, and also against *A. Fumigatus*, *Cryptococcus neoformans*.<sup>57</sup> The “gold standard” of antifungal chemotherapy, Amphotericin B, is produced by the aerobic actinomycete *Streptomyces nodosus*.<sup>49</sup> Because fungi need to defend themselves from antagonists in their environment, the investigation of fungal secondary metabolites should lead to the discovery of compounds with antifungal activity.

In addition, fungi offer a readily available, renewable, abundant and diverse resource that has barely been tapped.<sup>62</sup> Fungi are the second largest group of organisms in the world.<sup>62</sup> Of the

1.5 million species of fungi suspected to exist, 100,000 species are currently known.<sup>29</sup> Only 5000 of these known species have secondary metabolites that are documented.<sup>62</sup> Within fungal ecosystems, endophytic fungi seem likely to be a potential source of antifungal compounds. Endophytic fungi inhabit the spaces between plant cells.<sup>29</sup> These endophytes appear to protect their hosts against pathogens.<sup>63</sup> Because it is accepted that virtually all plants have fungal associates, selective investigation of the fungi of those plants known to be resistant to fungal infection could yield compounds with antifungal activity.<sup>63</sup> For example, mycorrhizal fungal associates of plants provide their hosts with protection against plant pathogens. Dreyfuss isolated xylotrophic endophytes—those that live with in woody plants—and they demonstrated biological activity.<sup>7</sup>

These reasons led to our selection of endophytic fungi as organisms with potential production of biologically active secondary metabolites. Dr. Kate Graham, The College of St. Benedict, and Dr. Ulrich Mueller, University of Maryland, collected large numbers of novel fungi from The Archbold Biological Preserve in Archbold, Florida. These fungi were tested for an ability to inhibit the growth of several known pathogenic yeasts of the genus *Candida*. The thirty fungi which produced antifungal activity were kept for further analysis. KG146A, one of the library endophytic fungi found in the wild rosemary/tree shrub plant *Ceratiola ericoides*, demonstrated activity against the following yeasts: *Candida albicans* 406, *Candida albicans* wisconsin, *Candida albicans* 109, and *Saccharomyces cerevisiae*. Using bioassay-guided fractionation, we sought to isolate, purify, and obtain the structure of the antifungal compound(s) from the fungus, KG146A.



## RESULTS

Initial screening revealed that the endophytic fungus, KG146A, demonstrated activity against the following yeasts: *Candida albicans* 406, *Candida albicans* MEN, *Candida albicans* wisconsin, *Candida albicans* 109, and *Saccharomyces cerevisiae* (Table 1).

**Table 1:** Activity of plug assays of KG146A

Yeast	Diameter of Zone of Inhibition in mm
<i>Saccharomyces cerevisiae</i>	8 (clear)**
<i>Candida albicans</i> wisconsin	5 (opaque)
<i>Candida albicans</i> 406	4 (opaque)
<i>Candida albicans</i> C109	8 (opaque)

\*\*Zones of inhibition are described as either clear (no yeast growth) or opaque (resistant yeast growth)  
 ---- indicates no activity

Fermentation conditions were developed to optimize the production of antifungal compounds. The fungus was cultured Sabouraud's Dextrose Broth with additional dextrose at room temperature in baffled flasks and shaken at 200 rpm to provide enough aeration. The liquid cultures of KG146A began to show mycelial balls after the third day of growth and gradually produced more mycelial balls throughout the fermentation period. The cultures of KG146A were extracted with EtOAc on the eighth day (because previous work determined extracts after eight days of growth possessed the greatest antifungal activity) and tested for antifungal activity via disc diffusion assay (Table 2).

**Table 2:** Disk Diffusion Assay of the EtOAc Extracts (5mg/disk)

Yeasts	Diameter of Zone of Inhibition (mm)
<i>Saccharomyces cerevisiae</i>	20 (clear/opaque)**
<i>Candida albicans</i> wisconsin	13 (clear)
<i>Candida albicans</i> 406	15 (clear)
<i>Candida albicans</i> MEN	10 (clear)

\*\*Zones of inhibition are described as either clear (no yeast growth) or opaque (resistant yeast growth)

The crude fungal extracts were separated into multiple fractions on a 78cm x 3cm column packed with Sephadex LH-20 using 1:1 MeOH:CH<sub>2</sub>Cl<sub>2</sub> as the eluent. Thin-Layer Chromatography (TLC) of the fractions afforded fraction combination based on TLC R<sub>f</sub> value patterns. The combined fractions were tested for antifungal activity. Fraction 3 was found to have antifungal activity (Table 3).

**Table 3:** Sephadex LH-20 fraction combinations and assay for activity (5mg/disk)

Fraction	Activity (Diameter of Zone of Inhibition, mm)	
1: test tubes 1-16	-----	
2: test tubes 17-22	-----	
3: test tubes 23-33	<i>Saccharomyces cerevisiae</i>	29 (clear)**
	<i>Candida albicans</i> wisconsin	15 (clear)
	<i>Candida albicans</i> MEN	11 (clear)
	<i>Candida albicans</i> 406	18 (clear)
4: test tubes 34-49	-----	

\*\*Zones of inhibition are described as either clear (no yeast growth) or opaque (resistant yeast growth)  
 ----- indicates no activity

Fractions containing activity were combined and were further purified via HPLC. HPLC fractionation using an analytical Lichrosorb RP-C18 analytical column with an isocratic solvent system of 30:70 MeOH:H<sub>2</sub>O resulted in 8 new fractions. Previous work demonstrated that fraction 6 possessed slight antifungal activity against the yeast *Saccharomyces cerevisiae*, so fraction 6 was collected, concentrated, and tested for activity by disc diffusion assay.

**Table 4:** Disk Diffusion Assay of Fraction 6 (0.5 mg/disk)

Yeasts	Diameter of Zone of Inhibition (mm)
<i>Saccharomyces cerevisiae</i>	-----
<i>Candida albicans</i> wisconsin	-----
<i>Candida albicans</i> 406	-----
<i>Candida albicans</i> MEN	-----

\*\*Zones of inhibition are described as either clear (no yeast growth) or opaque (resistant yeast growth)

----- indicated no activity

Fraction 6 demonstrated no antifungal activity (Table 4). Therefore, ten new fractions were generated and collected from HPLC separation of the remaining LH-20 fungal compounds using the semi-preparatory RP C-18 column. These fractions were concentrated and tested for activity using disc diffusion assay. None of the ten fractions demonstrated antifungal activity (Table 5).

**Table 5:** Disc Diffusion Assay of HPLC fractions from C-18 Semi-Preparatory Column (0.3 mg of each fraction (1-10) per concentration disc)

<b>Yeasts</b>	<b>Diameter of Zone of Inhibition (mm)</b>
<i>Saccharomyces cerevisiae</i>	-----
<i>Candida albicans</i> wisconsin	-----
<i>Candida albicans</i> 406	-----
<i>Candida albicans</i> MEN	-----

## DISCUSSION

The optimal growth conditions developed by previous work were employed: baffled flasks; Sab Dex broth + Dextrose, room temp, shaking for 8 days, and extracting on the eighth day of growth. The test tube fractions from the Sephadex LH-20 separation into larger fractions, and fraction three (~test tubes 26-30) reproducibly demonstrated activity against *Saccharomyces cerevisiae* (19 mm radius zone of inhibition), and also demonstrated activity against several strains of *Candida*. However, fractionation by RP C-18 HPLC resulted in fractions with no activity.

The disk diffusion bioassay was utilized to assure that antifungal activity increased with purification by monitoring the size and clarity of the zone of inhibition. Ideally, after each step the compound being screened should exhibit a larger and less opaque zone of inhibition of the yeast growth. Results showed increased antifungal activity of the purified compounds. As the

compound(s) gets progressively more pure, it would be possible to perform the bioassay with less extract dispensed onto the concentration discs, because potency should increase with purity, and therefore less compound should be required to inhibit the test strains to the same degree.

The chromatography used to separate a solution of the fungal isolate into its components exploited the concepts of polarity and size exclusion. Sephadex LH-20 is a gel packing that has both hydrophilic and lipophilic properties useful for gel filtration in organic solvents.<sup>66</sup> The bead-like Sephadex molecules swell in polar organic solvents by taking up the solvent, an effective property for stable and reproducible separations via partition chromatography. It was introduced as a tool for the separation of natural products in polar organic solvents, something that had previously been a difficult task.<sup>66</sup> Sephadex LH-20 can be used to separate substances by size, partition chromatography, or based on gel-solute interactions—certain solvents cause reversible adsorption of certain substituents.<sup>66</sup> This separation with 1:1 MeOH:CH<sub>2</sub>Cl<sub>2</sub> was most likely achieved through partition chromatography effects and size exclusion effects.<sup>66</sup>

Reverse-phase (RP) chromatography was used in the second purification step. In RP HPLC, the stationary phase is non-polar (such as C-18, octadecyl chains covalently bonded to a silica gel matrix) and molecules are eluted with a polar mobile phase. Lipophilic type molecules are retained on the column as the more polar organics elude more rapidly. The primary interactions involved in separations on C18 columns are nonpolar, and polar interactions and cation exchange interactions play secondary roles in the separation. Molecules that are very polar are not retained on the column at all, but C18 packing is very useful when the compounds to be separated consist of structural diversity, because it is the least selective sorbent. However, the converse side of its non-selectivity and effective separation of structurally diverse

compounds is the separation into fractions that are not as pure as they would be had another stationary phase been used.

All of the chromatography conditions were selected in previous work on KG146A by Ryan Jense and Dr. Kate Graham. Therefore, when the chromatographic separations were performed in this project, these previously developed conditions were duplicated in order to reproduce the active compound, however, collection of the specific fraction containing the active components of the fungal extracts was not reproducible. HPLC purification and collection of the fraction previously shown to be active against *Saccharomyces cerevisiae* yielded fractions with no antifungal activity. One or more of the following factors are speculated to have produced the anomalous results. Slight changes in the C-18 column could have shifted the elution of active components outside of the range that was collected. Fractionation of eluents could have spread the active components between one or more fractions, causing their concentration (% composition) in each of the fractions to be insufficient to produce antifungal activity.

If the compound is not extremely stable, prolonged contact with the methanol/water solvents could lead to nucleophilic interactions with the active compound(s) resulting in structural degradation. If the compound is air, light, or temperature sensitive, too much exposure to any one of these variables could alter the structure in a way significantly enough to disrupt the antifungal activity.

To eliminate these factors, several precautions can be taken. Performing disc-diffusion assays on all HPLC fractions as soon as sufficient material is obtained will reveal potential changes in elution, stripping the solvents from all fractions as rapidly as possible should prevent structural degradation, and storing all dried compounds in a regulated environment (i.e. a cold

room) in opaque, sealed containers are all means in which to control deviation from the known procedure and yield reproducible results.

Additionally, it is possible that the correct fraction was collected, but that not enough material was used in the disc-diffusion bioassay. In response to this potential problem, large quantities of KG146A were harvested and are being put through the Sephadex column in order to obtain a much greater amount of material for HPLC separation. The most recent bioassay used about a 1.5 fold reduction (0.75 mg to approximately 0.5 mg) in amount of fungal compound loaded onto the discs, which could have resulted in the loss of activity. Another recent change was the switch from the analytical HPLC column to a semi-preparative C18 column. The procedural change was made in an attempt to separate larger amounts of material. However, further investigation is necessary to determine whether enough resolution is maintained in switching to a larger column bed, as none of the fractions collected from the semi-preparative column were observed to have antifungal activity. Another factor was the 3 fold reduction in the amount of material concentrated onto the diffusion disk (0.75 mg reduced to 0.3 mg). Furthermore, in order to continue the HPLC fractionation using the semi-preparative RP C-18 column a more efficient way to remove solvent from eluent is necessary.

Pending the final purification of greater amounts of the fungal compounds, NMR, Infrared and Mass Spectroscopy will be used to elucidate the structure of the active compound. This compound, if proven to have relevant antifungal capabilities, will then be approached with medicinal chemistry and combinatorial chemistry to synthesize a large array of derivatives to optimize specificity, efficacy, potency, and to minimize toxicity and adverse interactions. Lead compounds derived from natural products have great potential in the pharmaceutical industry.

## CONCLUSION

The most needed tool for improved control of fungal infections, primarily in the immunocompromised patient, remains the development of new antifungal agents.<sup>49</sup> Creative means of screening for sources of novel antifungal drugs should yield compounds with toxicity to fungi via alternate mechanisms than those of current drugs. One method of finding new lead compounds that has past evidence to be very effective, is the use of chemical ecology and biorationale to select certain natural ecosystems as producers of compounds with fungicidal activity. Specifically, fungi, because of their known antagonistic interactions with other fungi to compete for nutrients and survival, should be thoroughly evaluated for production of antifungal compounds. The past has provided examples of successful production of antifungal agents from fungi, and chemotaxonomy would predict that other fungi should also produce vast arrays of novel metabolites with biological use. Several fungi produce antimicrobials that are currently employed everywhere: Cephalothin came from the fungus *Cephalosporium*, griseofulvin, the topical antifungal agent, came from the fungus *Penicillium griseofulvum*, and Penicillin is produced by the fungus *Penicillium notatum*.

Endophytic fungi were investigated as potential sources of antifungal compounds due to their known prolific production of secondary metabolites, their role in defending their plant hosts from fungal pathogens, and their abundance in nature. The endophytic fungus, KG146A has been demonstrated to be a novel source of antifungal activity. Organic extracts from KG146A contain compounds with inhibitory activity against several strains of *Candida*, a yeast responsible for severe opportunistic infection in immuno-compromised patients. Using bioassay-guided fractionation, a procedure for purification of the antifungal compound has been partially developed. Further purification will be achieved using HPLC.

The frequent incidence of fungal infections, their ability to be unaffected by therapy, and their tendency to relapse and become chronic, have accompanied the growing advances in modern medicine. Because a decline in medical advances is unlikely, a decrease in serious fungal infections in the patients left with compromised immune function from such advances is equally unlikely.<sup>50</sup> It has been stated that we have entered the golden age of mycology and antifungal chemotherapy, and that “no doctor can neglect mycology, no matter what their specialty.”<sup>28</sup> If this is the case, we are entering a battle against fungi and fungal infections with insufficient ammunition, and therefore, it is imperative that the future provides novel, safe, and effective antifungal drugs. Rare incidences of fungal infection caused by fungi that are expected to not be human pathogens, have even led to the statement that it may be necessary to conclude that there no longer exists a non-pathogenic fungus.<sup>28</sup>

## **EXPERIMENTAL**

Instrumentation All solutions and inoculation utensils were sterilized using a Yamato Sterilizer SM 300. Liquid cultures were shaken on the Labline Orbit Environ Shaker at 200 rpm. A 78cm x 3cm column packed with Sephadex LH-20 was used to separate crude compounds with a mobile phase of 1:1 MeOH:CH<sub>2</sub>Cl<sub>2</sub>. The ISCO Model 328 Fraction Collector was used to collect column fractions. High Performance Liquid Chromatography was performed with the Varian 5010 Liquid Chromatograph connected to a Shimadzu C-R3A Chromatopac Integrator. HPLC was performed on an analytical lichrosorb C18 column (250mm x 4.6mm; 10μ bead size), and then a semi-preparatory lichrosorb C-18 column (250 mm x 10 mm; 10μ bead size). Culture media was purchased from Fisher Scientific.

Fermentation conditions KG146A was maintained on Sabouraud’s and potato dextrose agar plate cultures. Pieces of the agar from these parent cultures were used to inoculate 250 mL



Sabouraud's Dextrose and Potato Dextrose broth solutions (plus 20g dextrose per liter to account for the nutrient difference in agar and broth) in 500 mL baffled Erlenmeyer flasks. Cultures were grown for 8 days at room temperature (22°C) while shaken at 200 rpm.

Extraction KG146A broth was centrifuged at 15,000 rpm for 30 minutes and then filtered (Whatman #1, Whatman #41) using vacuum filtration. Filtrate was neutralized to a pH 7 with 3M NaOH (~1.5mL per 1L broth). Organics were extracted three times with EtOAc (250 mL EtOAc per 500 mL broth), backwashed with a saturated NaCl solution (250 mL per 500 mL broth), dried with anhydrous MgSO<sub>4</sub>, and concentrated using rotary evaporation.

Disc Diffusion Bioassay Initial tests for activity in KG146A were plug assays (using pieces of agar inoculated with KG146A against the yeasts *Candida albicans* 406, *Candida albicans* 109, *Candida albicans* wisconsin, and *Saccharomyces cerevisiae* to confirm the presence of antifungal compounds. Concentrated organics extracted from KG146A were tested against four yeast test strains. Liquid cultures of *Candida albicans* 406, *Candida albicans* MEN, *Candida albicans* wisconsin, and *Saccharomyces cerevisiae* were prepared in 75 mL Sabouraud's Dextrose broth. Yeasts were grown for 24 hours at room temperature without shaking. Sabouraud's dextrose agar (50 mL/test strain) was inoculated with ~1 mL of liquid yeast culture and 10-15 mL was poured into each plate. Concentrated fungal extracts were redissolved in EtOAc, the compounds were dispensed onto blank concentration disks (5mg/disk). Occasionally, small white particles remained insoluble in the EtOAc. Bioassay revealed antifungal activity regardless of whether these white particles were included on the concentration disks. The positive control was a concentration disk of Nystatin. Disks were placed on yeast-embedded agar plates and grown at room temperature without light for 24 hours before observing the zones of inhibition.

Column chromatography Crude extracts (0.25 g dissolved in 2 mL 1:1 MeOH:CH<sub>2</sub>Cl<sub>2</sub>) were eluted on a 78 cm x 3 cm Sephadex LH-20 column with a mobile phase of 1:1 MeOH:CH<sub>2</sub>Cl<sub>2</sub> into approximately 40 (4 mL) fractions. TLC of the fractions on silica with a 95/5 CH<sub>2</sub>Cl<sub>2</sub>/MeOH solvent system was performed (60 mL in the developing chamber); plates were first analyzed under short and long wave UV light and then the plates were developed with 5% PMA in 95% EtOH and heated. Fractions with similar distributions of R<sub>f</sub> values were combined to yield five final fractions which were condensed and tested for antifungal activity.

Second Disk Diffusion Assay Dried column fractions were redissolved in CH<sub>2</sub>Cl<sub>2</sub> and dispensed onto blank concentration disks (5 mg/disk). Occasional residual white particles were again encountered, and bioassay displayed antifungal activity regardless of the transfer of these particles to the disks. Fractions 3 and 4 demonstrated activity and were combined so that in future separations four final fractions were combined and condensed. Fraction 3 was found to contain antifungal activity. (Characteristic R<sub>f</sub> values: Fraction 1: no spots; Fraction 2: 0.6, 0.5, 0.28, 0.15, 0.06; Fraction 3: 0.28, 0.34, 0.18; Fraction 4: no spots)

Purification of active components The active fraction from column chromatography was further separated using reversed-phase HPLC with an Alltech Lichrosorb RP-C18 analytical column (250mm x 4.6mm; 10μ bead size) using an isocratic separation with the mobile phase of 30:70 MeOH:CH<sub>2</sub>Cl<sub>2</sub>, detector sensitivity at 0.02 AU/MV, UV detector set at 254 nm, and a flow rate of 2 mL/min. Previous work by Ryan Jense under these conditions indicated the collection of eight fractions from an injection of 60μL of 158 mg/mL MeOH solution of fraction 3. His results showed that HPLC fraction 6 demonstrated slight antifungal activity (0.75mg/disk) against *Saccharomyces cerevisiae* (Retention times (min.): 1.2-1.5, 1.51-2.3, 2.31-3.4, 3.41-5.4, 5.41-10, 10.01-13.5, 13.51-16, 16.01-35).

Duplicating the previously developed HPLC conditions, 215 injections of Sephadex column fraction 3 were performed. Initial injections were 5  $\mu$ L and increased in increments of 10 $\mu$ L up to 60 $\mu$ L. Most injections were 40 $\mu$ L. One fraction was collected off the column for each elution (Retention times 13-19 minutes). The dried compounds from the combined collected fraction were not completely soluble in MeOH, even when a small amount of water was added to the solution. The solution containing the undissolved particles was transferred to concentration disks for bioassay and demonstrated no antifungal activity against *Candida* or *Saccharomyces cerevisiae*. Assay was performed by dispensing 0.5 mg dried HPLC fraction per disk. No antifungal activity was observed.

Summary of batches of KG146A A total of seven batches of KG146A (29.7 L liquid cultures) were used to isolate the compounds analyzed in the research. This volume yielded approximately 7g solid crude fungal extracts. Separation of 4.398g of the fungal extracts via Sephadex LH-20 chromatography yielded 1.346 g of active material, which was further separated using the analytical C-18 column. The combined collected fraction yielded 30mg dried compound with no demonstrated antifungal activity.

Separation of the remaining 0.05 g of Sephadex fraction 3 was then attempted using an Alltech RP-C18 semi-preparative column (250 mm x 10 mm; 10 $\mu$ ) at the same detector conditions, and solvent system, with the flow rate increased to 5 mL/min. Injected fraction three (15 total injections initially of 10 $\mu$ L increasing up to 75 $\mu$ L of 158mg/mL MeOH solution of fraction 3) yielded ten fractions that were collected in 500mL RBF (Retention times (minutes): 0-2.1, 2.12-2.85, 2.86-4.99, 5.0-7.22, 7.23-10.0, 10.01-16.0, 16.01-17.7, 17.71-19.0, 19.01-21.0, 21.01-24.5). Fractions were dried for several days via lyophilization. The ten dried fractions were of the following amounts (**fraction #**: mass (mg): **1**: 1.1, **2**: 0.5, **3**: 1.9, **4**: 3.3, **5**: 2.01, **6**: 4,

7: 1.1, 8: 1.4, 9: 1.1, 10: 1.4). Approximately 2.602g crude fungal extracts remain to be separated with Sephadex LH-20 chromatography and with further purification via HPLC.

Dried column fractions were not completely soluble in MeOH, H<sub>2</sub>O, ACN, EtOAc, CH<sub>2</sub>Cl<sub>2</sub>, DMSO, or combinations of two or more of these solvents. Insoluble white residual particles remained in solution. These particles were transferred with the rest of the solution to concentration disks (0.3 mg/disk). Antifungal activity was not observed for any of the collected fractions.

#### **ACKNOWLEDGEMENTS**

Great appreciation is expressed to Ryan Jense (CSB/SJU '98) for preliminary methods and results, and to the thesis advisor Dr. Kate Graham, and the thesis committee members Fr. John Klassen (CSB/SJU Department of Chemistry) and Dr. Ellen Jensen (CSB/SJU Department of Biology) for their advice and instruction. Special thanks to Mrs. Nikki Jochman, the CSB/SJU chemistry department stockroom manager for providing chemicals and materials at moments notice. Also gratitude is expressed to The College of St. Benedict/St. John's University for funding attendance to NCUR 99.

## WORKS CITED

- <sup>1</sup> Conant, M.A. **Fungal infections in immunocompromised individuals.** *Cutaneous Mycology.* 1996, 12(1):155-162.
- <sup>2</sup> Stevens, D. **Problems in antifungal chemotherapy.** *Infection.* 1987, 15(2):87-92.
- <sup>3</sup> Martino, P. & Girmenia, C. **Are we making progress in antifungal therapy?** *Current Opinion in Oncology.* 1997, 9:314-320.
- <sup>4</sup> van't Wout, J.W. **Fungal infections and antifungal drugs: has the age of antifungal resistance dawned?** *Current Opinion in Infectious Diseases.* 1996, 9:63-66.
- <sup>5</sup> Dupont, B. **Azole antifungal agents: emerging and inherent resistance.** *Current Opinion in Infectious Diseases.* 1995, 8:424-427.
- <sup>6</sup> Gloer, J.B. **The chemistry of fungal antagonism and defense.** *Can. J. Bot.* 1995, 73(Supp.1):S1265-S1274.
- <sup>7</sup> Dreyfuss, M.M.; Chapela, I.H. **Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals,** In *The Discovery of Natural Products with Therapeutic Potential.* V.P. Gullo, Ed., Butterworth-Heinemann: Boston, 1994, 49-73.
- <sup>8</sup> Klepser, M.E.; Lewis, R.E.; Pfaller, M.A. **Therapy of *Candida* infections: susceptibility testing, resistance, and therapeutic options.** *The Annals of Pharmacotherapy.* 1998, 32:1353-1361.
- <sup>9</sup> Flanagan, P.G.; Barnes, R.A. **Fungal infection in the intensive care unit.** *Journal of Hospital Infection.* 1998, 38:163-177.
- <sup>10</sup> Clark, A. **The need for new antifungal drugs.** In *Recent Progress in Antifungal Chemotherapy.* Yamaguchi, H.; Kobayashi, G.S.; Takahashi, H. (eds). 1992, Marcel Dekker, Inc.:1-19.
- <sup>11</sup> Mendoff, G. **New challenges to the therapy of systemic fungal infections.** *Transactions of the American Clinical and Climatological Association.* 1995, 107:146-458.
- <sup>12</sup> Alexander, B.D.; Perfect, J.R. **Antifungal resistance trends towards the year 2000.** *Drugs.* 1997, 54(5):657-678.
- <sup>13</sup> Conant, M.A. **Fungal infections in immunocompromised individuals.** *Dermatologic Clinics.* 1996, 14(1):155-162.
- <sup>14</sup> Dupont, P.F. ***Candida Albicans*, the opportunist: a cellular and molecular perspective.** *Journal of the American Podiatric Medical Association.* 1995, 85(2):104-115.
- <sup>15</sup> Odds, F.C. **Resistance of yeasts to azole-derivative antifungals.** *Journal of Antimicrobial Chemotherapy.* 1993, 31:463-471.

- 
- <sup>16</sup> Anaissie, E.; Bodey, G.P. **Nosocomial fungal infections: old problems and new challenges.** *Infectious Disease Clinics of North America.* 1989, **3**(4):867-882.
- <sup>17</sup> Lipman, J.; Saadia, R. **Fungal infections in critically ill patients.** *British Medical Journal.* 1997, **315**:266-267.
- <sup>18</sup> Levitz, S.M. **Overview of host defenses in fungal infections.** *Clinical Infectious Diseases.* 1992, **14**(Suppl.1):S37-S42.
- <sup>19</sup> Antrum, J. **Meeting the challenge of systemic fungal infections in cancer: nursing implications.** *European Journal of Haematology.* 1996, **56**:7-11.
- <sup>20</sup> Richardson, M.D. **Opportunistic and pathogenic fungi.** *Journal of Antimicrobial Chemotherapy.* 1991, **28**(Suppl. A):1-11.
- <sup>21</sup> Chimenos, E.; Puy, D.; Lopez, J. **Antifungal drugs in the management of mycoses.** *Dermatologic Clinics.* 1996, **14**(1):155-162.
- <sup>22</sup> Romagnoli, M. **Host factors in human fungal infections.** *Fungal Infections of the Head and Neck.* 1993, **26**(6):941-948.
- <sup>23</sup> Reiss, K.; Tanaka, G. et al. **Molecular diagnosis and epidemiology of fungal infections.** *Medical Mycology.* 1998, **36**(Suppl. 1):249-257.
- <sup>24</sup> Walsh, T.J.; Chanock, S.J. **Diagnosis of invasive fungal infections: advances in nonculture systems.** *Current Clinical Topics in Infectious Diseases.* 1998, **18**:101-153.
- <sup>25</sup> Diamond, R.D. **Immune response to fungal infection.** *Reviews of Infectious Diseases.* 1989, **11**(Suppl. 7):S1600-S1604.
- <sup>26</sup> Donnelly, J.P. **Unusual infections and development in the use of existing antimicrobial agents.** *Current Opinion in Infectious Diseases.* 1995, **8**: 261-267.
- <sup>27</sup> Deepe, G.S.; Bullock, W.E. **Immunological aspects of fungal pathogenesis.** *Eur. J. Clin. Microbiol. Infect. Dis.* 1990, **9**(8):567-579.
- <sup>28</sup> Rinaldi, M.G. **Controversies in medical mycology.** *Dermatology.* 1997, **194**(suppl 1):45-47.
- <sup>29</sup> Prescott, L.M., Harley, J.P. Klein, D.A. *Microbiology* (4th ed.). 1999. WCB McGraw-Hill: Madison, WI.
- <sup>30</sup> Azelson, K. (1998). "A Tale of Two Yeasts". Presented at the April 15<sup>th</sup> 1998 Biology Department Seminar, St. John's University, Collegeville, MN.
- <sup>31</sup> Peters, B.S. et al. **Adverse effects of drugs used in the management of opportunistic infections associated with HIV infection.** *Drug Safety.* 1994, **10**(6):439-454

- 
- <sup>32</sup> Negroni, R. et al. **Teaching medical mycology in the year 2000.** *Medical Mycology*. 1998, **36**(suppl. I):106-108.
- <sup>33</sup> Dismukes, W.E., Bennett, J.E., Drutz, D.J., Graybill, J.R., Remington, J.S., Stevens, D.A. **Criteria for evaluation of therapeutic response to antifungal drugs.** *Reviews of Infectious Diseases*. 1980, **2**(4): 535-544.
- <sup>34</sup> Pfaller, M.A. **Antifungal susceptibility testing: *in vitro* and *in vivo*.** In *Recent Progress In Antifungal Chemotherapy*. Yamaguchi, H.; Kobayashi, G.S.; Takahashi, H. (eds). 1992, Marcel Dekker, Inc.:415-426.
- <sup>35</sup> Andriole, V.T. **Current and future therapy of invasive fungal infections.** *Current Clinical Topics in Infectious Diseases*. 1998, **18**:19-36.
- <sup>36</sup> Richardson, M.D.; Kokki, M.H. **Diagnosis and prevention of fungal infection in the immunocompromised patient.** *Blood Reviews*. 1998, **12**:241-254.
- <sup>37</sup> Stevens, D.A. **Antifungal drug susceptibility testing: A critical review.** *Mycopathologia*. 1984, **87**: 135-140.
- <sup>38</sup> Burnie, J.P. **Early diagnosis of systemic fungal infection.** *Current Opinion in Infectious Diseases*. 1995, **8**: 258-260.
- <sup>39</sup> Stevens, D.A. **Historical perspectives and state-of-the-art treatment of systemic mycoses compared to newer problems in management of fungal infections in debilitated and immunosuppressed hosts.** In *Recent Progress in Antifungal Chemotherapy*. Yamaguchi, H.; Kobayashi, G.S.; Takahashi, H. (eds). 1992, Marcel Dekker, Inc
- <sup>40</sup> Georgiev, V.S. **Fungal cell envelope and mode of action of antimycotic drugs.** In *Recent Progress in Antifungal Chemotherapy*. Yamaguchi, H.; Kobayashi, G.S.; Takahashi, H. (eds). 1992, Marcel Dekker, Inc 11-23.
- <sup>41</sup> Clark, J.M. **Amphotericin B phospholipid formulations.** In *Recent Progress in Antifungal Chemotherapy*. Yamaguchi, H.; Kobayashi, G.S.; Takahashi, H. (eds). 1992, Marcel Dekker, Inc.:189-195.
- <sup>42</sup> Lyman, C.; Walsh, T.J. **Systemically administered antifungal agents: a review of their clinical pharmacology and therapeutic applications.** *Drugs*. 1992, **44**(1):9-35.
- <sup>43</sup> Bossche, H.V.; Marichal, P.; Odds, F.C. **Molecular mechanisms of drug resistance in fungi.** *Trends in Microbiology*. 1994, **2**(10):393-400.
- <sup>44</sup> Kauffman, C.A.; Carver, P.L. **Antifungal agents in the 1990's: current status and future developments.** *Drugs*. 1997, **53**(4):539-49.

- 
- <sup>45</sup> Munoz, A.J.; Tur-Tur, C. **Comparative study of activity of sertaconazole, terbinafine, and bifonazole against clinical isolates of *Candida* spp., *Cryptococcus neoformans*, and dermatophytes.** *Chemotherapy*. 1997, 387-392.
- <sup>46</sup> Walsh, T.J.; Lee, J.W.; Roilides, E.; Pizzo, P.A. **Recent progress and current problems in management of invasive fungal infections in patients with neoplastic diseases.** *Current Opinion in Oncology*. 1992, 4:647-655.
- <sup>47</sup> Rex, J.H.; Rinaldi, M.G.; Pfaller, M.A. **Resistance of *Candida* species to fluconazole.** *Antimicrobial Agents and Chemotherapy*. 1995, 39(1):1-8.
- <sup>48</sup> Monk, B.C.; Perlin, D.S. **Fungal plasma membrane proton pumps as promising new antifungal targets.** *Critical Reviews in Microbiology*. 1994, 20(3):209-223.
- <sup>49</sup> Alexander, B.D.; Perfect, J.R. **Antifungal resistance trends towards the year 2000: implications for therapy and new approaches.** *Drugs*. 1997, 54(5):657-678.
- <sup>50</sup> Male, O. **The significance of mycology in medicine.** In *Frontiers in Mycology*. 1991. Hawksworth, D.L. (ed). Regensburg, CAB International.
- <sup>51</sup> Sheehan, D.J.; Hitchcock, C.A.; Sibley, C.M. **Current and emerging azole antifungal agents.** *Clinical Microbiology Reviews*. 1999, 12(1):40-79.
- <sup>52</sup> Caporale, L.H. **Chemical ecology: a view from the pharmaceutical industry.** *Proc. Natl. Acad. Sci. USA*. 1995, 92:75-82.
- <sup>53</sup> Williams, D.H.; Stone, M.J.; Hauck, P.R. and Rahman, S.K. **Why are secondary metabolites (natural products) biosynthesized?** *Journal of Natural Products*. 1989, 52:1189-1208
- <sup>54</sup> Miller, J.D. **Mycology, mycologists, and biotechnology.** In *Frontiers in Mycology*. D.L.Hawksworth (ed). *Agriculture Canada*. 1990:225-240.
- <sup>55</sup> Waterman, P.G. **Searching for bioactive compounds: various strategies.** *Journal of Natural Products*. 1990, 53(1):13-22.
- <sup>56</sup> Hanafi, M. et al. **UK-2A,B,C and D, novel antifungal antibiotics from *Streptomyces* sp.** 517-02.
- <sup>57</sup> Furumai, T.; Hasegawa, T.; et al. **Pradimicins t1 and t2, new antifungal antibiotics produced by an actinomycete.** *The Journal of Antibiotics*. 1993, 46(4):589-597.
- <sup>58</sup> Gloer, J.B. **New antifungal and antiinsectan natural products from fungi: an ecology-based approach.** *Rev. Latinoamer. Quim.* 1996, 24(3-4):191-208.
- <sup>59</sup> Patel, M.; Conover, M. et al. **Sch 31828, a novel antibiotic from a *Microbispora* sp.: taxonomy, fermentation, isolation and biological properties.** 1988, 41(6):794-797.



- 
- <sup>60</sup> Yamashita, M.; Tsurumi, Y. et al. **Chryscandin, a novel peptidyl nucleoside antibiotic: taxonomy, fermentation, isolation and characterization.** *The Journal of Antibiotics.* 1984, **37**(11):1279-1283.
- <sup>61</sup> Landecker, E.M. (1990). *Fundamentals of the Fungi.* (3<sup>rd</sup> edition). Englewood Cliffs, New Jersey: Prentice-Hall, Inc.
- <sup>62</sup> Hawksworth, D.L. **The fungal dimension of biodiversity: magnitude, significance, and conservation.** *Mycol. Res.* 1991, **95**(6):641-655.
- <sup>63</sup> Petrini, O. **Taxonomy of endophytic fungi of aerial plant tissues.** In *Microbiology of the phyllosphere.* Fokkema, N.J; Van Den Heuvel, J. (eds). 1986, 175-187.
- <sup>64</sup> Vining, L.C. **Functions of secondary metabolites.** *Annu. Rev. Microbiol.* 1990, **44**:395-427.
- <sup>65</sup> Sephadex LH-20: Chromatography in Organic Solvents. Pharmacia Fine Chemicals AB.