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Studies of the structures, biosynthesis and function of fungal glycosphingolipids

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STUDIES OF THE STRUCTURES, BIOSYNTHESIS AND FUNCTION OF FUNGAL GLYCOSPHINGOLIPIDS

BY

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B.Ed., Kenyatta University, 2002

THESIS

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This thesis has been examined and approved.

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12-13-07

Date
DEDICATION

I would like to dedicate this work to all my family members who are here to see this happen. In particular, my three children Ricky, Suzzane and Crystal who, through their support, humor and love made it possible for me to sail through these years with ease. I would also like to dedicate this thesis to Peter, who for his constant love, understanding and cheer made this accomplishment easier than it was already difficult. To my father whose advice was undoubting, I say thank you. Together, my family cushioned me when times were tough and were also there to laugh with me in good times.

I would like to make a special dedication to my mother who did not live to see this accomplishment. Although your absence in this world may seem too void to be filled, your teachings of determination and achievement no matter the circumstance have been my driving force. You touched the lives of many in a special way and your name is mentioned with joy in many lips. There is no greater joy, than the joy in knowing that although you did not see the end of this journey, you were there to bless its beginning.

Finally, I dedicate this work back to my creator, who promised that weeping may last in the night, but joy cometh in the morning. I thank you for bringing a bright and clear morning with the completion of this work.
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ABSTRACT

STUDIES OF THE STRUCTURES, BIOSYNTHESIS AND FUNCTION OF FUNGAL GLYCOSPHINGOLIPIDS

by

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University of New Hampshire, December 2007

Glycosphingolipids (GSLs) are ubiquitously distributed among all eukaryotes. In fungi, GSLs have also been implicated in a variety of cellular processes, including growth, cell signaling, differentiation and morphogenesis. Detailed knowledge of fungal GSL structure and biosynthesis is therefore expected to yield valuable insights into both their functions within the organism and their interactions with other organisms, such as with the immune system, of an infected host as well as to provide targets for diagnostic and therapeutic agents.

Research described in this thesis focused on fungal GSL structure and biosynthesis. Two projects are described. The first was aimed at elucidating the functions of GSLs in Neurospora crassa. The isolation and structural analysis of glycolinositolphosphoryl ceramides (GIPCs) from N. crassa wild type (ATCC 18889 and 24698) and a defensin-resistant mutant strain (SP 10) were attempted, using NMR, MS and other techniques. Although no GIPCs were ultimately isolated in this work, phosphatidylinositol, the donor substrate for inositolphosphorylceramide IPC synthase, was detected and characterized. In addition, a preliminary study of the effects of an
inhibitor of GSL biosynthesis, 4'-ethylenedioxy-1-phenyl-2-palmitoyl-3-pyrrolidinopropanol (EDO-P4), was performed. The results of this experiment were consistent with inhibition of *N. crassa* glucosylceramide synthase (GCS) by EDO-P4.

In the second project, studies of the structures and biosynthesis of glycosphingolipids of the pathogenic model fungus, *Cryptococcus neoformans* were performed on both the wild type (JEC 21) and a mutant, termed *ctx1ctx2Δ*, in which the genes for *C. neoformans* β1,2-xylosyltransferases, Cxt1 and Cxt2 had been disrupted. Our results demonstrated an altered expression in GIPC structures in the absence of the two xylosyltransferases, characterized by the expression of normal *C. neoformans* GIPC core structure, but without addition of characteristic Xylβ1,2 residue. We detected no residual xylose-containing GIPCs in the *ctx1ctx2Δ* mutant sample, confirming that no other enzyme performs the function of xylose addition during GIPC synthesis.
SIGNIFICANCE OF GLYCOSPHINGOLIPIDS

Sphingolipids, along with cholesterol and phosphoglycerolipids, are the three major types of lipids found in biological membranes (Dickson et al., 1997). Sphingoids are long-chained hydrocarbon bases with a common 1,3-dihydroxy-2-amino-backbone motif that comprises the defining structural unit of sphingolipids (Figure 1.1).

![Figure 1.1](image)

Figure 1.1 The general core structure of a sphingolipid incorporating a long chain amino alcohol, commonly (4E)-1,3-dihydroxyl-2-amino-4-octadecene (sphingosine), where X = H, R = H: sphingosine; X = H, R = fatty acid: ceramide; X = phosphocholine, R = fatty acid: sphingomyelin; X = glycan containing head group, R = fatty acid: glycosphingolipid (GSL).

The chain length of the sphingoid is variable, although in mammals and fungi it is generally 18-20 carbons. Modifications such as additional hydroxylation, acetylation, esterification, unsaturations, and alkyl branching are common features of sphingoids. A sphingoid base amide linked to a fatty acid chain is called a ceramide, and the fatty-N-acyl chain may also have variations of carbon number and additional modifications such as hydroxylation and unsaturation. Ceramides are usually modified (as will be discussed later in this section) and very small amounts of free ceramides are found in organisms.
Mammals and other animal phyla for example, exhibit only one phosphorylated ceramide derivative, sphingomyelin, or choline phosphorylceramide, found in quantity in their tissues.

Glycosiphingolipids (GSLs) are the O-glycosides of ceramides (Cer) or myo-inositol-1-phosphoryl-1-ceramides (IPC), (Figure 1.2 and 1.3 respectively), and are ubiquitously distributed among all eukaryotes (Kolter and Sandhoff, 1999) and in some bacteria (Olsen and Jantzen 2001).

![Figure 1.2](image)

Where R₁ and R₂ can be H or OH interchangeably and R₃ can either be;

\[
R₃ = \begin{cases} 
\text{H} \\
\text{OH} 
\end{cases}
\]

or

\[
R₃ = \begin{cases} 
\text{H} \\
\text{OH} 
\end{cases}
\]

**Figure 1.2** Structure of a prototypical fungal cerebroside with numbering of sphingosine (Sph), hexose (Hex), and R₃; fatty acid (Fa) moieties.

Found mainly in biological membranes, particularly in the plasma membrane, GSLs are amphiphilic conjugates, since each is comprised of a polar head group attached
to an \( N \)-acylated sphingoid or ceramide. In GSLs, variations in both glycan and ceramide structure may depend on phylum, species, tissue stage of differentiation and development and pathology (Levery et al., 1998). The heterogeneity within this class of lipids is therefore robust.

![Figure 1.3 IPC, the obligate intermediate for the biosynthesis of glycosylinositol phosphorylceramide or GIPC found in plants, fungi and other organisms but not in mammals (Lester and Dickson 1993). Where \( R_1=H \), spinganines or \( R_1 = \text{OH} \), 4-hydroxysphinganine, commonly referred to as phytosphingosine in the phytoceramide. The fatty-N-acyl chain may be of typical chain lengths of C22-C26 and be non-hydroxy (\( R_2 = R_3 = H \)), 2-hydroxy (\( R_2 = \text{OH}, R_3 = H \); most common in fungi and plants), 2,3-dihydroxy (\( R_2 = R_3 = \text{OH} \)).

Sphingolipids, like glycerophospholipids and sterols, can play both structural and regulatory roles, and their basic lipid building block, ceramide, is considered to be a signaling molecule in mammals (Toledo, M.S., et al 2001, Dickson and Lester., 1993, 1999). Investigations of GSL biosynthesis and function in \textit{Saccharomyces cerevisiae} have established that GSLs are essential components of the fungal membrane. GSLs have also been implicated in many cellular processes including growth, differentiation and
morphogenesis in *S. commune* (Kawai and Ikeda 1982), *Cryptococcus neoformans* (Rodrigues et al 2000), *P boydii* (Pinto et al 2002), *Aspergillus nidulans, Aspergillus fumigatus* (Levery, S., et al 2002) amongst others. GSLs may also modulate cell signaling by controlling the assembly and specific activities of plasma membrane proteins (Hakomori 1993, Kasahara and Sanai 2000). GSLs on the cell surface are involved in cell type specific adhesion processes where they form cell type specific patterns on the cell surface, which changes with the differentiation stage and with viral oncogenic transformations (Kolter, T. and Sandhoff, K., 1999). Previous studies have shown that GSLs can serve as binding sites for toxins, viruses and bacteria. Interactions of GSLs with receptors and enzymes, which like themselves are located in the same membrane, have been described and are of possible physiological relevance. The ganglioside GM1 for example, activates the nerve growth factor while the ganglioside GM3 inhibits tyrosine phosphorylation of the epidermal growth factor receptor. It is proposed that in the attempt to elucidate the functional role of fungal GSLs, systematic isolation and characterization of these GSLs should be of interest.

GSLs fall mainly into two categories, neutral and acidic GSLs. Monohexosylceramides (CMHs) commonly known as cerebrosides are examples of neutral GSLs that contain a monosaccharide, usually glucose or galactose in the beta-glycosidic linkage to the 1-hydroxyl of a ceramide. Transfer of a sugar to the ceramide is catalyzed by a glycosyltransferase enzyme, yields a monoglycosylceramide, such as β-galactosylceramide (GalCer) or β-glucosylceramide (GlcCer) (Figure 1.2). The ceramide moieties in fungal cerebrosides differ structurally in a number of ways that distinguish them from mammalian GSLs. (E)-Δ^8 unsaturation and 9-methyl group branching of the

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sphingoid base as well as variable levels of (E)-Δ^3 unsaturation of the fatty acid are common. Ceramides of fungal cerebrosides are also different from those found in fungal GIPCs suggesting that their assembly is from structurally distinct pools of sphingoids. In animals, galactose is the common monosaccharide though this may vary with the tissue, and the sphingoid is usually sphingosine or dihydrosphingosine.

Glycosylation of ceramide to yield GlcCer or GalCer is of significant importance to eukaryotes. It has been proposed, for example, that it protects the mammalian cells from cancer drug-triggered apoptosis. Overexpression of GlcCer synthase in MCF-7 breast cancer cells showed resistance to adriamycin and TNF alpha while in multidrug-resistant cells, accumulation of GlcCer was observed. Expression of GlcCer is particularly widespread, and inhibition of GlcCer synthase has also been shown to be fatal for *A. nidulans* and *A. fumigatus*. This suggests that the synthesis of GlcCer is essential for growth in fungi. Most importantly, in mammals GlcCer and GalCer are intermediates in the biosynthesis of a wide variety of more complex GSLs. In fungi, by contrast, these are generally accumulated as terminal products.

A unique class of GSLs found in plants, yeasts, filamentous fungi and protozoans, based on the glycosylation of myo-inositol-1-phosphoryl-1-ceramides (IPC), has hitherto not been found in animal cells, which apparently lack the capacity to synthesize IPC, the obligate intermediate in glycosylinositolphosphoryl ceramide (GIPC) biosynthesis (Lester and Dickson 1993). GIPCs are a class of acidic GSLs widely distributed among fungi and not in mammalian cells or tissues. This group of sphingolipids have also been detected in protozoa (including the soil amoeba *Acanthamoeba castellanii*), plants, and in parasitic nematode *Ascaris suum* (Sugita et al., 1996). Researchers have shown that
fungal GIPCs (or its metabolites) can interact with the mammalian immune system, in particular where they present oligosaccharide epitopes not shared with mammalian glycoconjugates (Lester and Dickson 1993).

Sphingolipid synthesis begins with the condensation of palmitoyl-CoA and serine in the endoplasmic reticulum (Scheme 1.1). This essential irreversible reaction occurs in all eukaryotes and is catalyzed by the enzyme serine palmitoyl-transferase (SPT) to generate the intermediate 3-ketodihydrosphingosine (3-ketosphingosine). Three genes are required for the optimal activity of SPT in *S. cerevisiae*, LCB1, LCB2 and TSC3, which is essential only at elevated temperatures. Other eukaryotes have homologues of LCB1 and LCB2 and mutations in the mammalian LCB1 homologue (SPTLC1) has been linked to hereditary sensory neuropathy type 1 (Dickson and Lester, 2002, Dawkins et al., 2001 and Bejaoui et al., 2001) suggesting that the synthesis of sphingolipids is regulated in the neurons. The second step involves the reduction of 3-ketosphingosine to the long chain base, erythro-dihydrosphingosine (DHS, sphinganine) by 3-ketosphingosine reductase, an enzyme encoded by the gene TSC10 (Dickson and Lester 2002, Obeid et al., 2002). NADPH is also needed for this reaction. The catalytic effects of Tsc10p, a membrane-bound protein, is essential for this step and its expression in *Escherichia coli* confirms that NADPH is required for the reduction of 3-ketosphingosine to DHS. The third step involves the formation of a ceramide, a sphingoid base amide linked to a fatty acid chain. Mammalian cells N-acylate DHS using ceramide synthase to generate ‘dihydroceremide’; this is later oxidized by the introduction of a trans-4,5 double bond to form ‘ceramide’. A first branching point in fungal sphingolipid synthesis is observed after the generation of DHS. Instead of being converted to ceramide, the long chain base can be hydroxylated to
yield phytosphingosine (PHS) and then N-acylated to form 'phytoceramide', the type of ceramide found in inositolphosphorylceramide (IPC), as shown in Scheme 1.1.

*S. cerevisiae* can only hydroxylate DHS at C-4 to form phytosphingosine (PHS), so this is the primary sphingoid base observed in all these cells (85% PHS vs 15% DHS in yeast) (24). Hydroxylation of DHS is catalyzed by SUR2, an enzyme found not to be necessary for growth. Addition of long chain fatty acids, commonly 16-24 carbons, to DHS or PHS then follows, and is catalyzed by the enzyme ceramide synthase. This enzymatic activity is encoded by the two genes LAC1 and LAG1; the initial product referred to as N-acylphytosphingosine (Cer-1). This ceramide can then be α-hydroxylated on the fatty acid moiety to form phytoceramide (Cer-2); this activity is encoded by the SCS7 gene, and has been found to take place in the endoplasmic reticulum of yeasts. Further hydroxylation on the fatty-N-acyl 3-carbon yields Cer-3 (not shown).

In Plants and fungi, addition of phosphorylinositol to form inositolphosphorylceramide (IPC) is an essential step. The inositol phosphate group of phosphatidylinositol is transferred to the C-1 OH group of ceramide by IPC synthase, a membrane-bound enzyme that is encoded by the AUR1 gene, and inhibition or disruption of IPC synthase has been found to be fatal for most fungi. In *S. cerevisiae*, for example, mutants that do not synthesis IPC are not viable (Dickson and Lester 2002; Nagiac et al., 1997), and pathogenic fungi are killed when treated with compounds such as khafrefungin (Mandela et al., 1997), rustmicin (Mandela et al., 1998) and aureobasidin A (Takesako et al., 1993; Zhong et al., 2000), inhibitors of IPC synthase. As such, enzymes catalyzing the synthesis of phosphorylinositol-containing sphingolipids have been considered potential targets for antifungal drugs.
Scheme 1.1 Biosynthetic pathway in the yeast *S. cerevisiae* starting with palmitoyl CoA and serine and ending at the MIPC. The structure of the head-group of M(IP)_{2}C has been omitted in this scheme.
In yeast, IPC is then mannosylated to yield mannose inositol-P-ceramide (MIPC) a reaction that requires two genes: \textit{CSG1} and \textit{CSG2}. Mutants in either gene are viable, but their deletion prevents MIPC formation and causes IPC accumulation. This step appears to be shared by most other fungi.

The derivatives of IPC, mannose-inositol-phosphorylceramide (MIPC), and mannose-di(inositolphosphoryl)-ceramide (M[IP]$_2$C) were first discovered in \textit{S. cerevisiae} as the major GIPCs (Smith and Lester, 1974). Since then, many other studies performed by different groups have provided insight into the structures of GIPCs found in other fungi. Detailed knowledge of GSL structure and biosynthesis is expected to yield valuable insights into both their functions within the organism and their interactions with the host immune system, as well as to provide targets for diagnostic and therapeutic agents. It has been proposed that enzymatic digestion of the fungal cell wall takes place when fungal cells come into contact with that of the host’s. This consequently exposes the antigens of the cell membrane and cytosolic contents. GSLs have been shown to trigger strong immunological responses in the host when they present different structural features from those of the host (Kannagi, R., and Hakomori, S., 1986). GIPCs from fungal cell membranes almost always present glycan structural features distinct from those of mammals, and can therefore be expected to produce some immunological response. Sera of patients with aspergillosis, for example, exhibit strong a reaction with GIPCs of \textit{A. fumigatus} (Toledo et al 2007). It is envisaged that systematic isolation and characterization of fungal GSLs will help elucidate their functional roles as well as help in understanding their impact in the host’s immune system. Research described in this thesis focused on fungal GSL structure and biosynthesis. Two projects are described. The
first was aimed at elucidating the functions of GSLs in *Neurospora crassa*. The isolation and structural analysis of GIPCs from the filamentous fungus *N. crassa* wild type (WT) and a defensin-resistant mutant strain were attempted using NMR, MS and other techniques. In addition, a preliminary study of the effects of an inhibitor of GSL biosynthesis, 4'-ethylenedioxy-1-phenyl-2-palmitoyl-3-pyrrlidinopropanol (EDO-P4), was performed. In the second project, studies of the structures and biosynthesis of GSLs of the pathogenic model fungus, *Cryptococcus neoformans* were performed on both the wild type (JEC 21) and a mutant, termed *cxt1cxt2Δ*, in which the genes for *C. neoformans* β1,2-xylosyltransferases, Cxt1 and Cxt2 had been disrupted.
CHAPTER II

STUDIES OF THE STRUCTURES AND FUNCTIONS OF GLYCOSPHINGOLIPIDS OF THE SAPROPHYTIC MODEL FUNGUS, NEUROSPORA CRASSA

Introduction

Fungi, plants and animals represent three phylogenetic kingdoms within the eukaryotes (non bacteria) having similar cellular, genomic and metabolic organizations. Fungi rank in importance with bacteria as ubiquitous agents in the biosphere for biochemical transformation and environmental housekeeping. In addition, as with bacteria and viruses, fungi take part in a multitude of complex interspecies interactions as either symbionts or pathogens. As pathogens (http://biology.unm.edu/biology/ngp/Whitewater.html, 2005), fungi can be problematic for development of therapeutic reagents, because of the genetic similarity shared among eukaryotes. These shared characteristics, on the other hand, mean that fungi can in many respects be used as models for studying cellular processes in other eukaryotes, where the degree of genetic and functional similarity is high. For example, like plants and animals, the saprophytic filamentous fungus Neurospora crassa exhibits circadian rhythms, and thus the sequencing and functional analysis of the Neurospora genome should contribute to the understanding of this phenomenon. In general, the numerous genes identified by function in N. crassa have enhanced the determination and interpretation of other
eukaryotic genomic DNA sequences. Although it is not considered a pathogen (Ferket, et al., 2003), a number of genes in *N. crassa* are similar to those identified in other fungi as being required for plant pathogenesis. This makes *N. crassa* a suitable model for studying some plant-phytopathogen interactions, including those involving plant-derived antimicrobial peptides called defensins that target fungal membrane components.

Defensins are small (45-54 amino acids), highly basic, cysteine-rich peptides found in a variety of eukaryote species, including plants, and are one family of antimicrobial peptides either synthesized in direct response to pathogenic attack or constitutively expressed in certain tissues and released or dispersed as a consequence of microbial attack or injury. Defensins are also found in other type of organisms (Thomma, B.P et al., 2002), including insects and humans, and are important components of innate immunity. Plant defensins are thought not to be toxic to either mammalian or plant cells, and some of these defensins do not display any antimicrobial activities, while others demonstrate anti-fungal and antibacterial activities in vitro. Among those exhibiting anti-fungal activities, some may have hyper branching effects on specific target fungi (Ferket, et al., 2003), while others inhibit growth of fungi without causing morphological changes. Studies of *Dahlia merckii* (dahlia) and *Raphanus sativus* (radish) defensins indicate that they induce rapid responses in fungal membranes, such as alteration of normal membrane potential and membrane-permeabilization. Some plant defensins have been shown to induce rapid increase of potassium efflux and calcium uptake in *N. crassa* hyphae. Plant defensin membrane permeabilization of *N. crassa* and *S. cerevisiae* cells has been correlated with inhibition of growth in these species (Thomma et al., 2002; Park et al., 2005; Thevissen et al., 1996).
The existence of high affinity binding sites for these defensins on fungal cells and plasma membrane fractions has been demonstrated (Park et al., 2005; Thevissen et al., 1996; Thevissen et al., 1998). Considerable evidence has accumulated that in some cases these binding sites are GSLs, or at least include GSLs as essential components (Ferket et al., 2003; Thomma et al. 2002). Mannosylinositol phosphorylceramide (MIPC) and mannose-di(inositolphosphoryl)-ceramide (M[IP]₂C) are the major GIPCs in *S. cerevisiae*, and the latter is thought to be a target of the *D. merckii* defensin DmAMP1. On the other hand, the *R. sativus* defensins RsAFP1 and RsAFP2 appear to interact with GlcCer in *N. crassa*.

Previous studies suggested that there were major differences between wild type (WT) and mutant *N. crassa* strains with respect to expression of both neutral and acidic GSLs (Park et al., 2005). Characterization of the neutral GSL, β-glucosyl-ceramide, as well as a sterol glycoside, 3-O-β-glucosyl-ergosterol, from both strains, has been carried out previously and several differences were identified. These differences correlate with the resistance of the mutants to RsAFP2 and other defensins, but the causal relationship of these changes, if any, to defensin resistance remains to be elucidated. In the current study, it was expected that the *N. crassa* model would be used further to elucidate functions of GSLs in two ways. First, the isolation and structural analysis of GIPCs from *N. crassa* WT and defensin-resistant mutant strains were to be carried out using NMR, MS and other techniques. Secondly, a study of the functions of GSLs in *N. crassa* using an inhibitor of GSL biosynthesis, 4’-ethylenedioxy-1-phenyl-2-palmitoyl-3-pyrrplidinopropanol (EDO-P4), was to be performed. EDO-P4 is an inhibitor of glucosylceramide synthase (GCS). The inhibitor experiment would allow correlation of
biological effects with changes in expression of GSLs, other lipids, and proteins. In order to carry out this study, it was first necessary to determine the viability of the WT strains when challenged with increased EDO-P4 concentrations. The point was to determine a sub-lethal dose that would enable us to study the effects of GlcCer depletion on lipid and protein expression. The hypothesis is that the affected proteins and lipids will be known components of transmembrane and other signaling pathways. Preliminary results are reported herein.

**Materials and Methods**

**Fungal isolates and growth conditions**

Wild type isolates of *N. crassa* strains (ATCC numbers 18889 and 24698), were each dispersed in approximately 30 mL sterile deionized water in 100 mL bottles and left to stand overnight in a biological safety cabinet at room temperature. 1 mL of each, in suspension, was inoculated to six plates containing solid potato dextrose agar (PDA, 39g/L). The plate cultures were incubated for 4-5 days at room temperature. Cultures of a mutant strain termed SP10 were already plated prior to this work, by Steven B. Levery as described above. For the purpose of lipid extraction, the plate cultures were transferred to 500 mL liquid potato dextrose broth (PDB 39g/L) media in 1 L Fernbach flasks and shaken at 25°C, 250 rpm, for 24 h. Aliquots of each culture were kept at 4°C and used for re-plating purposes every three months.

Fresh 10 mL aliquots of each strain in 24 h liquid culture were then inoculated to four flasks each containing 1 L of PDB medium, and left to shake at 25°C, 250 rpm, for
2-3 days. Mycelia were harvested by filtration through cheese cloth, washing off excess media with deionized water. Excess water was removed by gentle pressure and mycelia were either stored at –80°C until extraction, or processed immediately as described below.

**Solvents for extraction, anion exchange chromatography, and high performance thin layer chromatography**

Solvent A, chloroform/methanol (1:1; v/v); Solvent B, isopropanol/hexane/water (55:25:20 v/v/v, upper phase discarded); solvent C, chloroform/methanol/water (30:60:8 v/v/v); solvent D, isopropanol/hexane/water (55:40:5 v/v/v); solvent E, chloroform/methanol/water (60:35:5 v/v/v); solvent F, chloroform/methanol/water (50:47:14 v/v/v, containing 0.035% w/v CaCl₂). All solvents were HPLC grade.

**Extraction and fractionation of glycosphingolipids**

Extraction and purification of glycosphingolipids were carried out as described previously with minor modifications. Briefly, GSLs were extracted by homogenizing mycelia (40-80g wet weight) in a glass-walled blender, once with 200 mL of solvent A, two times with 200 mL of solvent B and once more with 200 mL of solvent A. The four extracts were pooled and dried on a rotary evaporator at 40°C. The extracted lipids were then transferred to a 16x100 screw cap test tube, conserving all material by repeatedly (3x) adding ~ 4 mL of solvent B, sonicating for ~ 2 min, and transferring. The lipids were dried after each transfer under N₂ stream at 35-40°C. A final transfer was made using solvent A. The dried crude samples of the two WT strains were each taken up in 2 mL of
solvent C, sonicated, centrifuged, and analyzed by HPTLC (silica gel 60; solvent E) as described below.

Crude total lipids from each strain were separately dried down under N₂-stream, re-suspended in solvent C, and applied to a column of DEAE-Sephadex A-25 (Ac-form, in solvent C). Neutral lipids, including putative glucosylceramides (GlcCer) and steryl glucosides (GlcSte) were eluted with five bed volumes of solvent C, dried, taken up in 1 mL of solvent D and analyzed by HPTLC (solvent E). Acidic GSLs were eluted with five bed volumes of 0.5M sodium acetate in methanol, dried, resuspended and dialyzed for 18h against deionized water, changing the water every 6h. The lipid suspension was then recovered, redried, taken up in 1 mL of solvent B, and analyzed by HPTLC (solvent F).

**High performance thin layer chromatography (HPTLC)**

All HPTLC analysis was performed on silica gel 60 HPTLC plates (E. Merck, Darmstadt, Germany). The lipid samples were applied by streaking from 5 μL Microcaps (Drummond, Broomall, PA). For analytical HPTLC, detection was made by Bial’s orcinol reagent (orcinol 0.55% [w/v] and H₂SO₄ 5.5% [v/v] in ethanol/water 9:1 [v/v]; the plate was sprayed and heated briefly to ~200-250°C; violet staining is positive for the presence of hexose). In some cases, a preliminary observation was made by a lipid-specific stain, primulin (Aldrich; 0.01% in 80% aqueous acetone, observed under uv-light), prior to orcinol detection. Any coloration that appeared bright blue/purple, confirming the presence of lipids, was circled, and the plate sprayed further with orcinol for analytical comparison. In other cases, the acidic samples were subjected to staining by
Dittmer-Lester reagent, a molybdenum spray specific for phospholipids (any dark blue coloration against a light blue background confirms the presence of phosphate) (50).

**High performance liquid chromatography (HPLC)**

Normal phase preparative HPLC was performed on 5 μ micron porous silica (250 x 4.6 mm Sphereclone, Phenomenex, Torrance, CA). The mobile phase was a 2-propanol-hexane-water gradient programmed from 55:40:5 to 55:25:20 (v/v/v) over 120 min, followed by isocratic elution for 40 min; flow rate, 0.5 mL/min. The injection volume was 1.0 ml and generally, 160 x 0.5 mL fractions were collected in each run and the identity and purity of each fraction were assessed by analytical HPTLC (solvent F). Fractions that displayed comparatively similar HPTLC Rf-values were combined in 16x100 screw cap test tubes, dried under N₂, and prepared for further analysis by 1-D ¹H-NMR spectroscopy (as described below).

**¹H-Nuclear magnetic resonance spectroscopy (NMR)**

Samples of underivatized acidic lipid fractions (~ 0.5–1.0 mg) were deuterium exchanged (3x) by repeated evaporation from 99.98% D₂O using a rotary evaporator, and then dissolved in 0.5 mL DMSO-δ6/2% D₂O for 1-D ¹H-NMR analysis. Spectra were acquired at 35°C on a Varian Unity Inova 500 MHz spectrometer using standard acquisition software available in the Varian VNMR software package.
Mass spectrometry (MS)

Mass spectrometry was performed in the positive ion mode via matrix assisted laser desorption ionization (MALDI), and electrospray ionization-mass spectrometry (ESI-MS). Briefly, ESI-MS was performed on a Thermo-Finnegan (San Jose, CA) LCQ with a Pico-View nanospray source (New Objectives, Inc., Woburn MA) and LCQ-Tune acquisition software. Dried GSL samples were dissolved in methanol, loaded into a 100 μL syringe and introduced into the instrument by direct infusion through a nanospray capillary tip at an estimated flow rate of 0.5 μL/min. Lithium iodide (LiI) (10 mM in methanol) was added to samples for the purpose of generating [M(Li)+Li]+ adducts of the molecular species. LiI was added until the observed ratio of [M(Li)+Li]+ adducts to mixed Na+/Li+ adducts in the MS profile mode was approximately > 5:1. Nominal, monoisotopic m/z values are used in the labeling and description of the ESI-MS spectra, which represent a summation of 50-150 scans (30 scans /min) for both MS profiles of [M(Li)+Li]+ species and MS/CID MS analysis.

Lipidomic analysis of the effects of glucosylceramide synthase inhibitor on N. crassa

A few modifications were made to the procedures described above in order to determine the viability of the N. crassa WT strain ATCC 18889, when challenged with increased EDO-P4 concentrations. Briefly, mycelia from plated cultures of ATCC 18889 were harvested and transferred to 500 mL liquid potato dextrose broth (PDB 39g/L) media in 1L Fernbach flasks and shaken at 25°C, 250 rpm, for 48 h. Four flasks each containing 1L PDB medium was prepared. GlcCer synthase inhibitor EDO-P4 dissolved in ethanol (4mmol/L), was added to the four flasks consistent with the concentrations 0, 0.5, 1.0 and 20.0μmol/L respectively. 10 mL aliquots of ATCC 18889 were then added into each
flask and were left to shake at 25°C, 250 rpm, for 2-3 days. Mycelia from each flask were harvested, and the extraction, purification and HPTLC analysis of glycosphingolipids were carried out in parallel as described earlier.

Results and Discussion

A. Detection, isolation and structural analysis of *N. crassa* WT and mutant GIPCs resistant to plant defensins

HPTLC and NMR results

The HPTLC profiles from the crude total lipids obtained from *N. crassa* wild type strains ATCC 24698 and 18889 are represented in Figure 2.1. As expected, GlcCer was initially detected in all the crude total lipid fractions, recognized in the HPTLC analysis by their orcinol staining and comigration with an authentic standard, and subsequently in all the neutral fractions (Figure 2.2) from the WT *N. crassa* strains. There did not appear to be any striking quantitative differences between the two WT strains. A component characterized previously as sterol glycoside having an Rf-value just above GlcCer, is known to have been present in low concentrations in WT *N. crassa*, and is barely detectable in these HPTLC profiles. A number of other orcinol+ bands (with lower Rf values than GlcCer), which did not co-migrate with any standard compound, were observed in these experiments. The neutral fractions from ATCC 18889 that were stained with primulin and subsequently by orcinol (Figure 2.3), showed the presence of glycolipids at two different Rf-values (bands 1 and 2), besides GlcCer. Some orcinol-positive but primulin negative bands were also detected at lower Rf-values and were
tentatively proposed to be free oligosaccharides. The neutral fractions obtained from the ATCC 18889 and SP 10 were then fractionated by preparative-scale HPLC and those compounds with similar Rf-values from HPTLC analysis were combined for further analysis. The components isolated by HPLC were characterized initially using 1-D $^1$H-NMR and MS, and the identity of a combined neutral fraction (9-12), confirmed as GlcCer similar to authentic GlcCer, was in agreement with previously observed results (Park et al., 2005). Reproduced in Figure 2.4 Panel A, is the HPTLC profile of the crude acidic lipid fractions from N. crassa strains ATCC 18889, 24698 and SP 10, stained with orcinol for detection of hexose-containing material, compared with a reference standard containing MIPC from P. squamosus.

![HPTLC profile](image-url)

**Figure 2.1** Profiles of crude lipids from N. crassa WT detected using orcinol. Lanes 2 and 3 represent the ATCC 18889 strain while lanes 4 and 5 represent ATCC 24698 strain. Standards of MIPC (from *Polyporus squamosus*) and GlcCer (from *Candida albicans* yeast) are represented in lanes 1 and 6.
Figure 2.2 Profiles of crude neutral lipids from \textit{N. crassa} WT detected using orcinol. Lanes 1, 2 and 3 represent three different batches of the ATCC 18889 strain, while lane 4 represents the ATCC 24698 strain. GlcCer standard (from \textit{Candida albicans} yeast) lane 5 was used for comparison.

Two orcinol stained bands appeared to co-migrate with MIPC in all the three strains. These appeared in considerably lower abundance than previous analyses,
however (Park et al., 2005). The crude acidic lipid fractions were then fractionated by preparative-scale HPLC. Analytical HPTLC of the fractions, using orcinol and Mo spray was used for detection of the relevant compounds, and those with similar Rf-values were combined for further analysis.

The partial profile of the HPLC purified acidic fraction number 43-47 from the ATCC 18889, (Figure 2.4 Panel B), stained with Mo spray shows the presence of an acidic phospholipids (a similar trend was observed in the HPTLC profiles of purified SP 10 acidic lipids, data not shown). These fractions were combined and subjected to further analysis by $^1$H-NMR spectroscopy.

Figure 2.4 Profiles of acidic lipid fractions from *N. crassa* WT. Panel A, detected using orcinol. Lanes 2, 3 and 4 represent ATCC 18889, 24698 and SP10 strains respectively. MIPC standard (from *P. squamosus*) was used for comparison. Panel B, Lanes 2-6, consecutive fractions 43-47 from HPLC purified *N. crassa* (ATCC 18889) acidic lipids, detected using Mo spray. These were combined for further analysis.
The 1-D $^1$H-NMR spectrum (DMSO-$d_6$/2% D$_2$O; 35°C) of acidic fraction 43-47 of the ATCC 18889 WT strain is shown in Figure 2.5. Our preliminary assessment was that it was not a GIPC, but phosphatidylinositol (myo-inositol-1-phosphoryl-diacyl glycerol). In the 2-D TOCSY (Appendix B) spectra, spin systems characteristic for myo-inositol, glycerol, and fatty acyl groups were detected; no resonance characteristic for a sphingoid moiety were observed. In the absence of GIPCs from the WT and mutant strains, no further analysis was made using MS of the acidic fractions.

![Figure 2.5](image)

*Figure 2.5* 1-D $^1$H-NMR spectrum (DMSO-$d_6$/2% D$_2$O; 35°C) of the acidic fractions 43-47 of the ATCC 18889 wild type strain.
B. Lipidomic analysis of the effects of glucosylceramide synthase inhibitor on \textit{N. crassa}

HPTLC results on the effect of EDO-P4 addition on GlcCer expression

In order to determine a sub-lethal dose that would enable us study the effects of GlcCer depletion on lipid and protein expression, it was first necessary for us to determine the viability of the WT strain ATCC 18889 when challenged with increased EDO-P4 concentrations (0 μM, 0.5 μM, 1.0 μM and 20 μM). There was an inverse relationship between the increasing concentrations of EDO-P4 and the wet weights of mycelia that were harvested after 48 hours. The wet weights of the mycelia were 4.13g, 2.83g, 1.60g and 0.63g for the inhibitor concentrations 0, 0.5, 1.0 and 20 μM respectively. This information is presented graphically in Figure 2.6. These results clearly indicate that EDO-P4 has an effect on viability of \textit{N. crassa}.

![effect of inhibitor concentration on fungal mass](image)

Figure 2.6 A graph relating the wet weight of the mycelia obtained from \textit{N. crassa} WT 18889 and increasing concentrations (0 μM, 0.5 μM, 1.0 μM and 20 μM) of EDO-P4
The HPTLC profiles of crude total lipids in which a range of EDO-P4 concentrations was added can be found in Figure 2.7. We observed a band (band 3) in all samples that co-migrated with the authentic GlcCer standard, although it’s staining appeared at decreasing intensities as EDO-P4 concentrations in the media increased. Interestingly enough, certain bands with low mobility (bands 4 and 5), that tested positive with orcinol staining showed an increase in staining intensity with increase in inhibitor concentration. Additionally, the lowest migrating compound, (band 6) was also orcinol positive but the staining intensity of the bands decreased with increased EDO-P4 concentrations.

In order to analyze further the effect of EDO-P4 on the lipid components, each of the four total lipid extracts, representing growth at different concentrations of EDO-P4, were fractionated using DEAE sephadex-anion exchange and further analyzed by HPTLC (Figure 2.8).

![Figure 2.7](image)

**Figure 2.7** Profiles of crude lipids from *N. crassa* ATCC 18889 detected using orcinol. Lanes 2 and 3 represents lipids form original culture, lanes 4, 5, 6 and 7 represent inhibitor concentration 0 μM, 0.5 μM, 1.0 μM and 20 μM respectively. The streaked amounts were adjusted according to wet weight mycelia.
The HPTLC profiles exhibited a significant decrease in the amount of GlcCer (band 3) in samples that had been inoculated with EDO-P4 compared to the control (without inhibitor), with a noticeable decrease in the amount of GlcCer with increased inhibitor concentrations. This decrease in band intensities suggests that the GCS inhibitor, EDO-P4 developed for the mammalian enzyme, also affects GlcCer synthesis in fungi. The migration of bands marked 4, were similar to those found in the crude total lipid (bands 4 and 5). These bands having low mobility tested positive with orcinol staining and showed an increase in the amount of components with increase in inhibitor concentration. This may have been as a result of accumulation of metabolic intermediates upstream of the blocked biosynthetic stage. We did not observe a consistent trend with the lowest migrating compounds, bands 5 and 6 (that were also orcinol positive) with increased EDO-P4 concentrations. These could be secondary effects of GlcCer depletion or Cer accumulation, mediated by signaling networks dependent on normal GlcCer metabolism. It is also possible that EDO-P4 has direct effects on other enzymes not related to GCS.

The HPTLC profiles of the crude acidic fractions obtained from ATCC 18889 are represented in Figure 2.9. Although there were several bands that were orcinol positive, none of these co-migrated with a fungal Mana2InsPCer (MIPC) standard. Notably, the set of bands marked 2 increased in intensity as inhibitor concentration increased. This may suggest that there are shared intermediates in the biosynthetic pathways of the two fractions (neutral and acidic) and that the inhibition of GCS may have the effect of increasing one or more intermediates in the biosynthetic pathways of the acidic fraction.
Figure 2.8 Profiles of crude neutral lipids from *N. crassa* ATCC 18889 detected using orcinol. Lanes 2, 3, 4 and 5 represent inhibitor concentration 0 μM, 0.5 μM, 1.0 μM and 20 μM respectively.

Figure 2.9 Profiles of crude acidic lipids from *N. crassa* ATCC 18889 detected using orcinol. Lanes 2, 3, 4 and 5 represent inhibitor concentration 0 μM, 0.5 μM, 1.0 μM and 20 μM respectively.
These could also be secondary effects of altered metabolism in cells responding to the GCS inhibition. It is also possible as mentioned earlier, that the direct effects of EDO-P4 are not confined to GCS. Results from HPTLC done using Mo-staining (results not shown), were consistent with those of orcinol staining. No bands co-migrated with the authentic MIPC standard.

While isolation and analysis of GlcCer from fungi is straightforward, direct assays for GCS activities are more difficult to establish (Levery et al., 2003). The effects of EDO-P4 on the biosynthetic pathway of GlcCer synthesis may for now be determined by analyzing the levels of this cerebroside in the presence or absence of sub-lethal amounts of inhibitor. While the results of this experiment were consistent with inhibition of GCS by EDO-P4, there were effects on the concentrations of other components in its presence. These compounds may be competing for the same ceramide acceptor substrate, although a variety of other explanations have not been ruled out, including the possibility that EDO-P4 affects the function of other enzymes in fungi that are not affected in mammals. Some observed changes could be secondary effects of GlcCer depletion or ceramide buildup. The results of this project appear to be in agreement with those observed with other fungal models (namely *A. fumigatus* and *A. nidulans* (Levery et al., 2003)), suggesting that EDO-P4, an inhibitor of mammalian GCS, could be a useful tool in the analysis of the physiological functions of GCS in fungi, and may provide important target for development of novel classes of antifungal agents, especially as its function(s) become better understood.
CHAPTER III

STUDIES OF THE STRUCTURES AND THE BIOSYNTHESIS OF GLYCOSPHINGOLIPIDS OF THE PATHOGENIC FUNGUS, CRYPTOCOCCUS NEOFORMANS

Introduction

Over the last two decades there have been rising incidence rates of fatal mycosis associated with the growing population of immunosuppressed or -compromised individuals, including those with acquired immune deficiency, organ and tissue transplant recipients as well as certain cancer cases. Despite attempts at development of new models for fungal pathogenicity and strategies for diagnosis and treatment, reports on the resistance of mycotic infection to available antifungal agents are on the increase. This is correlated with the emergence of non-susceptible variants within populations of sensitive strains. Coupled with the fact that many of the drugs available to treat fungal diseases are difficult to administer, are not effective, or are toxic, the emergence of resistant strains has made opportunistic fungi an increasing threat to immunosuppressed individuals.

Cryptococcus neoformans is a basidiomycetous fungus ubiquitous in the environment, often found in soil, in association with certain trees, and in bird guano (Peled et al., 2001). As an opportunistic fungal pathogen, it can infect humans and other mammals. Infection begins with inhalation of airborne spores or yeast cells that infiltrate the alveoli in the lungs, where it remains dormant in individuals with a competent immune system until an immune imbalance triggers further development. Further
dissemination takes place in immunocompromised hosts, especially those with AIDS, with a particular attraction to the central nervous system, where it causes meningoencephalitis, a fatal condition if left untreated. (Bose et al., 2003). Even when treated with the most effective antifungal drugs, cryptococcal infections can be fatal if the host does not have adequate T-cell dependent immune function (Mitchelle et al., 2006). The pathogenicity of \textit{C. neoformans} is dependent on various factors. The most notable of these include the large polysaccharide capsule; the small cell diameter of 2.5 - 10\textmu m that enable the yeast cells to penetrate the alveolar spaces of the lungs, where it establishes a pulmonary infection; the synthesis of melanin; and its ability to grow at 37\textdegree C, the normal temperature within a human host (Moyrand et al., 2002; Klutts et al., 2007). It has been demonstrated that melanin protects the fungus from the host immune response, and melanin-deficient mutants of the fungus are avirulent in animals. Although there are various factors that make \textit{C. neoformans} a pathogen, its distinguishing feature, the extensive polysaccharide capsule that surrounds its cell walls, is strictly required for its virulence in humans and other mammals (Peled et al., 2001). Strains that lack this capsule have been shown to be avirulent in animal models, and thus the synthesis of this polysaccharide structure can be targeted therapeutically. Three components constitute the major part of this enormous capsule. Two xylose-containing polysaccharides, namely glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) (Figure 3.1), make up about 97\% of the capsule mass, while a third component, a polysaccharide conjugate called mannoprotein, constitutes the remaining part (Bose et al., 2003).

GXM is a polymer whose backbone consists of mannose residues that are $\alpha$-1,3-linked. This is glycosylated with xylose and glucuronyl side groups the ratio of
xylose/glucuronic acid/mannose range from 1:1:3 to 4:1:3, depending on the strain, thus accounting for four known cryptococcal serotypes, A-D. The backbone of GalXM is built on an α-1,6-linked galactose polymer glycosylated with variable side chains containing galactose, mannose and xylose residues (Peled et al., 2001; Bose et al., 2003; Klutts et al., 2007).

![Figure 3.1](image_url)

**Figure 3.1** Structures of the cryptococcal capsule components. (A) GXM of serotype B; (B) GalXM. The structures show the linkages between sugars where blue, mannose; gray, glucuronic acid; red, xylose; and green, galactose. (39).

Three biosynthetic precursors are required for the synthesis of GXM, namely GDP-mannose, UDP-xylose, and UDP-glucuronic acid; GalXM also requires UDP-galactose. Studies have shown that UDP-xylose (UDP-Xyl) is not only a biosynthetic precursor, but is also significant for conferring virulence via incorporation into GalXM and the four serotypes of GXM. Mutant strains that lack UDP-xylose cannot form the capsule and are avirulent in mice. Although the entire biosynthetic pathway of the capsule is yet to be elucidated, it has been established that the construction of GXM and
GalXM require an array of glycosyltransferases, and up to seven xylosyltransferases have been found (Klutts et al 2007).

Recent studies on fungal GIPCs obtained from *C. neoformans*, have reported the expression of a variety of xylose branched glycan structures for the first time (34, 40). Mendonca-Previato and co-workers recently showed, structural variation between The GIPCs of the wild type (KN99a strain) and mutant strains that lack UDP-xylose, brought about by disruption of either UDP-glucose dehydrogenase (NE321 strain) or UDP-glucuronic acid decarboxylase (NE178 strain). The structures of the GIPCs they characterized are represented in Figure 3.2 below. This study further showed that mutants that lacked the xylose side chain were unable to complete the elongation of the GIPC glycans suggesting that this residue is required for further mannosylation of the core glycosylinositol moiety (Gutierrez et al., 2007).

A. KN99 a

\[
\begin{align*}
\beta-Xylp \\
1 \\
\downarrow \\
2 \\
\alpha-Manp-(1\rightarrow6) - \alpha-Manp-(1\rightarrow3) - \alpha-Manp-(1\rightarrow4) - \beta-Galp-(1\rightarrow6) - \alpha-Manp-(1\rightarrow2)-Ins-1PCer
\end{align*}
\]

**Figure 3.2** Structures of PI-oligosaccharides of GIPCs isolated from *C. neoformans*. (A) WT (KN99 a); (B) and (C) mutant strains (NE321 and NE178) that lack UDP-xylose.
Figure 3.2 Continued

B. NE321

\[ \alpha\text{-Manp-(1\rightarrow3)}\alpha\text{-Manp-(1\rightarrow4)}\beta\text{-Galp-(1\rightarrow6)}\alpha\text{-Manp-(1\rightarrow2)}\text{-Ins-1PCer} \]

\[ \alpha\text{-Manp-(1\rightarrow2)}\alpha\text{-Manp-(1\rightarrow4)}\beta\text{-Galp-(1\rightarrow6)}\alpha\text{-Manp-(1\rightarrow2)}\text{-Ins-1PCer} \]

C. NE178

\[ \alpha\text{-Manp-(1\rightarrow2)}\alpha\text{-Manp-(1\rightarrow4)}\beta\text{-Galp-(1\rightarrow6)}\alpha\text{-Manp-(1\rightarrow2)}\text{-Ins-1PCer} \]

The striking similarities between the backbone of GXM, GalXM and the GlPC structure of \textit{C. neoformans} WT is of significant interest to this research. Further, pathogenic fungi have been killed by inhibitors of inositol phosphoryl ceramide (IPC) synthase (Heise et al., 2002), suggesting that the biosynthesis of phosphoryl containing sphingolipids is a promising target for the development of more effective antifungal drugs. The focus of this research is in the studies of the structures and biosynthesis of glycosphingolipids of the pathogenic model fungus, \textit{C. neoformans}. Isolation and structural analysis was performed on both the WT and the mutant termed \textit{cxt1cxt2}A, in which the xylosyltransferases, Cxt1 and Cxt2 have been disrupted. The structural analysis was accomplished by a variety of techniques including HPLC, NMR and MS.

**Materials and Methods**

All experimental methods conducted in this chapter are similar to those in chapter 2 part A but with minor additions. First, all solvents were the same with an addition of solvent G, saturated Butanol; H, methylamine, methanol/water/n-butanol (4/3/1 v/v/v).
Second, cells of *C. neoformans* WT (JEC 21) and mutant (cxt1cxt2Δ) were obtained from our collaborators. Extraction and purification of glycosphingolipids were carried out as described previously with minor modifications. Briefly, frozen cells were removed from the freezer and left to sit at room temperature for about five minutes in order to just thaw. The cells were then immediately transferred to a glass-walled blender and GSLs were extracted by homogenizing yeast cells ~40-80g with 200 mL of solvent A. The extract was then dried on a rotary evaporator at 40°C. This was followed by butanol extraction and base, amine treatment.

**Butanol extraction**

The dried crude lipid was partitioned between water and butanol pre-saturated with water as follows. 200ml of distilled water and 200ml of saturated butanol were added to the crude total lipid in the round bottomed flask. The mixture was shaken vigorously for about five minutes and eventually transferred into a separating funnel and the aqueous layer that settled at the bottom was collected into the original flask and similarly extracted four more times with equal volume of water saturated butanol. The upper organic butanol layer was collected in a separate clean round bottomed flask. This last step was repeated four more times, and the five butanol extracts were pooled and dried on a rotary evaporator at 40°C.

**Base treatment**

The dried butanol extract was treated with 20ml of solvent H containing 25-30% methylamine at 55°C for 4 h (flask tightly stoppered), with occasional agitation in order
to destroy the ester linkage due to the diacyl glycerol in the phospholipid. The base treated sample was then dried on a rotary evaporator at 55°C and finally transferred to a 16x100 screw cap test tube, conserving all material by repeatedly (3x) adding ~ 4 mL of solvent B, sonicking for ~ 2 min, before transferring. The lipids were dried after each transfer under N₂ stream at 35-40°C. A final transfer was made using solvent A. The dried crude samples of JEC 21 and ext1/ext2Δ were each taken up in 2 mL of solvent B, sonicated, centrifuged, and analyzed by HPTLC (solvent F) before fractionation into neutral and acidic lipids as described in chapter 2. The two fractions were analyzed by HPTLC and detection was made by Bial’s orcinol reagent.

**High performance liquid chromatography (HPLC)**

The dried acidic lipid was taken up in 120ml of a mixture of 1:1 vol/vol of solvents B and D. Preparative HPLC was then performed as described previously (in chapter 2) over 160min; flow rate, 0.5 mL/min. Generally, 80 x 1.0 mL fractions were collected in each run and the identity and purity of each fraction were assessed by analytical HPTLC (solvent F). Fractions that displayed comparatively similar HPTLC Rf-values were combined in 16x100 screw cap test tubes, dried under N₂, and prepared for further analysis by I-D ¹H-NMR spectroscopy (as described in chapter 2).

**Mass spectrometry (MS)**

Mass spectrometry was performed in the positive ion mode on an Electrospray Ionization-Mass Spectrometer (ESI-MS) instrument. Briefly, ESI-MS was performed on an LTQ equipment with a LCQ-Tune acquisition software. The dried GSL samples were
dissolved in 200μL methanol, vortexed and centrifuged, and 40 μL of sample introduced into the instrument by direct infusion through a nanospray capillary tip at an estimated flow rate of 0.5 μL/min. Addition of Lithium Iodide (LiI) (10 mM in Methanol) to samples was done for the purpose of generating [M(Li)+Li]⁺ adducts of the molecular species. Although LiI was to be added until the observed ratio of [M(Li)+Li]⁺ adducts to mixed Na⁺/Li⁺ adducts in the MS profile mode was approximately > 5:1, this was difficult to achieve in most cases given the small sample size. Nominal, monoisotopic m/z values are used in the labeling and description of the ESI-MS spectra which represent a summation of 50-150 scans (30 scans /min) for both MS [M(Li)+Li]⁺ and MS/CID MS profiles.

Results and Discussion

**HPTLC analysis of GIPCs from *C. neoformans***

The HPTLC profiles from crude GIPCs obtained from wild type (JEC 21) and mutant (cxt1cxt2Δ) cells are represented in Figure 3.3 below. Both strains exhibit a pair of bands (Cn-1) that co-migrates with a fungal Manα2InsPCer standard with an observable difference in the relative distributions of the bands between monohydroxy- (upper) and dihydroxy- (lower) N-acyl forms. More significantly, the GIPCs from JEC 21 cells express major, low mobility components that are completely absent in the cxt1cxt2Δ strain. As described in the next section, MS data for these compounds were found to be consistent with previously characterized WT GIPCs, Manα3(Xylβ2)Manα4Galβ6Manα2InsPCer (Cn-5), and Manα6Manα3(Xylβ2)Manα4Galβ6Manα2InsPCer (Cn-6) (Heise et al., 2002, Gutierrez

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et al., 2007). These structures were confirmed by mass spectrometry as described in the subsequent section.

![HPTLC profiles of GIPCs](image)

**Figure 3.3** HPTLC profiles of GIPCs (orcinol stained acidic lipid fractions) from *C. neoformans* wild type, WT (JEC 21), and mutant (*cxtlcxt2A*) strains. Lane 1 represents authentic MIPC standard from *Aspergillus fumigatus*, lanes 2 and 3 represent crude GIPCs from the WT and mutant strains respectively.

In contrast, a dark band representing a pair of components with higher mobility, whose Rf is consistent with a less polar GIPCs than the major wild type species, was observed as the dominant product in the *cxtlcxt2A* strain. Based on prior findings (Gutierrez et al., 2007; Castle et al, manuscript submitted), these compounds were proposed to be xylose-free GIPCs with glycan sequence \( \text{Mana}_3\text{Mana}_4\text{Gal}\beta_6\text{Mana}_2\text{InsPCer} \) with two ceramide lipoforms (Cn-4). We hypothesized that the *cxtlcxt2A* cells are unable to transfer xylose from donor UDP-xylose due to deletion of two xylose transferases (Cxt1 and Cxt2) and therefore the GIPCs do not possess the xylose branch in their glycan structure. Our HPTLC results appeared to indicate that in the absence of these xylosyltransferases, the *cxtlcxt2A* cells are unable to transfer xylose to cryptococcal GIPCs. In order to confirm this, it was necessary to isolate and analyze the GIPCs by mass spectrometry. This was also done to obtain wild type GIPCs for comparison.
The crude acidic fractions of both the JEC 21 and \textit{cxt1cxt2Δ} were further fractionated by HPLC and the identity and purity of each fraction were assessed by analytical HPTLC (Figures 3.4 and 3.5 respectively). Fractions that displayed comparatively similar HPTLC Rf-values were combined in 16x100 screw cap test tubes, dried under \textit{N}_2, and later analyzed by NMR (results not shown) and mass spectrometry. Results of the HPTLC of the relevant HPLC purified fraction are shown below.

\textbf{Figure 3.4} HPTLC profiles of GIPCs (orcinol stained acidic lipid fractions) from \textit{C. neoformans} WT (JEC 21) strain. Lane 1 represents authentic MIPC standard from \textit{Aspergillus fumigatus} (S) and the subsequent lanes represent HPLC purified GIPCs fractions from the WT strain (Panel A; HPLC purified fractions numbered 5 through 23, Panel B; HPLC purified fractions numbered 24 through 42).
Figure 3.5  HPTLC profiles of GIPCs (orcinol stained acidic lipid fractions) from C. neoformans mutant (cxt1cxt2Δ) strain. Lane 1 represents crude acidic fraction of GIPC from the mutant (C) and the subsequent lanes represent HPLC purified GIPCs fractions from cxt1cxt2Δ cells (Panel A; HPLC purified fractions numbered 10 through 26, Panel B; HPLC purified fractions numbered 27 through 41).
ESI-MS and -MS" analysis of GIPCs from C. neoformans

A. GIPCs from JEC 21

HPLC purified GIPC fractions from JEC 21 and cxt1cxt2Δ cells were further analyzed using electrospray-ionization-linear ion trap mass spectrometry (ESI-LIT-MS and -MS\textsuperscript{"}), in the positive ion mode (\textsuperscript{+}ESI) on a linear ion trap instrument (LTQ, Thermo-Finnigan, San Jose, CA). Interpretation of the spectra was consistent with previously described reports of purified GIPC components (Levery et al., 1998, 2001, 2005; Bennion et al., 2003; Toledo et al., 2007). Molecular profiles of C. neoformans GIPCs as [M(Na)+Na\textsuperscript{+}] salt-adducts were first acquired using +ESI-MS. The major molecular species in the JEC 21 profile of one of the earlier eluted combined HPLC fractions, 24-29 (Figure 3.6), was more complex with two main molecular adduct groups observed at nominal, monoisotopic \textit{m/z} 1132, 1146, 1148, 1160, 1176; and 1618, 1632, 1634, 1646, 1662 consistent with compositions Hex\textsubscript{4}lnsPCer and Hex\textsubscript{4}lnsPCer, respectively. These compositions correspond to the previously characterized GIPC sequences Mana\textsubscript{2}lnsPCer (C\textsubscript{n}-1) and Mana\textsubscript{3}Mana\textsubscript{4}Galβ6Mana\textsubscript{2}lnsPCer (C\textsubscript{n}-4), respectively. The difference of \textit{m/z} 16 between the most abundant peaks within each cluster is consistent with lipoforms differing in the degree of hydroxylation of the ceramide fatty-N-acyl group (t18:0 4-hydroxy-sphinganine with h24:0 and h\textsubscript{2}24:0 fatty acids, respectively). The differences of 14 or 28 amu between molecular ion signals in each group are consistent with one or two additional \textendash CH\textsubscript{2} groups on the sphingoid or fatty acid chain in the ceramide. The molecular ions at \textit{m/z} 1262 and 1485 are not consistent with GIPCs, and are attributed to impurities. Overall this fraction appears to contain non-xylosylated intermediates in the WT GIPC biosynthetic pathway.
Figure 3.6 $^+$ESI-MS$^1$ spectra (as [M(Na)+Na]$^+$ salt adducts) of purified GIPC fraction (24-29) from JEC 21. The peaks labeled X and Y are correspond to groups of GIPC adducts with the sequence, \text{Mana$_2$InsPCer (Cn-1)} and \text{Mana$_3$Mana$_4$Gal$_6$Mano$_2$InsPCer (Cn-4)}, respectively. Ions marked with asterisk are attributed to impurities. The values in bold are nominal, monoisotopic m/z.

Figures 3.7 and 3.8 (representing profiles of later eluting fractions) are consistent with GIPCs having formula Hex$_4$PenInsPCer ($m/z$ 1750, 1766; Figure 3.7 Panels A and B) and Hex$_5$PenInsPCer ($m/z$ 1912, 1928; Figure 3.8 Panels A and B). These correspond to the previously characterized GIPC sequences \text{Mana$_3$(Xylp$_2$)Mana$_4$Gal$_6$Mano$_2$InsPCer (Cn-5)}, and \text{Mana$_6$Mano$_3$(Xylp$_2$)Mana$_4$Gal$_6$Mano$_2$InsPCer (Cn-6)} respectively (Heise et al 2002). Again the 16 amu difference between each molecular adduct pair is consistent with lipoforms differing in the degree of hydroxylation of the ceramide fatty-N-acyl group (t18:0 4-hydroxy-sphinganine with h24:0 and h24:0 fatty acids, respectively). These results show that the low Rf band in the wild type HPTLC profile of JEC 21 GIPCs (Figure 3.3) corresponds to 4 components: (Cn-5) and (Cn-6), each bearing two types of ceramide.

In order to facilitate fragmentation, LiI was added to the GIPC fractions (Levery et al 2001, Levery 2005). The addition of Li$^+$ to the GIPCs caused a shift of 32 amu.
(Table 3.1); corresponding to replacement of two Na⁺ by two Li⁺ thus the major molecular species at m/z 1718 and 1734 in Figure 3.7 Panels C and D correspond to m/z 1750, 1766 in Figure 3.5 Panels A and B. Likewise the m/z of 1880 and 1896 in Figure 3.8 Panels C and D correspond to 1912 and 1928 in Figure 3.8 Panels A and B. The molecular ion increments of m/z 16 in Figure 3.7 Panels C and D and Figure 3.8 Panels C and D correspond to mixed [M(Na)+Li]+ salt adducts arising from insufficient lithiation, and not to an additional hydroxyl group on the ceramide. (The way this is confirmed is discussed in the ext1ext2A section).
Figure 3.7  Continued (Panel C and D)

Figure 3.8  Mass spectrometry of GIPC fractions of $^+$ ESI-MS$^1$ profiles of purified fractions from JEC 21 consistent with the GIPC sequence, Man$_6$Man$_3$(Xyl)$_2$Man$_4$Gal$_6$Man$_2$InsPCer (Cn-6). Panel A, fraction 41; Panel B, fraction 42; (as [M(Na)+Na]$^+$ salt adducts). Panel C and D are [M(Li)+Li]$^+$ salt adducts after addition of Lil to fractions 41 and 42, that correspond to the [M(Na)+Na]$^+$ salt adducts in Panels A and B. The values in bold are nominal, monoisotopic $m/z$. 

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B. GIPCs from cxt1cxtΔ

The MS¹ profiles obtained from GIPC fractions 29 and 30 of the cxt1cxtΔ mutant are shown in Figure 3.9 (Panels A and B, respectively). Each of the major molecular adduct species [M(Na)+Na]⁺ observed, m/z 1132 (fraction 29) and, m/z 1148 (fraction 30), is consistent with the composition HexInsPCer, and corresponds to the previously characterized GIPC with the sequence Mana2InsPCer. The 16 amu difference 

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between these molecular adduct pair m/z 1132 and 1148 is again consistent with lipoforms differing in the degree of hydroxylation of the ceramide fatty-N-acyl group (t18:0 4-hydroxy-sphinganine with h24:0 and h224:0 fatty acids, respectively). Further, the major molecular adduct species observed at m/z 1132, 1146 and 1160 in panel A are consistent with HexInsPCer with ceramides containing t18:0 4-hydroxysphinganines h24:0, h25:0 and h26:0 fatty N-acylation respectively. This pattern is also observed with the peaks at m/z 1148, 1162 and 1176 in panel B. As before the fractions were treated with LiI to facilitate fragmentation in MS^n modes. Figure 3.9 Panel C exhibits the major molecular adduct species [M(Li)+Li]^+ after lithiation m/z 1100 (corresponding to [M(Na)+Na]^+ m/z 1132). In this case the adduct at m/z 1116 represents underlithiation. The ESI-MS^2 spectra (Figure 3.9, Panel D) acquired from the molecular adduct: at m/z 1100 exhibits ceramide containing ions [Z_0PO_3(Li)+Li]^+ at m/z 758, [Y_0PO_3(Li)+Li]^+ at m/z 776, [Y_0 +Li]^+ and [Z_0+Li]^+ at m/z 690 and 672 respectively. ESI-MS^3 of the ion at m/z 690(Appendix C) provides evidence on the mass of the sphingoid [O/ Y_0 +Li]^+ and by subtraction from [Y_0 +Li]^+, the mass of the fatty acid as well. Glycosidic cleavages with or without phosphate are well represented in the fragmentation of the glycolinositol. An abundant pair of phosphoryl glycolinositol fragments is observed at m/z 417 an 435 for [HexInsPLi]^+, ([B_2PO_3(Li)+Li]^+ and [C_2PO_3(Li)+Li]^+ respectively). A pair of non-phosphorylated fragments representing the non-reducing end of glycosylinositol are observed at m/z 331 and 349 for [Hex.Ins+Li]^+, ([B_2+Li]^+ and [C_2+Li]^+ respectively). These cleavages are summarized in Scheme 3.1, Table 3.2 and Appendix A.

On the whole, lithiation of samples reduces the m/z of molecular species by 32 (Table 3.1), and is expected to simplify MS parent ion profiles as well as increasing the overall signal to noise (S/N) compared to [M(Na)+Na]^+ adducts in MS^n profiles (Levery et al 2001; Levery et al 2005). It is sometimes difficult, however, to achieve complete lithiation before the signal becomes suppressed by the high salt concentration. As a result it is common to observe some residual mixed salt adducts of GIPCs in these spectra.
Levery et al. 2001, Levery et al. 2005). This is evident, for example, from the peak at $m/z$ 1116 in Figure 3.9, Panel C (corresponding to $[\text{M(Na)+Na}^+]$ $m/z$ 1132). At low resolution the shift of +16 amu could suggest either an addition of a hydroxyl group to the ceramide or insufficient lithiation. In order to establish the difference, an MS$^2$ of the +16 adduct was acquired (Figure 3.9, Panel E). Although the +16 increment to all the ceramide related ions e.g., at $m/z$ 758 (from the parent ion $m/z$ 1100) and $m/z$ 774 (from the parent ion 1116), might suggest an extra hydroxyl group attached to the ceramide, the same +16 amu increment on the glycosylinositol phosphate ions, $m/z$ 433 and 451 from the $m/z$ 1116 parent ion compared to $m/z$ 417 and 435 from the $m/z$ 1100 parent ion demonstrates that it is a result of under-lithiation in this case.

**Figure 3.9** Mass spectrometry of GIPC fractions of $^+$ ESI-MS$^1$ profiles of purified fractions from *ctx/ctx2Δ* consistent with the GIPC sequence, Mano2InsPCer (Cn-1). Panel A, fraction 29; Panel B, fraction 30 (as $[\text{M(Na)+Na}^+]$ salt adducts ) Panel C, MS$^1$ of fraction 29 after addition of Lil. Panels D and E represent the MS$^2$ spectra of $[\text{M(Li)+Li}^+]$ at $m/z$ 1100 and 1116 respectively. The values in bold are nominal, monoisotopic $m/z$. 

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Figure 3.9  Continued

C

D

E

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Scheme 3.1 Fragmentation of \([\text{M}(\text{Li})+\text{Li}]^+\) at \(m/z\) 1100 consistent with the structure Man\(\alpha2\)InsPCer (C\(n\)-1) in \(^{+}\text{ESI-MS}^2\); fragment designations as in Costello and Vath (Costello, 1990) as expanded for GIPCs in Singh et al (Singh, 1991). The additional lithium ion has been omitted.

An \(^{+}\text{ESI-MS}^1\) profile of later a eluting HPLC purified GIPC fraction from cxt\(1\)cxt\(2\alpha\), that represent additional glycosylation on the Man\(\alpha2\)InsPCer GIPC structure, is presented in Figure 3.10, Panel A. The major molecular adduct species \([\text{M}(\text{Li})+\text{Li}]^+\) in the \(^{+}\text{ESI-MS}^1\) profile, consistent with the composition Hex\(\alpha2\)InsPCer \((m/z\) 1262, 1278 and 1290) are observed in Figure 3.10, Panel A. These values show a uniform increment of 162 to the first group of ions \((m/z\) 1100, 1116 and 1128), observed in Figure 3.9, Panel C consistent with the addition of a second hexose residue to (C\(n\)-1). The Hex\(\alpha2\)InsPCer composition corresponds to the previously characterized GIPC sequences Gal\(\beta6\)Man\(\alpha2\)InsPCer (C\(n\)-2) (Heise et al 2002). Again the 16 amu difference between each molecular adduct pair is consistent with lipoforms differing in the degree of
hydroxylation of the ceramide fatty-N-acyl group (t18:0 4-hydroxy-sphinganine with h24:0 and h22:4:0 fatty acids, respectively). ESI-MS^n spectra for the molecular adduct at m/z 1262 (Figure 3.10, Panels B, C and D) exhibit fragmentations consistent with Scheme 3.2. An abundant pair of glycosylinositol phosphate fragment is observed at m/z 579 ([B3PO3(Li)+Li]^+ and 597 ([C3PO3(Li)+Li]^+, respectively (Panel B).

![Graph A](image1)

**Figure 3.10** Mass spectrometry of GIPC fractions (as [M(Li)+Li]^+ salt adducts) of ESI-MS^1 profiles of purified fractions from cxtI/cxt2A. Panel A, fraction 33 is consistent with the GIPC sequence, Galβ6Manα2InsPCer (Cn-2). Panel B, ESI-MS^2 (m/z 1262 →) spectrum of selected [M(Li)+Li]^+ salt-adduct m/z 1262 (Hex2InsPCer); Panel C and D, ESI-MS^3 (m/z 1262 → 579 → and m/z 1262 → 417 →) spectrum originating from the same molecular species. The values in bold are nominal, monoisotopic m/z.

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A pair of phosphoryl-glycolinositol fragments is observed at m/z 417 and 435 for [HexInsPLi₂]⁺ ([Y₂/B₃PO₃(Li)+Li]⁺ and [Y₂/C₃PO₃(Li)+Li]⁺ respectively, Panel C). An additional pair [InsPLi₂]⁺ is observed in the MS³ spectrum (Panel D) at m/z 255 and 273 Y₁/[B₃PO₃(Li)+Li]⁺ and Y₁/[C₃PO₃(Li)+Li]⁺, respectively). A pair of non-phosphorylated fragments representing the non-reducing end of glycosyninositol is observed at m/z 493 and 511 for [HexIns+Li]⁺, ([B₃+Li]⁺ and [C₃+Li]⁺ respectively) (Panel B). Other non-phosphorylated fragments are observed at m/z 349 [C₂+Li]⁺, 331 [B₂+Li]⁺, 187 [C₁+Li]⁺ and 169 [B₁+Li]⁺. A ceramide containing ion [Z₀PO₃(Li)+Li]⁺ (Panel B) is observed at m/z 758 and O/([Z₀PO₃(Li)+Li]⁺ at m/z 392. Other ceramide related fragmentations were observed at m/z 776 ([Y₀PO₃(Li)+Li]⁺), [Y₀+Li]⁺ and [Z₀+Li]⁺ at m/z 690 and 672 respectively (Panel B).

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Scheme 3.2  Fragmentation of [M(Li)+Li]+ at m/z 1262 consistent with the structure Galβ6Mana2InsPCer (Cn-2) in ES-MS² and -MS³; Fragment designations as in Costello and Vath (Costello, 1990) as expanded for GIPCs in Singh et al (Singh, 1991). The additional lithium has been omitted.

In the ES-MS¹ profiles of the most complex GIPCs obtained from cxl cxlΔ cells, (Figure 3.11, Panels A and B). We observed the major molecular adduct species [M(Li)+Li]+ consistent with the composition Hex₄InsPCer (m/z 1586 and 1602 respectively). These values show a uniform increment of 324 to m/z 1262 and 1278 (Figure 3.10, Panel A), and is consistent with the addition of two hexose residues to (Cn-2). The Hex₄InsPCer composition corresponds to the previously characterized GIPC sequence Mana3Mana4Galβ6Mana2InsPCer (Cn-4). No traces of xylosylated GIPC products in the profile of cxl cxlΔ were detected. To confirm the lack of xylose in the
mutant GIPC molecular species, the major molecular adduct corresponding to \( m/z \) 1586 was selected for further fragmentation by \(^{+}\)ESI-MS\(^n\) mode (Figure 3.11 Panel D and E). \(^{+}\)ESI-MS\(^2\) spectrum acquired from the [M(Li)+Li]\(^+\) peak at \( m/z \) 1586 (corresponding to [M(Na)+Na]\(^+\) \( m/z \) 1618 (Na\(^+\) data not shown)) of the \( cx1/cx2\Delta \) mutant profile showed the predominant glycosylinositol phosphate fragments \([B_3PO_3(Li)+Li]\(^+\) and \([C_5PO_3(Li)+Li]\(^+\) pair (\( m/z \) 903 and 921) corresponding to Hex\(_4\)InsP, and other fragments from glycosidic cleavages (Scheme 3). A ceramide ion (Y\(_0\)+Li\(^+\)) was observable at \( m/z \) 690 (h\(_{24:0}/t\(_{18:0}\) lipoform). A \(^{+}\)ESI-MS\(^3\) spectrum acquired from the \([C_5PO_3(Li)+Li]\(^+\) ion at \( m/z \) 921 (\( m/z \) 1586 \( \rightarrow \) 921 \( \rightarrow \); Figure 3.11, Panel D and E) showed all of the glycosidic cleavages consistent with a linear Hex\(_4\)InsP primary fragment (Scheme 3.3). Essentially identical spectra were acquired from the [M(Li)+Li]\(^+\) salt-adduct at \( m/z \) 1602 (corresponding to [M(Na)+Na]\(^+\) \( m/z \) 1634) (not shown), except that the ceramide ion was observed in the MS\(^2\) spectrum at \( m/z \) 706 (h\(_{24:0}/t\(_{18:0}\) lipoform).

Figure 3.11  Mass spectrometry of GIPC fractions of \(^{+}\)ESI-MS\(^1\) profiles of purified fractions from \( cx1/cx2\Delta \) consistent with the GIPC sequence Man\(_3\)Man\(_4\)Gal\(_6\)Man\(_2\)InsPCer(Cn-4). Panel A, fraction 35-36; Panel B, fraction 37-38 (as [M(Li)+Li]\(^+\) salt adducts). Panel C, MS\(^2\) spectrum of [M(Li)+Li]\(^+\) at \( m/z \) 1586. Panels D and E represent the MS\(^3\) spectra of [M(Li)+Li]\(^+\) at \( m/z \) 1586 \( \rightarrow \) 921 \( \rightarrow \) and \( m/z \) 1586 \( \rightarrow \) 903 \( \rightarrow \). The values in bold are nominal, monoisotopic \( m/z \).
Figure 3.11  Continued
Scheme 3.3  Fragmentation of [M(Li)+Li]+ at m/z 1586 consistent with the structure Mana3Mana4Galβ6Mana2InsPCer (Cn-4) in ²ESI-MS² and -MS³; Fragment designations as in Costello and Vath (Costello, 1990) as expanded for GIPCs in Singh et al (Singh, 1991). The lithium adduct and ion have been omitted.

These results demonstrate an altered expression in GIPC structures in the absence of the two xylosyltransferase, Cxt1p and Cxt2p. Lack of xylose modification in cxt1cxt2Δ cells is accompanied by truncation of the terminal mannose residues distal to the branch. Furthermore, we detect no residual xylose-containing GIPCs in the cxt1cxt2Δ mutant sample, indicating that no other enzyme performs the function of xylose addition during GIPC synthesis. This study shows that either Cxt1p or Cxt2p, or both, are

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xylosyltransferases that are involved in the biosynthetic pathway of GIPCs in C. neoformans.
Table 3.1  Respective species from *C. neoformans* depicting both the Molecular adduct ion \([M(\text{Na})+\text{Na}]^+\) and \([M(\text{Li})+\text{Li}]^+\) observed in \(^{1}\text{ESI-LIT-MS}\) profiles

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<th>Specie</th>
<th>([M(\text{Na})+\text{Na}]^+)</th>
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<td>(Cn-2)</td>
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Table 3.2

(A) Molecular adduct ion \([\text{M(Li)}+\text{Li}]^+\) observed in ESI-LIT-MS profile, (B) and significant ceramides-containing, (C) glycosylinositol ions and (D) non-phosphorylated glycan derived from GIPC consistent with the \((\text{Cn-1})\) structure. Fragment designations as in Costello and Vath (Costello, 1990) as expanded for GIPCs in Singh et al (Singh, 1991) with modifications of ceramide-derived ions from Adams and Ann (Adams, 1993).

<table>
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<td>Fatty Acid</td>
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<tr>
<td>Sphingoid</td>
<td>t18:0</td>
</tr>
<tr>
<td>A. ([\text{M(Li)}+\text{Li}]^+)</td>
<td>1100</td>
</tr>
</tbody>
</table>

Product of \([\text{M (Li)}+\text{Li}]^+\):

**B. Ceramide related fragments**

- \([\text{Y}_0\text{PO}_3(\text{Li})+\text{Li}]^+\) 690
- \([\text{Z}_0\text{PO}_3(\text{Li})+\text{Li}]^+\) 758
- \([\text{Y}_0+\text{Li}]^+\) 672
- \([\text{Z}_0+\text{Li}]^+\) 392

**C. Glycosylinositol ions**

- \([\text{C}_2\text{PO}_3(\text{Li})+\text{Li}]^+\) 349
- \([\text{B}_2\text{PO}_3(\text{Li})+\text{Li}]^+\) 417

**D. Non Phosphorylated glycan**

- \([\text{C}_2+\text{Li}]^+\) 349
- \([\text{B}_2+\text{Li}]^+\) 331
Table 3.3

(A) Molecular adduct ion \([M(Li)+Li]^+\) observed in ESI-LIT-MS profile, (B) and significant ceramide-containing, (C) glycosylinositol ions, (D) inositol ions and (E) non-phosphorylated glycan derived from GIPC consistent with the \((Cn-2)\) structure. Fragment designations as in Costello and Vath (Costello, 1990) as expanded for GIPCs in Singh et al (Singh, 1991) with modifications of ceramide-derived ions from Adams and Ann (Adams, 1993).

<table>
<thead>
<tr>
<th>Fraction</th>
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<tr>
<td>Fatty Acid</td>
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<tr>
<td>Sphingoid</td>
<td>t18:0</td>
</tr>
<tr>
<td>A. ([M(Li)+Li]^+)</td>
<td>1262</td>
</tr>
</tbody>
</table>

Product of \([M (Li)+Li]^+\):

**B. Ceramide related fragments**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>([Y_0 PO_3 (Li)+Li]^+)</td>
<td>776</td>
</tr>
<tr>
<td>([Z_0 PO_3 (Li)+Li]^+)</td>
<td>758</td>
</tr>
<tr>
<td>([Y_0+Li]^+)</td>
<td>690</td>
</tr>
<tr>
<td>([Z_0+Li]^+)</td>
<td>672</td>
</tr>
<tr>
<td>([O/Z_0 PO_3 (Li)+Li]^+)</td>
<td>392</td>
</tr>
</tbody>
</table>

**C. Glycosylinositol ions**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>([C_3 PO_3 (Li)+Li]^+)</td>
<td>597</td>
</tr>
<tr>
<td>([B_2 PO_3 (Li)+Li]^+)</td>
<td>579</td>
</tr>
<tr>
<td>(Y_2/[C_3 PO_3 (Li)+Li]^+)</td>
<td>435</td>
</tr>
<tr>
<td>(Y_2/[B_2 PO_3 (Li)+Li]^+)</td>
<td>417</td>
</tr>
</tbody>
</table>

**D. Inositol ions**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Y_1/[C_3 PO_3 (Li)+Li]^+)</td>
<td>273</td>
</tr>
<tr>
<td>(Y_1/[B_2 PO_3 (Li)+Li]^+)</td>
<td>253</td>
</tr>
</tbody>
</table>

**E. Non Phosphorylated glycan**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>([C_3+Li]^+)</td>
<td>511</td>
</tr>
<tr>
<td>([B_3+Li]^+)</td>
<td>493</td>
</tr>
<tr>
<td>([C_2+Li]^+)</td>
<td>349</td>
</tr>
<tr>
<td>([B_2+Li]^+)</td>
<td>331</td>
</tr>
<tr>
<td>([C_1+Li]^+)</td>
<td>187</td>
</tr>
<tr>
<td>([B_1+Li]^+)</td>
<td>169</td>
</tr>
</tbody>
</table>
Table 3.4

(A) Molecular adduct ion \([\text{M(Li)}+\text{Li}]^+\) observed in ESI-LIT-MS profile, (B) are significant ceramide-containing, (C) glycosylinositol ions, and (D) non-phosphorylated glycan derived from GIPC consistent with the \((\text{Cn-4})\) structure. Fragment designations as in Costello and Vath (Costello, 1990) as expanded for GIPCs in Singh et al (Singh, 1991) with modifications of ceramide-derived ions from Adams and Ann (Adams, 1993).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>35-36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty Acid</td>
<td>h24:0</td>
</tr>
<tr>
<td>Sphingoid</td>
<td>t18:0</td>
</tr>
<tr>
<td>A. ([\text{M(Li)}+\text{Li}]^+)</td>
<td>1586</td>
</tr>
</tbody>
</table>

Product of [M (Li)+Li]^+:

**B. Ceramide related fragments**

| \([Y_0\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 776 |
| \([Z_0\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 758 |
| \([Y_0+\text{Li}]^+\) | 690 |
| \([Z_0+\text{Li}]^+\) | 672 |
| \([O/Z_0\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 392 |

**C. Glycosylinositol ions**

| \([\text{C}_5\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 921 |
| \([\text{B}_3\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 903 |
| \(Y_4/[\text{C}_5\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 759 |
| \(Y_4/[\text{B}_3\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 741 |
| \(Y_3/[\text{C}_5\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 597 |
| \(Y_3/[\text{B}_3\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 579 |
| \(Y_2/[\text{B}_3\text{PO}_3\text{(Li)}+\text{Li}]^+\) | 417 |

**D. Non Phosphorylated glycan**

| \([\text{C}_5+\text{Li}]^+\) | 835 |
| \([\text{C}_4+\text{Li}]^+\) | 673 |
| \([\text{C}_3+\text{Li}]^+\) | 511 |
| \([\text{C}_2+\text{Li}]^+\) | 349 |
CHAPTER IV

FUTURE STUDIES

In the present research, HPTLC and NMR were used to provide information about the structures of the acidic GSLs obtained from \textit{N. crassa} WT and the defensin-resistant mutant SP 10. Whereas no GIPCs were detected, further work needs to be performed on this fraction, using other techniques such as mass spectrometry for detailed characterization. Also, although characterization of the neutral GSL, \( \beta \)-glucosylceramide, as well as a sterol glycoside, 3-O-\( \beta \)-glucosyl-ergosterol, from both strains, had previously been carried out, and several differences that correlated with the resistance of the mutants to RsAFP2 and other defensins had been identified, we observed from the HPTLC profiles of the neutral fraction, a number of other orcinol\(^+\) bands (with lower Rf values than GlcCer), which did not co-migrate with any standard compound. Further work involving the identity of these compounds is called for.

While isolation and analysis of GlcCer from fungi is straightforward, direct assays for GCS activities are more difficult to establish. We however managed to determine that a concentration of 1 \( \mu \)M would provide a useful compromise between inhibitory effect and yield of mycelia. While the results of this experiment were consistent with inhibition of GCS by EDO-P4, there were effects on the concentrations of other components in its presence. A variety of possible explanations were provided in the text. The identities of these compounds still remain uncharacterized. Further research including characterization of these unknowns as well as performing a correlation study of biological effects with...
changes in expression of GSLs, other lipids, and proteins is needed. These changes would enable us learn more about possible pathways and functions of GlcCer and why inhibition of its synthesis is lethal to fungus.
Characteristic fragmentations of glycosylated IPCs. A: Nomenclature of Adams and Ann for fragmentation of the ceramide moiety, and of Singh, Costello, and Beach for the myo-inositol phosphoryl group. B: Sphingoid $d_{3b}$ ion, proposed by Hsu and Turk. C: Hydrated analog of $d_{3b}$ ion, proposed in this study as a product of t18:0 or t20:0 phytosphingosine-containing ceramides. D: Sphingoid $c_{1b}$ ion, proposed by Hsu and Turk. E: Hydrated analog of $c_{1b}$ ion.
Downfield expansions (5.48-2.06) of two-dimensional (2-D) 1H-NMR spectrum (500 MHz; 200msec; DMSO-$d_6$/2% D$_2$O; 35°C) of the acidic fractions 43-47 of the ATCC 18889 wild type strain of *N. crassa*. The acyl-chain and myo-inositol are represented by the spin systems at 5.1-3.7 and 3.8-2.9 respectively.
+ESI-MS$^3$ mass spectrometry profile of GlPC purified fraction 29 from $cxt1cxt2\Delta$ consistent with the GlPC sequence, \textit{Mana2InsP}Cer (Cn-1 (m/z 1100 $\rightarrow$ 690 $\rightarrow$) (as [M(Li)+Li]$^+$ salt adducts).
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(22) Levery, S. B., Toledo, M., Straus, A. Takahashi, K. 1998. Structure Elucidstion of Sphingolipids from mycopathogen Paracocccidiodes brasiliensis: An immunodorminant betta Galactofuranose Residue is carried by a Novel GIPC antigen Biochem 37,8764-8775


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