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Exploring the Design Space of Antifungal Peptides

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Exploring the Design Space of Antifungal Peptides

by

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We certify that we have read this thesis and that in our opinion it is sufficient in scientific scope and quality as a dissertation for the degree of Master of Science.

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Dedication

This Thesis is dedicated to my guardian angel, my grandmother. I also dedicate it to my family and friends, whose unconditional love and support have accompanied me to make this achievement possible. I am endlessly thankful for their guidance!

Abstract

The synthesis, characterization, and evaluation of antifungal activity of peptides FQ15 and FG10 will be conducted to address the hypothesis. The hypothesis of this thesis is that a peptide with similar properties to AS15 would have similar activity against *Cryptococcus neoformans.* This study addresses the challenge of drug- resistant microbes by developing inhibitors targeting lipid flippase and exploring their potential as antimicrobial agents against *Cryptococcus neoformans.* Chapter 1 provides a historical context of the background on the development of antimicrobials and common antifungal treatments, as well as an introduction about *C. neoformans* and its resistance mechanism. Chapter 2 introduces the materials and the experimental methods used for the drug discovery of the antifungal peptide designed to target the fungus pathogen *C. neoformans.* Chapter 3 reviews the preliminary results and discussion. Lastly, Chapter 4 is the conclusion of this study.

FQ15 and FG10 peptides demonstrated efficacy against *C. neoformans* in vitro. *C. neoformans* is a challenging and threatening fungal pathogen that is highly resistant to caspofungin drug, which poses a significant potential threat to cause severe meningitis in immunocompromised individuals like those with HIV/AIDS. This ongoing study aimed to investigate antifungal properties, focusing on exploring the elements within CDC50 loop region of the fungal pathogen *C. neoformans* to develop potential flippase inhibitors to design peptides as antifungal agents. Previous research has highlighted the essential role of Cryptococcus lipid translocase (flippase) in caspofungin resistance mechanism. 3B lab members focused on identifying peptides resembling the original AS15 peptide with similar biophysical characteristics, including hydrophobicity, net charge, isoelectric point, and molecular weight within the CDC50 loop sequence to demonstrate antifungal efficacy. Flippase inhibitors show promise in combating drug-resistant microbes.

Solid phase peptide synthesis (SPPS) was utilized to synthesize 15 amino acid-long sequences, FQ15 and FQ15MA, and 10 amino- acid long sequences, FG10 and FG10MA. The utilization of Mass Spectroscopy (MS) and High-Performance Liquid Chromatography (HPLC) confirmed the detection and isolation of the synthesized peptides. HPLC played a role in analyzing peptide detection and fractions isolation. Adding myristic acid on the N- terminus of the same amino acid sequence was done to explore its potential as an antimicrobial agent, and it showed efficacy at higher concentrations. FQ15MA demonstrated an MIC of 16 μg/mL against *H99* and FG10MA demonstrated an MIC of 8 μg/mL against *H99* wild type and 64 μg/mL against *ΔCDC50* mutant, and a fractional inhibitory concentration (FIC) index value of less than 0.5 when combined with caspofungin.

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Lastly, I want to offer special appreciation to my family, who have always encouraged and supported me to pursue my dreams and instilled in me a sense of courage and passion after migrating to the United Stated twelve years ago. I thank them for consistently standing by me through the joys and the challenges of this journey. Their love, support, and belief in me means everything. This journey would not have been possible without them!

Abbreviations

AA	Amino Acid
Ac	Acetic Acid
ACN	Acetonitrile
AMP	Antimicrobial Peptide
ATP	Adenosine Triphosphate
C'	Carboxy terminus of peptide
CFU	Colonies Forming Units
DCM	Dichloromethane
DIEA	N, N-Diisopropylethylamine
DMF	N, N- Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ESI	Electrospray Ionization
FA	Formic Acid
FAT	Fatty Acid Tail
FIC	Fractional Inhibitory Concentration
FMOC	Fluorenylmethyloxycarbonyl protecting group
g	Grams
HCTU	O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	High Pressure Liquid Chromatography
m/z	Mass to charge ratio
Ma, C14	Myristic Acid

MeOH	Methyl Alcohol
mg	Milligram
MIC	Minimum Inhibitory Concentration
min	Minutes
mol	Moles
MS	Mass Spectrometry
N'	Nitrogen terminus of peptide
N_2	Nitrogen gas
RPMI	Roswell Park Memorial Institute
SPPS	Solid phase peptide synthesis
TES	Triethyl silane
TFA	Trifluoroacetic acid
WT	Wild Type
YPD	Yeast Peptide Dextrose

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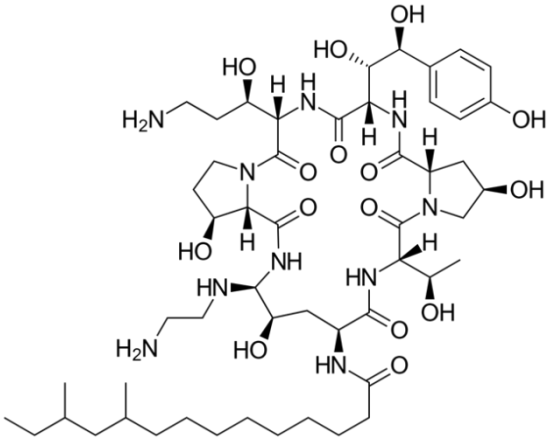
Chapter 1. Introduction

This study highlights the importance of understanding resistance mechanisms, addressing the challenges posed by *C. neoformans* and developing treatments targeting fungal infections in immunocompromised individuals. Peptides FQ15 and FG10 were developed as antifungal drugs, targeting a protein found in *C. neoformans.* When this drug binds to the fungal protein, it causes charged lipid molecules to accumulate on the cell surface. FQ15 and FG10 peptides work synergistically with the existing antifungal drug, caspofungin.

I. Caspofungin

Figure 1: Caspofungin Structure (ChemDraw).



There are three major classes of drugs that are currently approved to treat fungal infections. Caspofungin is a lipopeptide antifungal drug that belongs to the echinocandin drug class. Echinocandins consist of a cyclic peptoid linked via its N- terminus to a fatty acid.[4] Caspofungin is one of the limited options for antifungal treatments that inhibits the enzyme (1,3)- ß- D glucan synthase, which is a major component of the cell wall, so it disrupts the integrity of the cell wall,[12] however *C. neoformans* can resist them. Amino acid substitutions in the primary sequence of the glucan synthase allow the fungus to evade inhibition. The peptides could lower the concentration

of caspofungin needed for treating infections, potentially increasing the effectiveness of these antifungal drugs. Adding FATs to peptide backbones demonstrated the ability to increase a peptide's inhibitory activity against *C. neoformans*. The peptide with a lipid tail significantly enhanced caspofungin's activity, suggesting its potential as a therapeutic agent.

II. Cryptococcus neoformans

C. neoformans is a fungus that lives in the environment throughout the world. It usually affects the lungs or the central nervous system (brain and spinal cord). *C. neoformans* remains a prominent cause of meningitis in individuals who have weakened immune system, especially those who have HIV/AIDS,[6] posing a significant threat contributing to high mortality rates. Meningitis is the inflammation of meninges, which is the protective membrane covering the brain and spinal cord. It can be fatal if it is not treated early.[5] Since the fungi's resistance to caspofungin presents a challenge, especially for immunocompromised patients, *C. neoformans* needed to be investigated deeper due to the drug resistance. Existing antifungal treatments are limited and not effective against *Cryptococcus.* Given the limited treatment options for fungal infections, the goal is to enhance caspofungin's effectiveness against *C. neoformans.* Cryptococcal anti-fungal peptides, produced by *C. neoformans* fungus, act as a natural defense mechanism against fungal infection by penetrating cell membranes and inhibiting specific enzymes. The researchers investigated CDC50 loop region in *C. neoformans* and developed a series of flippase inhibitors to evaluate their potential microbial agents.

III. P4 ATPase

Flippase inhibitors play a crucial role in combating drug resistance, where the activity of the flippase enzyme is crucial for lipid disruption in bacterial cell membranes, which hold significance in the development of disease treatments. Recent studies identified a lipid flippase involving P4 ATPase and a regulatory subunit CDC50, where mutations in the CDC50 loop region increased sensitivity to caspofungin. P4 ATPases form heterodimers with members of the CDC50 protein family.[9] This loop region binds to the P4 ATPase, regulating flippase activity. By interfering with this activity using a peptide derived from the CDC50 loop region, researchers found caspofungin sensitivity in *Cryptococcus.* The function of CDC50 is not known in relation to P4 ATPase.

IV. Drug Resistance

Drug resistance is the ability of microbes to tolerate the drugs designed to kill them. Drug resistance is a major obstacle to treating infected individuals who are susceptible to antibiotics affected due to over-prescription of antibiotics. Fungal infections remain a significant global health concern, resulting in millions of deaths annually. The Center for Disease Control (CDC) identifies antibiotic-resistant microbes as major threats.[7] Although *C. neoformans* can be treated with the main classes of antifungals, Echinocandins, resistance is a growing concern in the infectious diseases' community. The rapid adaptation of microbes and the high cost of developing new pharmaceuticals pose significant challenges in combating drug resistance. Although factors that trigger resistance are not fully understood, substation in amino acid residues can target a specific enzyme and inhibit the flippase activity. Therefore, microbial agents show promise in controlling microbial growth, including fungi.

V. Antimicrobial Peptides (AMPs)

AMPs are a diverse class of compounds that are used to prevent or treat microbial infections. They are classified based on their targets such as antifungals are used to treat fungal infections.[8] They have a wide range of inhibitory effects against bacteria, fungi, parasites, and fungus. Since the emergence of antibiotic resistant microorganisms, the increase usage of antibiotics resulted in AMPs. It is hypothesized that cells do not easily acquire resistance to AMPs because of their membrane modifications.[1] AMPs are membrane active peptides (MAPs) that combat microbial threats by targeting organism membranes. These peptides interact and disturb the integrity of the cell membrane. AMPs have demonstrated effectiveness against viral illnesses. The effectiveness of antimicrobial activity of the peptides is mainly influenced by their charge, hydrophobicity, and isoelectric point. The development of these drugs has been limited and not studied enough.

VI. Drug Synergy

Drug synergy occurs when different components target the same biochemical pathway.[16] This synergy can be achieved through different methods. One approach involves mixing individual drug components in a solution, as seen in checkerboard assays/Fractional Inhibitory Concentration (FIC) assays. MIC assay is used to determine the lowest concentration of the peptides at which *C. neoformans* is susceptible. FIC assay determines the synergy between two compounds, in this study between Caspofungin and the peptide synthesized. The FIC index, a unitless value, serves as a mathematical measure of drug synergy (Equation 1).[3]

Equation 1. FIC Index formula

$$FIC\ Index = \frac{FIC_A}{MIC_A} + \frac{FIC_B}{MIC_B}$$

Table 5. In this study, only drug combinations resulting in FIC < 0.5 are considered synergistic, while those between 0.5 and 1 are considered additive, between 1 and 2 represent no effect or indifference. FIC exceeding 2 indicates anti- synergy.

Effect	**FIC Index Ranges**
Synergy	FIC index < 0.5
Additive effect	0.5 ≤ FIC index ≤ 1
No effect	1 < FIC index ≤ 2
Anti-synergy	FIC index > 2

VII. *Cell Membrane*

The cell membrane acts as a physical barrier, segregating the cell's internal environment from the external environment. Cells maintain high levels of organization, achieved through the metabolism of food molecules acquired via phagocytosis. These molecules are broken down into ATP and raw materials for building cellular structures. In eukaryotes, membrane bilayers exhibit distinct lipids predominantly located on either the inner or outer membrane. The lipid membrane bilayer is regulated by three types of lipid transporters. Flippases (transport toward the inside), use ATP (energy) to catalyze active transport. Some flippases have been identified as strong contributors against *C. neoformans.*[10] This lipid variation influences drug susceptibility and various physiological processes, including drug resistance in microbes.

VIII. *Peptide Design*

The main peptides utilized in this study were designed using a computational method probing further portions of the *C. neoformans* proteins. This work was performed as part of previous studies conducted by the Wiedman Laboratory- 3B Lab members (data unpublished). The CDC50 protein was analyzed at every fifteen amino acid intervals. These sequences were analyzed using the PepDraw program.[2] Values were generated through PepDraw for aspects such as mass, overall charge, isoelectric point (pI) and Hydrophobic moment. Each of these new segments was then compared to a previous peptide AS15 and ranked with respect to how close each of the values was related to AS15 (data unpublished). The peptides selected for this study were among those most closely related to AS15. We therefore hypothesized that these peptides would be good candidates that could potentially inhibit the activity of the target enzyme.

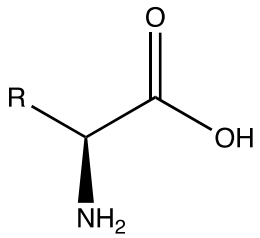
Table 6. Characteristics of peptides.

Sequence	**Mass (g/mol)**	**Isoelectric Point**	**Net Charge**	**Hydrophobicity (Kcal/mol)**	**Extinction Coefficient ($M^{-1}*cm^{-1}$)**
AS15	1579.80	9.73	1	18.31	6990
FQ15	1849.92	9.92	1	18.84	5500
QY15	1864.84	3.99	-3	23.65	1490

IX. Peptide Synthesis

SPPS was developed by chemist Robert Merrifield in 1963, involves coupling and deprotection of individual amino acids to produce a peptide bonded to an insoluble polymeric bead. Peptide synthesis played a pivotal role, enabling the isolation of short amino acid chains from the CDC50 loop region. The amino acids are attached to form a peptide chain, complemented by the addition of a lipid tail. SPPS was utilized to synthesize amino acid sequences which include resin beads. To synthesize the peptides, FMOC/t-Butyl strategy was employed with the Wang resin, which was used as the solid support by hand. The Wang resin is composed of functionalized polystyrene, which is insoluble and allows to wash out the byproducts and impurities efficiently. The Wang resin is used for the synthesis of peptides with a carboxyl terminus. The choice of beads is critical as it determines the type of carboxy terminus (C') present in the final peptide. Unlike cellular protein synthesis, which proceeds from N' to C', SPPS synthesizes peptides in the opposite direction. DIEA was used to activate the FMOC- protected amino acid through deprotonation of the carboxylic acid. HCTU was used as a coupling reagent to increase the rate of amide bond formation (activator). N' protecting group (FMOC) is removed using a piperidine solution, since the fluorenly proton in FMOC is acidic and is readily deprotonated by piperidine.[11] Products bound to the resin are removed through simple vacuum filtration and subsequent solvent wash steps, removing any residual traces of the previous reaction mixture.

Figure 2. The structure of an amino acid has an alpha carbon bonded to four atoms: an amino group (NH_2), a carboxyl group (COOH), and a hydrogen atom (H), and a side chain (R group) (ChemDraw).



X. Analytical Techniques

Techniques like MS and HPLC were important in synthesized peptide detection. These methodologies enhanced the comprehension of peptide structure and function.

Chapter 2. Materials

DCM, DMF, MeOH, Acetone, and ACN were purchased from Macron. TFA, Ac, and Piperidine were purchased from Sigma Aldrich. DIEA was purchased from Cero Salus. FMOC Amino Acids were purchased from Novabiochem/Cero Salus. HCTU was purchased from Quartzy/ Vivitide. TES was purchased from Alfa Azar. Lastly, the Wang Resin was purchased from Chem Pep Inc.

Chapter 3. Methods

I. Peptide Synthesis

The two peptides were synthesized FQ15 and FG10 in the N-terminus→ C-terminus. Peptides FQ15 and FG10 were coupled by hand directly onto the Wang resin through (SPPS) using HCTU and DIEA to initiate the reaction. Around 322 mg of the Wang resin was initially weighted out to make a 0.2 mmole scale synthesis. The resin was allowed to swell in DCM for 20 min. For both peptides, the resin was deprotected before coupling the second amino acid since the resins were pre-labeled with the first amino acid, Glutamine for FG15 and Glutamic acid for FG10. The synthesis process involved completing peptide sequences using Fmoc/tBu- protection scheme for all amino acids to couple the peptides to the Wang resin. FMOC is a common protection group for amines. FMOC protection is removed using 20% v/v piperidine base in DMF. FMOC- amino acids were coupled to the unprotected N-terminus. The coupling reagent HCTU was the activator which facilitated the amide bond formation and DIEA was a base that bonded the acidic proton from the C- terminus of the amino acid being coupled. The synthesis was done from C-terminus to N-terminus. The synthesis involved rounds of coupling and deprotection reactions, which were tracked and recorded. Each amino acid in the sequence was weighed out 3-fold scale coupling (0.2 mmol of each amino acid) beforehand and were saved in the fridge until it was time to couple the amino acids. 3-fold scale coupling of the coupling reagent HCTU (413.69 g/mol) and 6- fold scale of DIEA (129.24g/mol, 0.724g/mL) were prepared. When it was time to synthesize, 209 µL of DIEA and 1 mL of HCTU were added into the 1 DRAM vial that had the amino acid (amino acid solution with 1 mL of DMF). The mixture was vortexed well until there was no visible precipitate. When the amino acid solution was ready, it was poured onto the resin. The resin was placed on the shaker. Each coupling reaction was conducted at room temperature for 10 min. Larger and charged

amino acids were coupled twice for successful coupling. After each amino acid coupling, FMOC deprotection of the resin was performed by adding 1-2 mL of 20% piperidine in DMF solution for 20 min at room temperature. This process is typically repeated after every round of an amino acid coupling. After each reaction (coupling or deprotection) the peptide resin was washed with DMF twice, with MeOH twice, and with DCM twice before the next coupling. The coupling and deprotection reactions were repeated until the peptide chain sequence was complete.

A Ninhydrin-Kaiser test was performed to confirm if a coupling or deprotection was successful. Successful couplings will show clear beads and unsuccessful couplings will show blue beads as shown in **Figure 3A & 3B**, respectively. Several beads were removed using a spatula and were added into a 0.5-dram vial, and 200 µL of Ninhydrin reagent was added. The vial was heated using a heat gun to determine if there is any color change in the solution. It was made sure to handle the vials with tongs to avoid contamination which can give false positive results and for safety reasons due to the vial being hot. Unsuccessful Ninhydrin test (blue beads) after double coupling (10 min each) and coupling for one-hour, acetic anhydride was used to cap unreacted sites at room temperature for a duration of 20 minutes. Whenever there was an unsuccessful coupling even after double coupling, the amino acid was coupled for a longer period (30 min~ 60 min). If that did not work then the peptide beads were capped with acetic anhydride, where 378 µL of ac and 209 µL of DIEA were added. All the components were mixed, and the peptide resin was placed on the shaker for 20 minutes.

Figure 3. Kaiser Test. Successful coupling (clear beads) (**A**). Unsuccessful coupling (blue beads) (**B**).

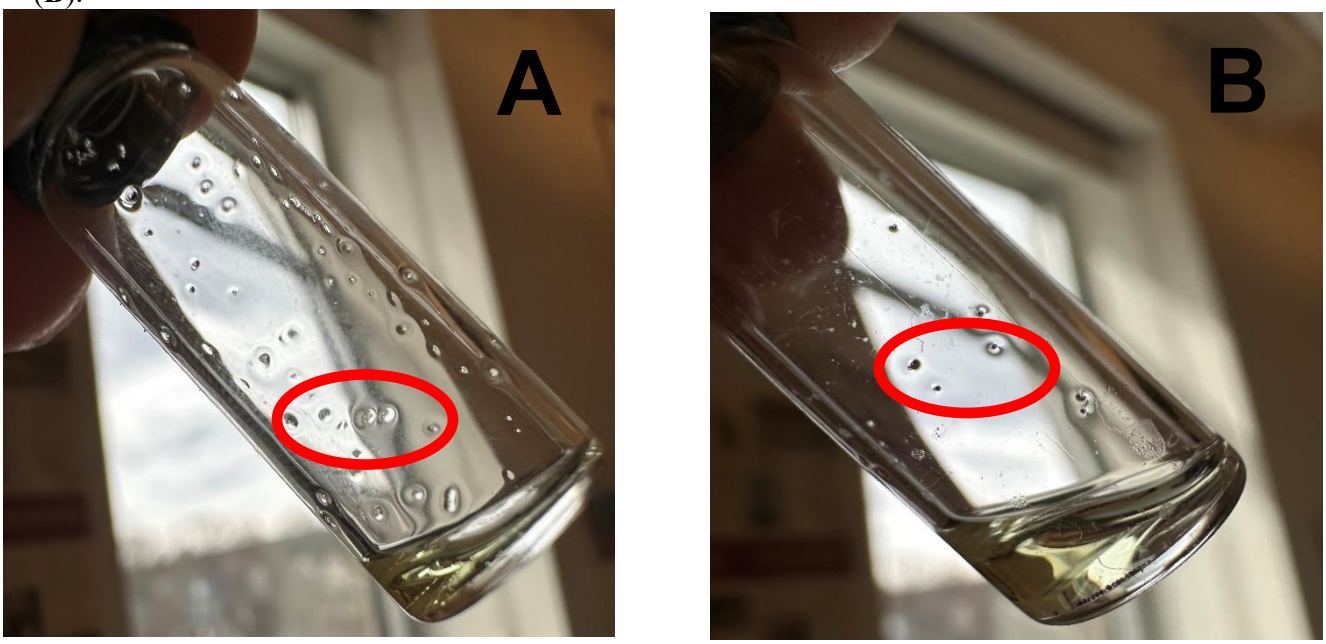


Table 7. Amino acids in both peptides that needed double couplings.

Glutamic Acid
Aspartic Acid
Arginine
Tryptophan
Lysine
Phenylalanine

II. Lipid Tail

FQ15MA and FG10MA are peptides with a conjugated fatty acid tail to the N- terminus. After splitting the resin in half, one of the resins underwent deprotection using 20% piperidine in DMF. The Kaiser test was done where blue color beads were detected confirming the presence of a free amino group. FAT was coupled using myristic anhydride acid with palmitic acid C16, HCTU and DIEA for a total volume of 6 mL. The microwave synthesizer was used to avoid the resin solidifying at room temperature.

Table 8. Microwave synthesizer conditions for coupling FATs.

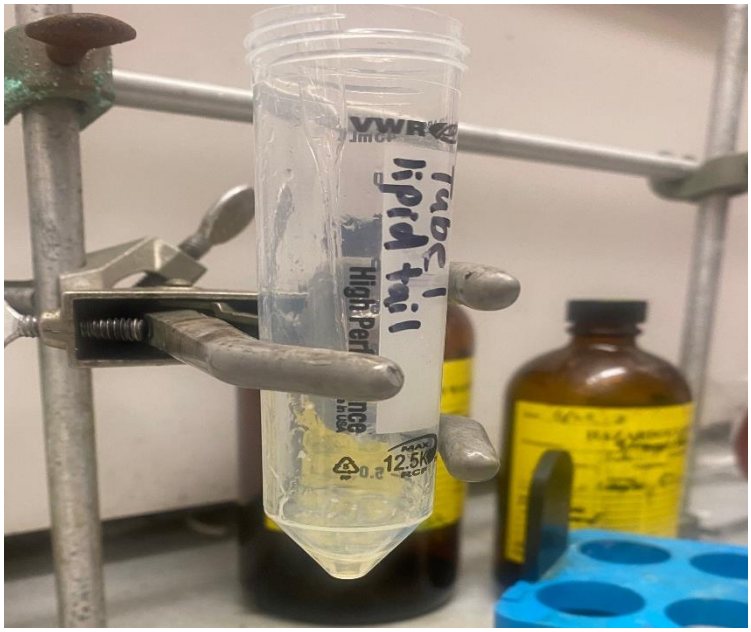
Stage	Temp	Hold time	Pressure	Power	Stirring
1	55 °C	00:10	0	120	Off
2	75 °C	05:00	0	30	Off

III. TFA Cleavage

Once the crude peptide sequences of FQ15 and FG10 were complete, the peptides were cleaved off from the resin using a reaction solution of 95% TFA. Since the synthesis has been carried out under basic conditions, the cleavage was carried out under acidic conditions using 95% TFA and 2.5% DI water, and 2.5% TES was also added to the cleavage cocktail for a 95:2.5:2.5 (v/v/v) mixture. The TFA was used to cleave the peptide from the resin and t-butyl groups from the side chains. TES was used to scavenge resulting carbocations.[16] The cleavage cocktail to cleave the peptide from the resin was prepared by the following: 3.8 mL total of the cleavage cocktail mixture where 2.5% DI water, and 95% TFA were added in a 1 DRAM vial and then was added onto the resin after vortexing the vial. Then, 2.5% TES was added directly to the resin because it

does not dissolve with water. The vapors were allowed to dissipate before capping the synthesis tube. The peptides were placed on a shaker for 2 hours at room temperature. The resin was then washed with DMF, MeOH and DCM twice. The liquid was collected in a 50 mL falcon tube by draining out the peptide, then it was rinsed with TFA. N_2 gas was used to evaporate and dry off TFA very subtly by hovering over the falcon tube to suction out the liquid. Once all the liquid evaporated, 5 mL of cold diethyl ether was added slowly to the cleavage mixture to precipitate the peptide. The precipitate (peptide) was dissolved/diluted in 10% ac in water. It was vortexed and sonicated until it dissolved. The sample was stored in the -80 °C freezer overnight tilted. The peptide was weighted out in the falcon tube. The peptide was lyophilized (frozen dried) over the weekend (~3 days) until dry.

Figure 4. TFA cleavage set up.



IV. Purification

The crude peptide was initially an off-white to yellow powder. The purification was achieved through reverse-phase semi prep High-Performance Liquid Chromatography (HPLC) - Varian Prep Start (Palo Alto, CA) controlled by Galaxy software using Luna C18 column and a 5%-95% ACN gradient. HPLC is a technique from analytical chemistry that is used to separate components in a liquid phase interacting with a solid phase column. The peptides were purified using reverse phase HPLC on a C18 column using a gradient elution. The solvent was used to separate components in a liquid sample (mobile phase), and the mobile phase is delivered to a separation column (stationary phase). If the component has stronger affinity with stationary phase, it moves slower through the column, if the components have stronger affinity with mobile phase, it will move quickly through the column.[15] The purified fractions were manually collected every 10 mL and were combined based on electrospray ionization (ESI) HP1100 mass spectroscopy and analytical Shimadzu *Nexera-I* LC-2040C 3D plus (Kyoto, Kyoto, JP) HPLC. The data of the samples were analyzed to determine purity and composition. The samples underwent rotary-evaporation and were stored at -80 °C overnight. So, the purified fractions containing the target analyte were combined, concentrated, acidified, frozen, and lyophilized to obtain a white to off-white dry powder.

V. Characterization

Yield and purity were recorded for both crude and purified peptides using the electronic balance and HPLC. Mass analysis (molecular identity) of the crude peptides was performed by using ESI HP1100 LC/MS (Spring, TX) with samples dissolved in 50% ACN in water and 0.1% FA. The peptides were characterized by ESI-MS. MS measures the exact molecular mass by breaking the molecule into smaller, charged fragments. It allows us to look at the movement of the

charged molecules through a magnetic field and based on their movement pattern, determine how massive they are. The movement pattern is proportional to the mass and the charge of the molecule.[14] MS was used to validate the product of the synthetic process. The samples were ensured they were dissolved by vortexing, sonicating and heating until the solution is completely dissolved.

VI. Fungal Cells Passage

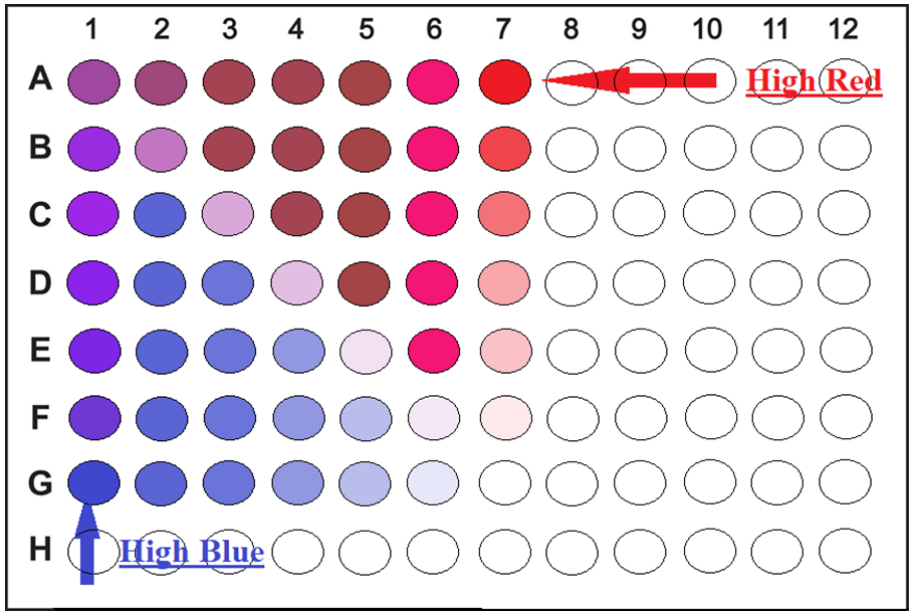
Fungal cells of *C. neoformans* wild type (*H99*) and mutant (Δ*CDC50*) were obtained from the -80 ℃ freezer and thawed for passage. All experiments were conducted with freshly passaged live cells, so it had to be done again if a month passes. The cultivation process involved diluting a 100 µL of the stock microbe solution of *C. neoformans* in 3 mL of YPD in a 10 mL tube, followed by incubation in an incubator shaker at 37 ℃ at 250 rpm. A second passage was done following the same procedure using the passage tube as the "stock microbe solution". Passages of *C. neoformans* were grown for 48 hours for each passage. For MIC assays and checkerboard assays, freshly passaged cells were grown on SDA plates and incubated at 37 ℃ for 3 days.

VII. Minimum Inhibitory Concentration Assay

The analysis to evaluate the antimicrobial activity was done by broth microdilution MIC Assay according to the CLSI M100 protocol. The sample preparation involved microbial preparation, setting up drug dilutions on a 96- well plate, inoculation with microbes, and incubation. Ensuring the appropriate cell number is crucial in microbiology experiments to determine the appropriate number of CFU. The fungal cell concentration was determined by pipetting 100 µL of PBS, McFarland standard and fungal cell solution in triplicate into nine wells of 96- well plate (column 12). Absorbance at 530 nm was recorded using a plate reader, and the

averaged measurements were calculated. The average absorbance of the PBS blank was subtracted from the average McFarland standard and average fungal cells. Subsequently, the ratio of corrected fungal cell absorbance to corrected McFarland standard absorbance was computed, and the product of this ratio and $1x10^7$ provided the cell concentration in CFU/mL. A 100 µL of RPMI media was added to each well. The peptide was measured and placed in an Eppendorf tube and was diluted using DMSO. Peptide-DMSO stock solution was diluted in column 1, and serially diluted to column 11 in rows A-C (peptide) and D-F (peptide with lipid tail). Cultured cells were harvested and suspended in PBS, the inoculum of the fungi was made. Caspofungin drug was added to rows G-I except column 11 (negative control), while column 12 served as the positive control. Different strains were done on different plates to avoid contamination. Plates were incubated at 37 °C, and the growth was visually inspected every 24, 36 and 72 hours. Results were compared to positive and negative controls. The MIC was determined to be the concentration of drug which resulted in no visible microbial growth.

Figure 5. MIC Assay (PowerPoint).

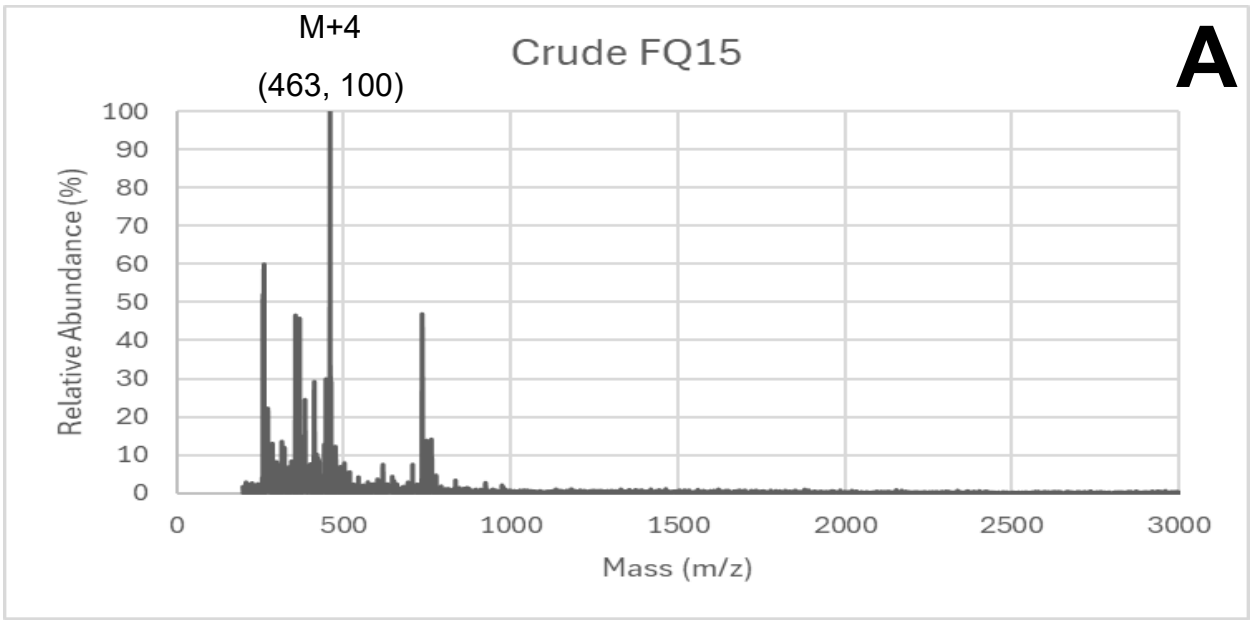


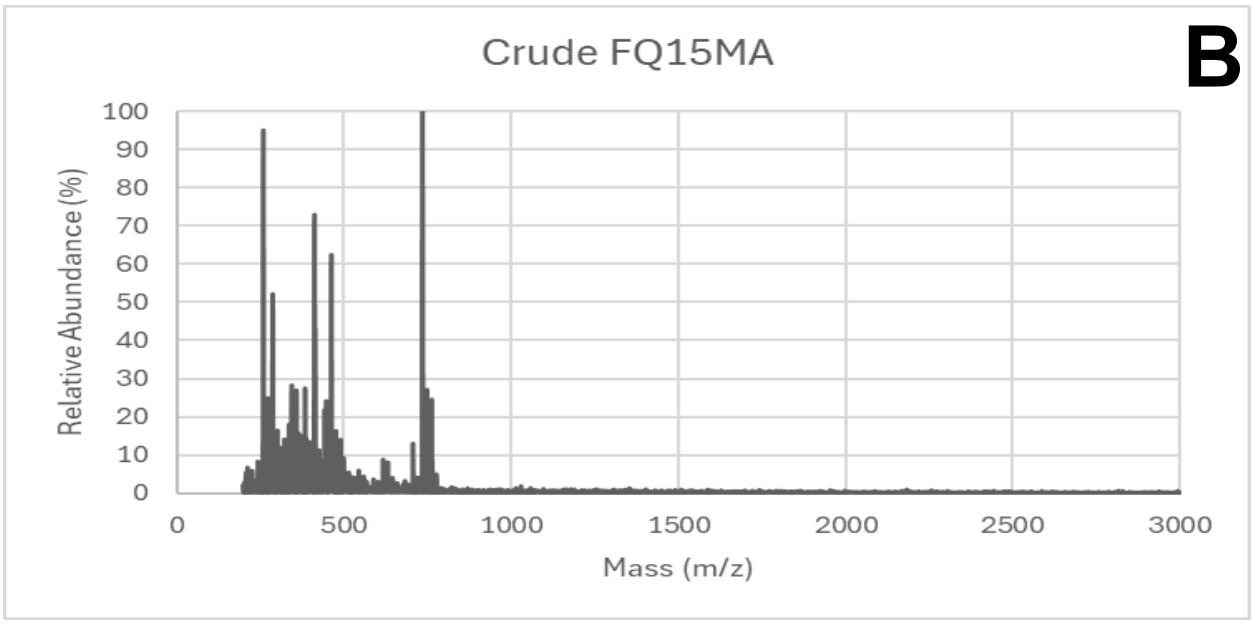
VIII. Checkerboard Assay

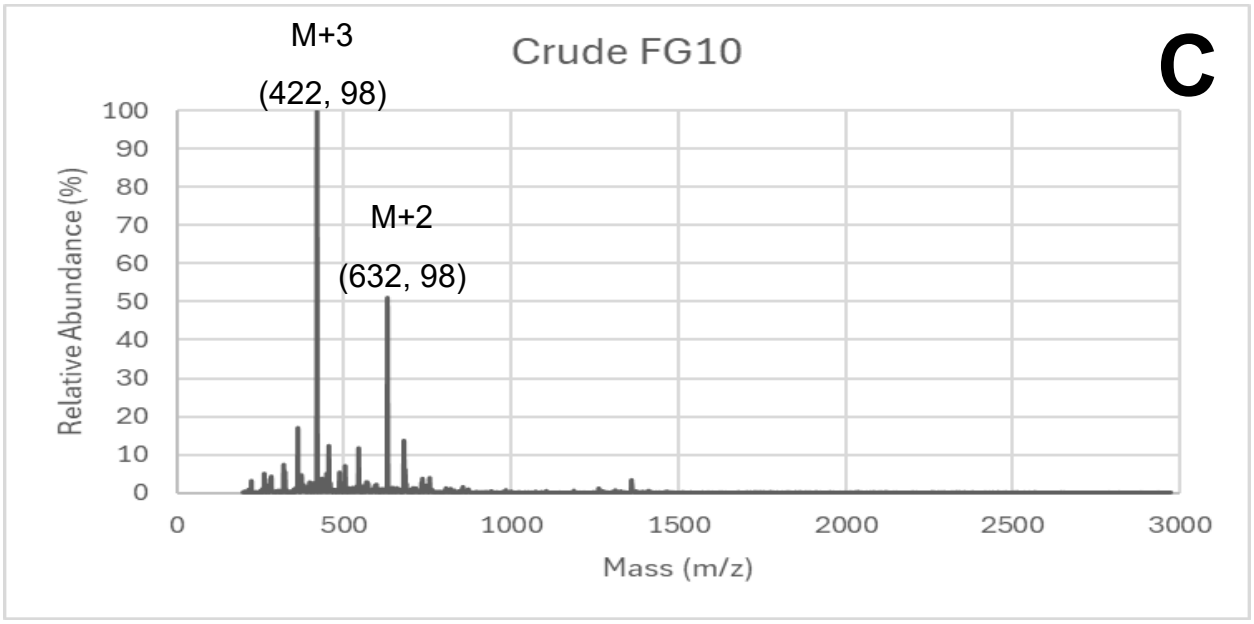
Cells were passed (2nd passage) from the -80 ℃ freezer, and plates were streaked out. The sample preparation closely resembles the MIC assay but involves two sets of drug serial dilutions instead of one. In a 96- well plate, each well received 100 µL of RPMI media except column 11. Both peptides were 2- fold diluted to 6.4 mg/mL in DMSO to make the peptide/DMSO stock solutions. An additional 100 µL of RPMI media was added to the first column. In the first 10 wells of row 1, an additional 100 µL of RPMI media was added. Fungal inoculants were prepared like the MIC experiment. A 100 µL fungal inoculant was added to every well except for column 11. Columns 9 and 10 served as controls for induvial drugs, column 11 as the negative control, and column 12 as the positive control. Caspofungin was pipetted into each well in column 1 and diluted it across. The specific peptide was pipetted into each well of row 1 and diluted it going down. So as a result, each well should have some mixture of the specific peptide and caspofungin. After incubation at 37 ℃, a visual inspection of the plate every 24, 26 and 72 hours was recorded comparing the fungal growth with the positive and negative controls. The FIC index was calculated by summing the ratios of MIC for both drugs.

Chapter 4. Results

I. *Mass Spectroscopy of crude peptides*







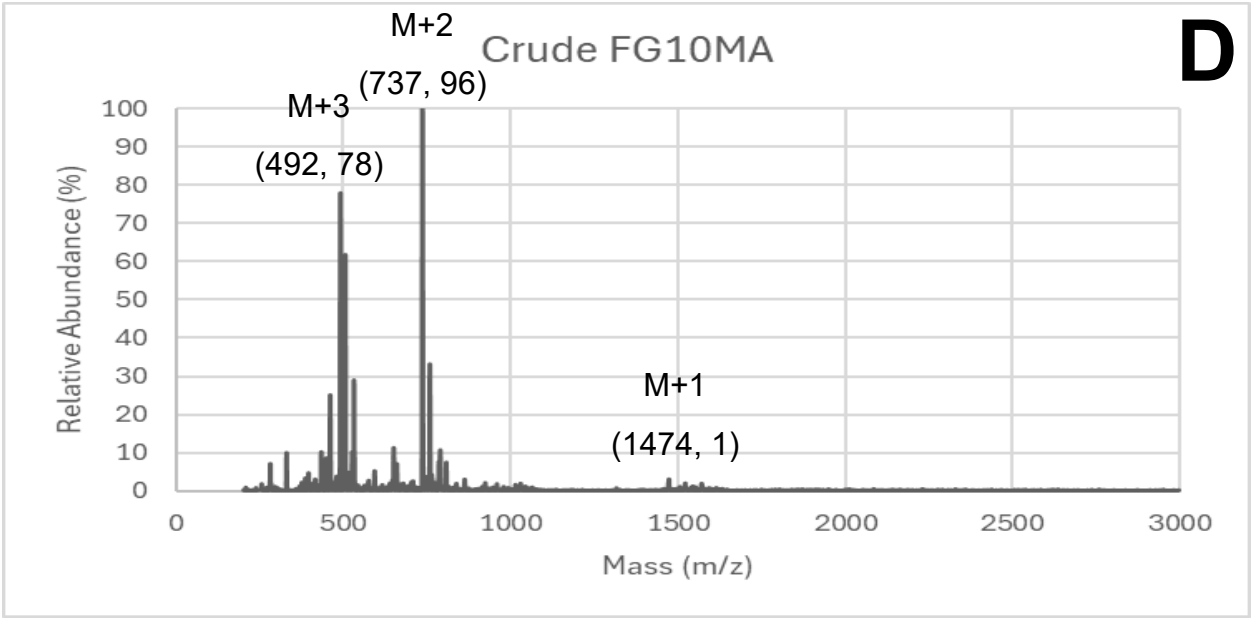
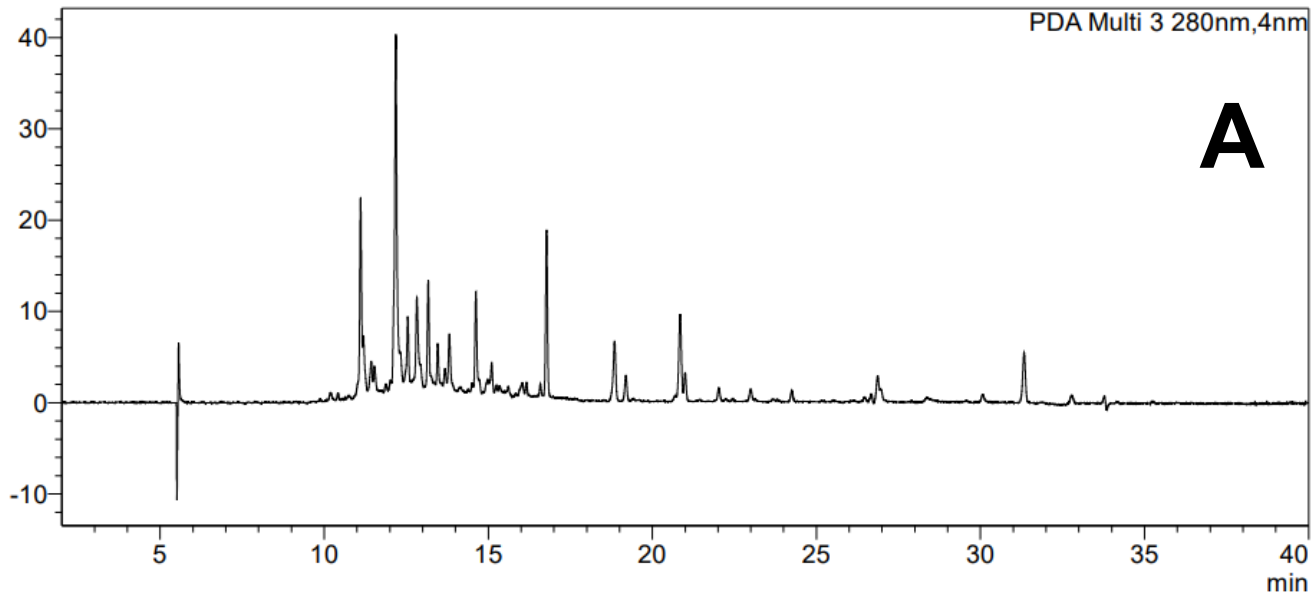
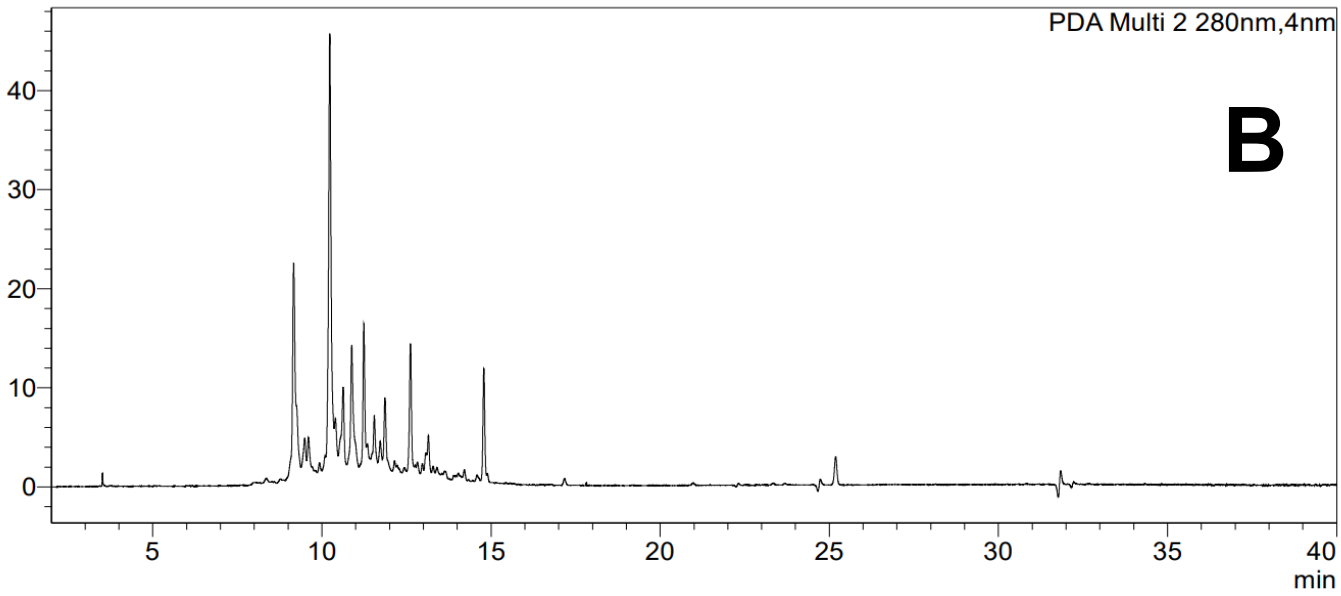


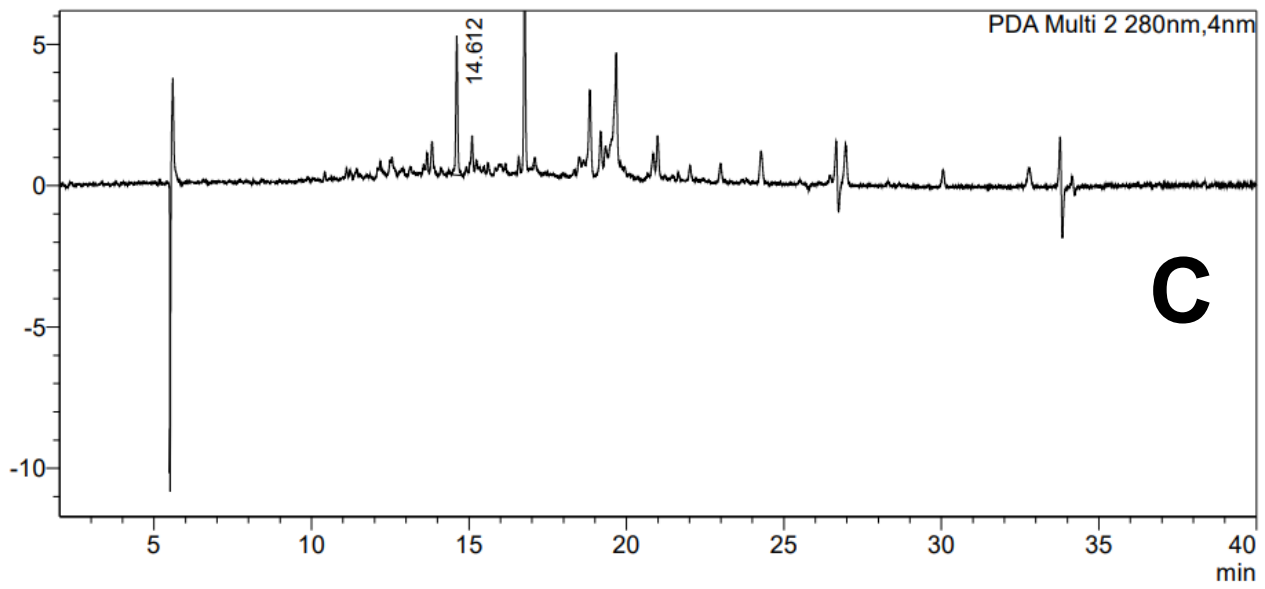
Figure 6. Presents MS data in the positive mode for the following crude peptides FQ15 (**A**) which has M+4 peak at m/z 463, FQ15MA (**B**), FG10 (**C**) which has peaks of M+2 at m/z 632 and M+3 at m/z 422 and FG10MA (**D)** which has peaks of M+1 at m/z 1474, M+2 at m/z 737 and M+3 at m/z 492. The expected mass for FQ15, FQ15MA, FG10 and FG10MA are 1849.92 g/mol, 2060.27 g/mol, 1262.44 g/mol and 1472.79 g/mol, respectively. Peaks M+2 and M+3 confirmed the molecular weight for peptides FG10 and FG10MA (Excel).

Line A contained a mixture of water with 0.1% FA, while line B consisted of MeOH with 0.1% FA. The flow rate was 0.5 mL/min. Injection columns ranged between 1-10 μL. MS confirmed the peptide's existence through the detection of the peaks and m/z mentioned in **Figure 6**. These spectra provide evidence for the presence of the peptide molecule. FQ15 and FG10 peptides and FQ15MA and FG10MA (peptides containing a lipid tail) were both synthesized using SPPS. Myristic Acid was added to the N-terminus of the FQ15 and FG10 to assess its antimicrobial effects. The FQ15MA and FG10MA samples needed a greater quantity of the 30% ACN/water solution and required heating for dissolving, owing to its hydrophobicity resulting from the elongated fatty acid chain compared to FQ15 and FG10.

II. *Analytical HPLC of crude peptides*



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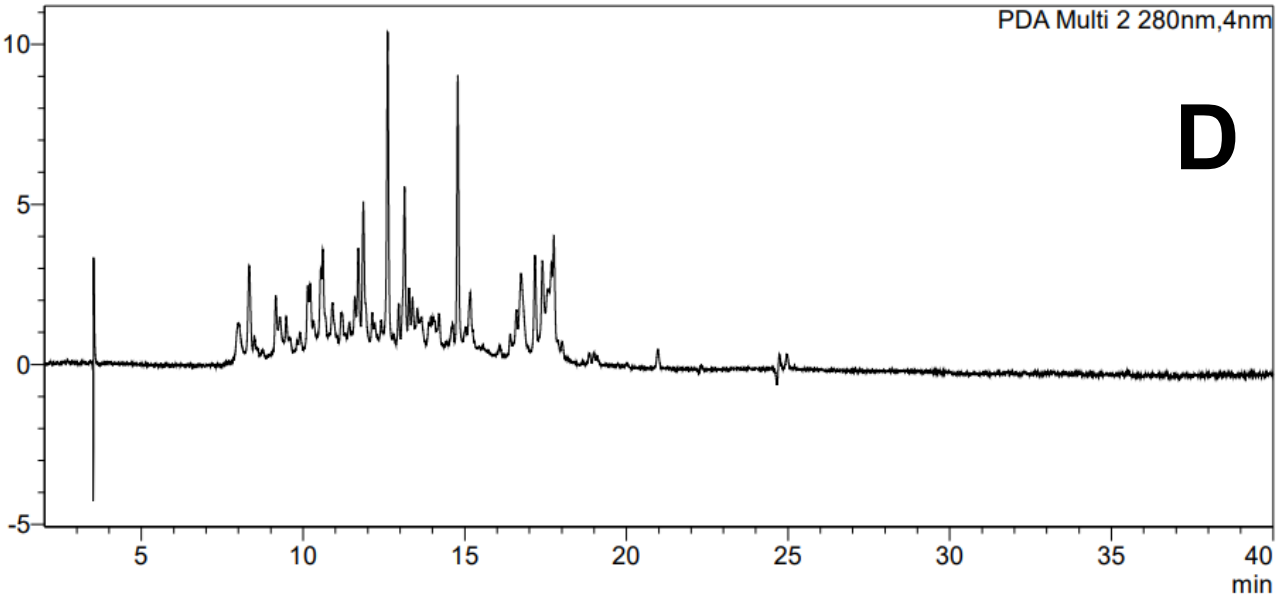
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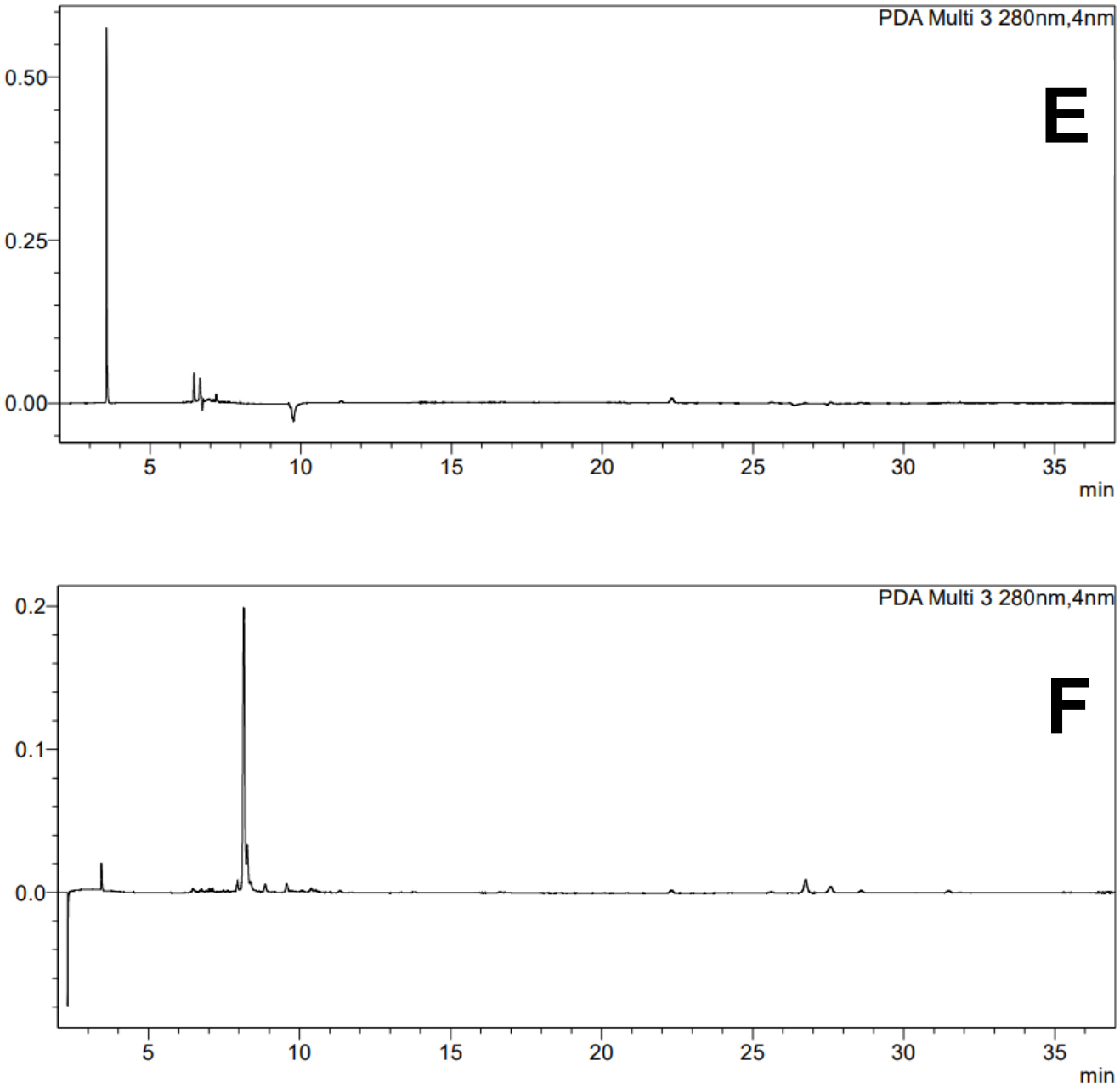


Figure 7. Analytical HPLC data for the following crude peptides: FQ15 (**A**), FQ15 (**B**), FQ15MA (**C**), FQ15MA (**D**), FG10 (**E**) and FG10MA (**F**). The concentration of each peptide was 1mg/mL. 30% of ACN + 70% of water solution was used to dissolve the peptides. Using 30/70 was the best option since 50/50 had some solubility problems and the peptides crashed before the solution was vortexed (Galaxy Software).

The flow rate remained constant at 1.0 mL/min. The autosampler initiated sample injection at the start of the run. The autosampler operated at 25 ℃ alongside the column oven, which was set at the range of 25 ℃ to 35 ℃. To improve accuracy and minimize noise, the signal obtained from a solvent blank injection was subtracted from the experimental sample injection. Channels of 220 nm and 280 nm were carried out. Significant peaks were identified in all figures during Analytical HPLC analysis at 280nm for the peptides containing tryptophan, which exhibits absorption at this wavelength.

Table 9. The HPLC gradient concentration, utilizing a mobile phase composed of water and ACN. % B indicates % concentration of ACN.

Time (min)	% B
0.01	5
2.00	5
18.00	35
30.00	70
40.00	95

III. *Semi- preparatory HPLC*

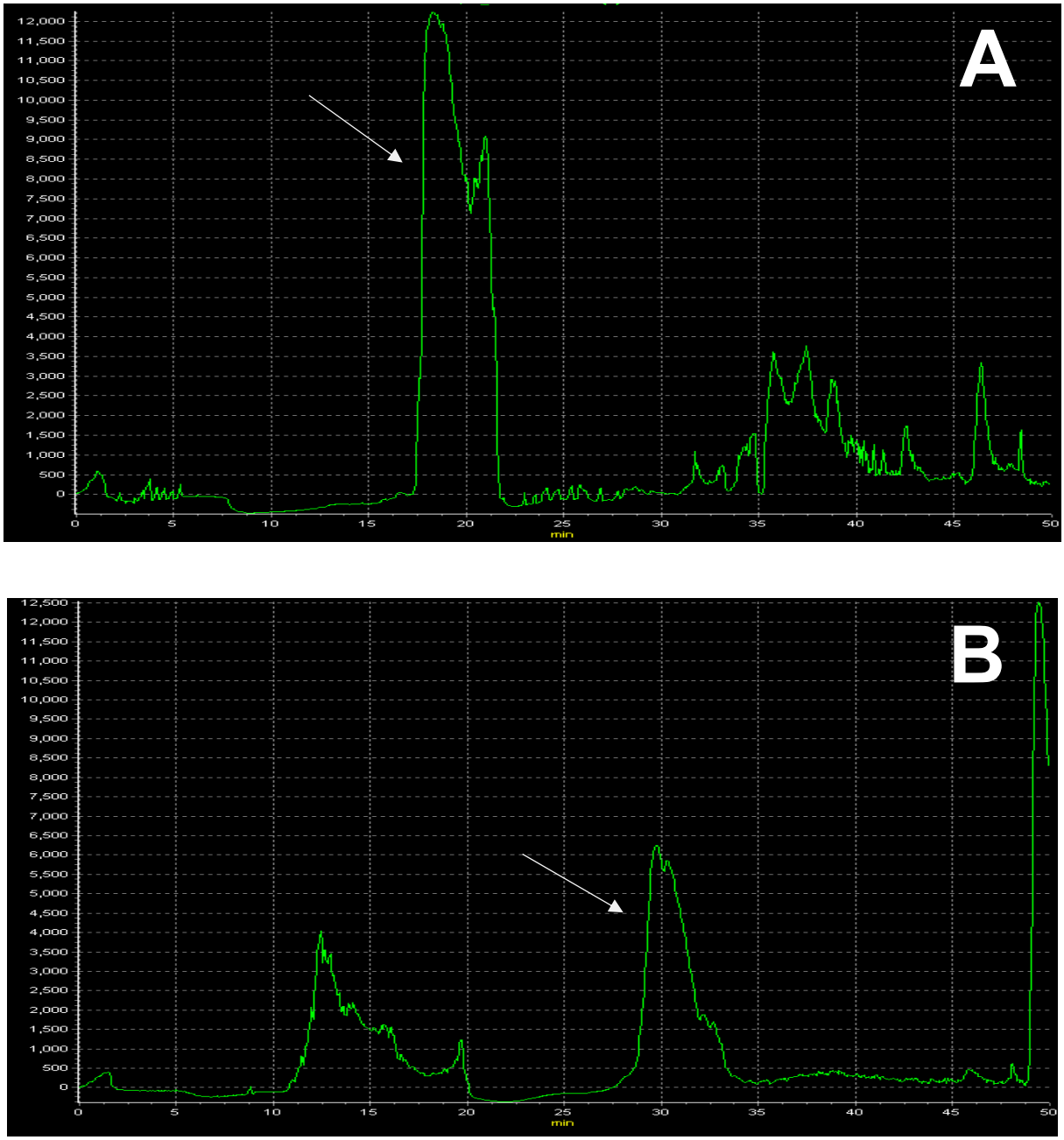


Figure 8A & 8B. Peptides FQ15 and FQ15MA were purified using semi- perparative HPLC. For peptide FQ15 (**A**), fractions of 10 mL were collected during the time intrevals of 17-22 min and 36-39 min. For peptide FQ15MA (**B**), factions of 10 mL were collected during the time intrevals of 11-18 min and 29-33 min (Galaxy Software).

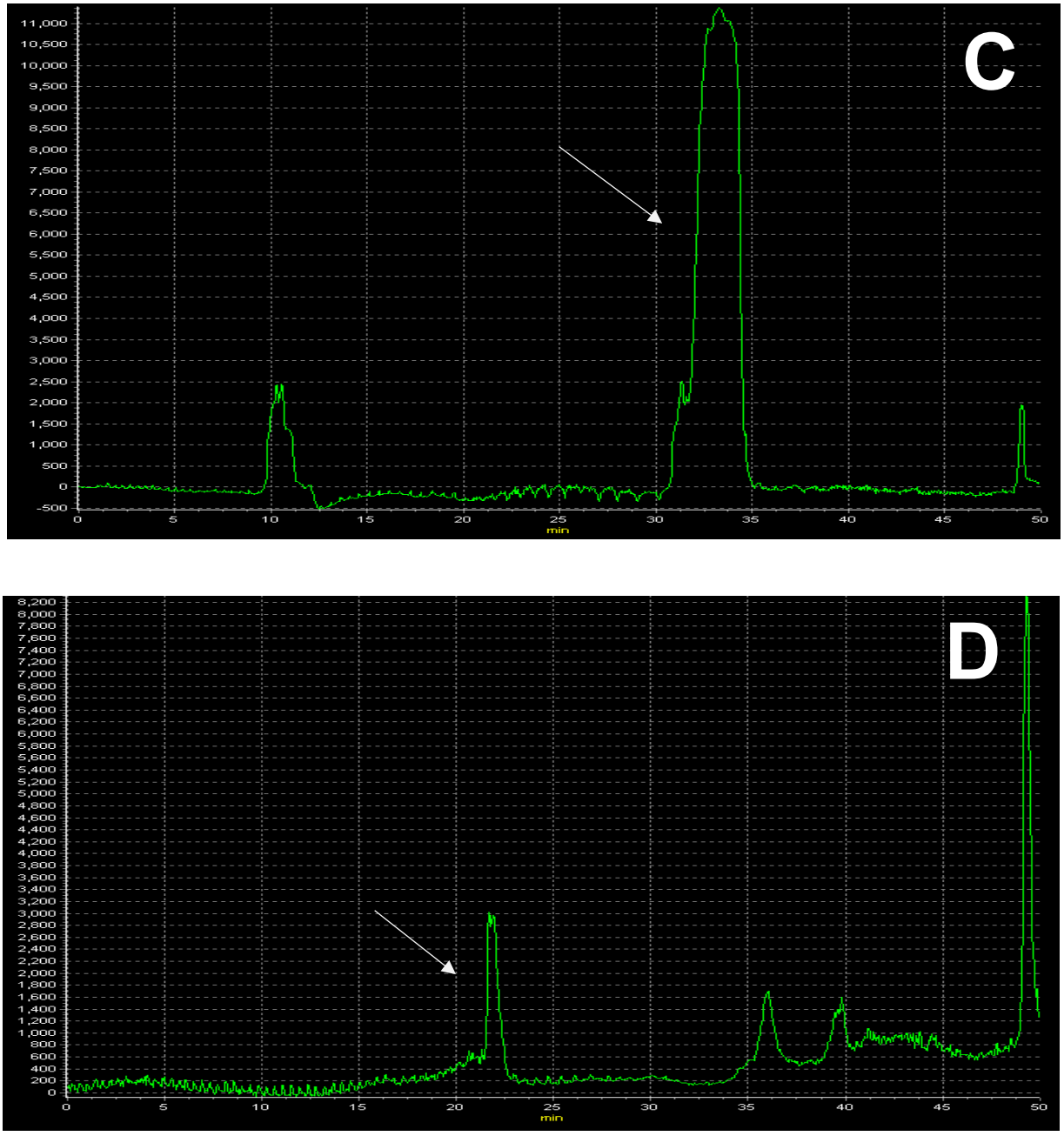
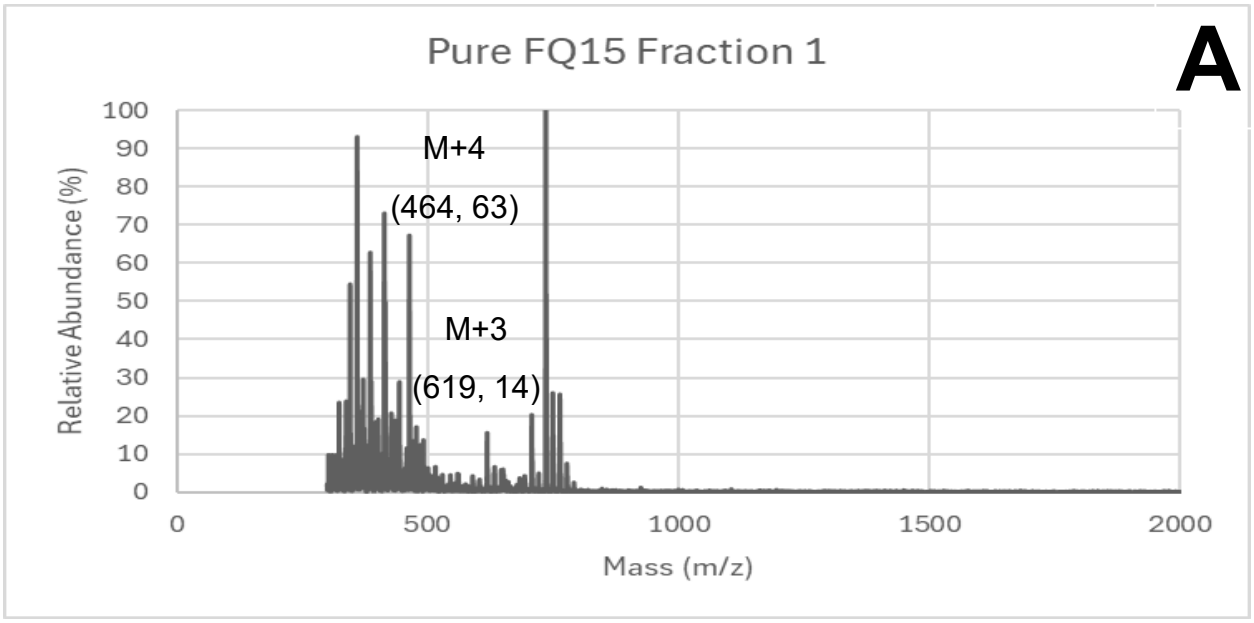
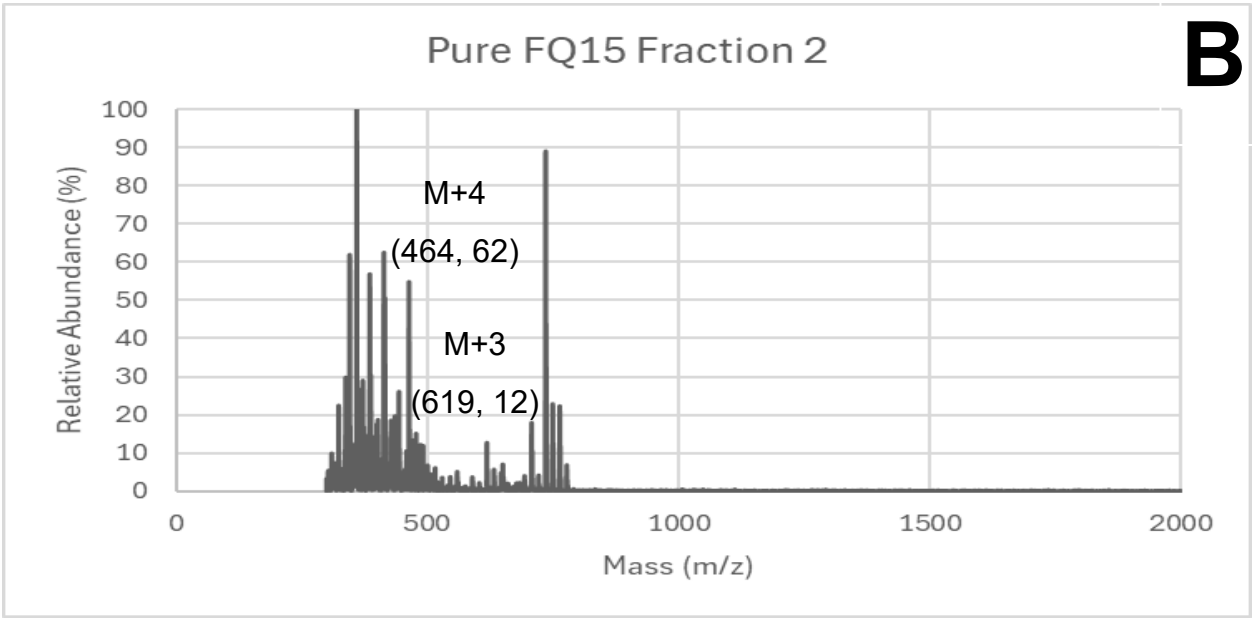


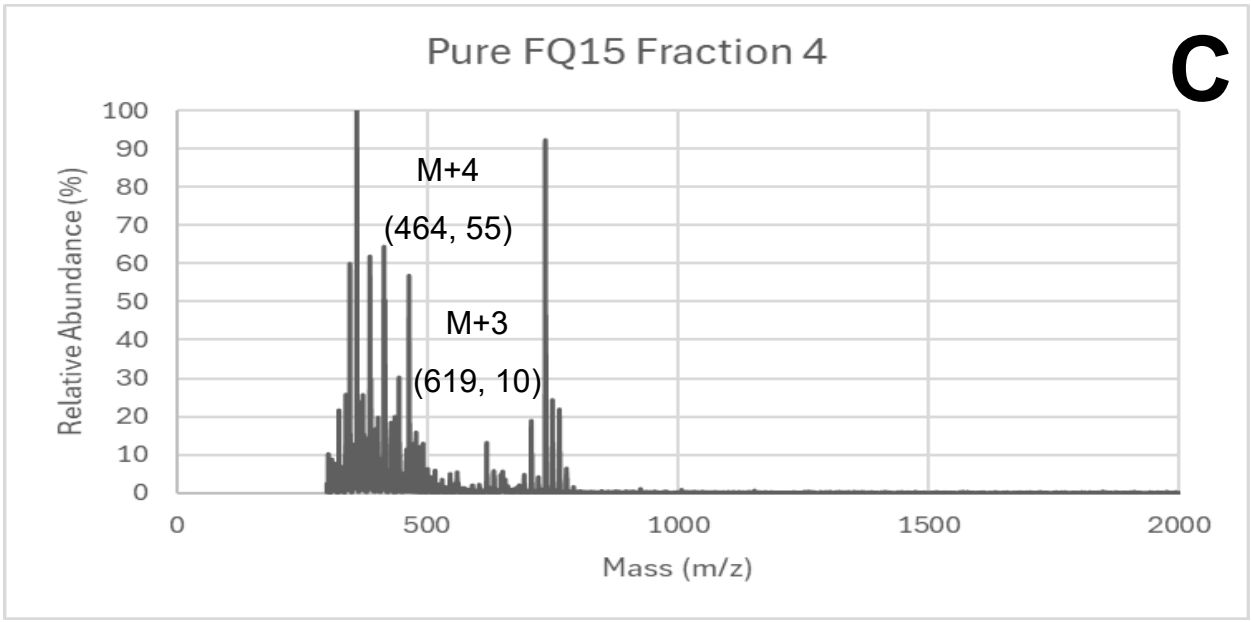
Figure 8C & 8D. Peptides FG10, and FG10MA were purified using semi- perparative HPLC. For peptide FG10 (**C**), fractions of 10 mL were collected during the time intrevals of 9-13 min, 30-35 min and 49-50 min. For peptide FG10MA (**D**), fractions of 10 mL were collected during the time intervals of 21-24 min, 35-40 min, and 48-50 min (Galaxy Software).

Line A contained milli- Q water with 0.1% v/v FA, while line B contained ACN with 0.1% FA. Whenever significant peaks were observed, they were manually collected into 50 mL falcon tubes for further examination and additional procedures. Both peptides were dissolved in a mixture of 30% ACN and water (v/v) with 0.1% v/v FA. Samples went through a gradual solvent titration until dissolved. The sample was gently warmed for a few seconds to aid in dissolving. Filtration was carried out using a 0.5 μm pore filter tip. The resulting filtrate was transferred into a 10 mL syringe and loaded into the injection loop for further processing. The experiment proceeded with a flow rate set at 6.0 mL/min, following the gradient concentration in **Table 9**. Data acquisition occurred at both 220 nm and 280 nm wavelengths, with a frequency of 1 Hz.

IV. *Mass Spectroscopy of Purified Peptide Fractions*







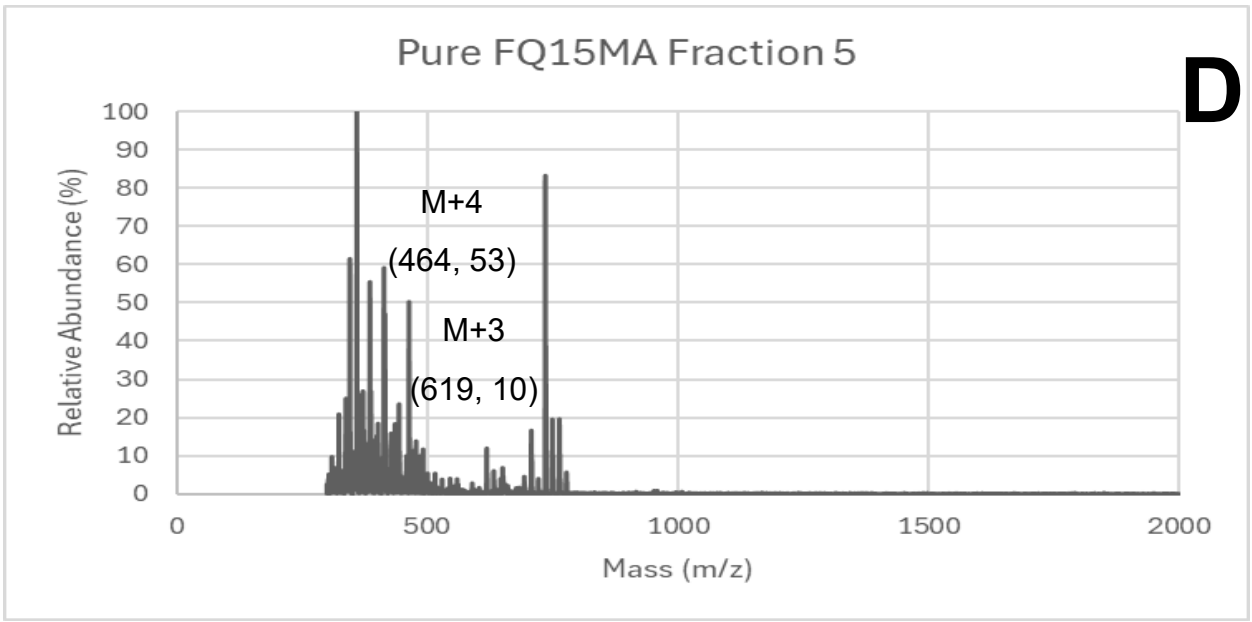


Figure 9. Presents MS data for the following pure peptides FQ15 fraction 1 (**A**), FQ15 fraction 2 (**B**), FQ15 fraction 4 (**C**) and FQ15MA fraction 5 **(D)**. Peptides were tested on MS to find multiple peaks with the same mass that will be combined for lyophilization. All the figures showed the M+3 and M+4 peaks. The expected mass for FQ15, and FQ15MA are 1849.92 g/mol and 2060.27 g/mol, respectively. Peaks M+3 and M+4 confirm the molecular weight for peptides FQ15 and FQ15MA (Excel).

After completing semi- preparatory HPLC, individual fractions underwent characterization using MS. A small amount of the peptide was dissolved in a very small amount of 50/50 A/B buffer (ACN+ 0.1% FA and water + 0.1% FA). These results guided the combination of fractions. Then following the process of rotary evaporator with dry ice, acidification, freezing at -80 ℃ and lyophilization into a dry powder using a freeze dryer.

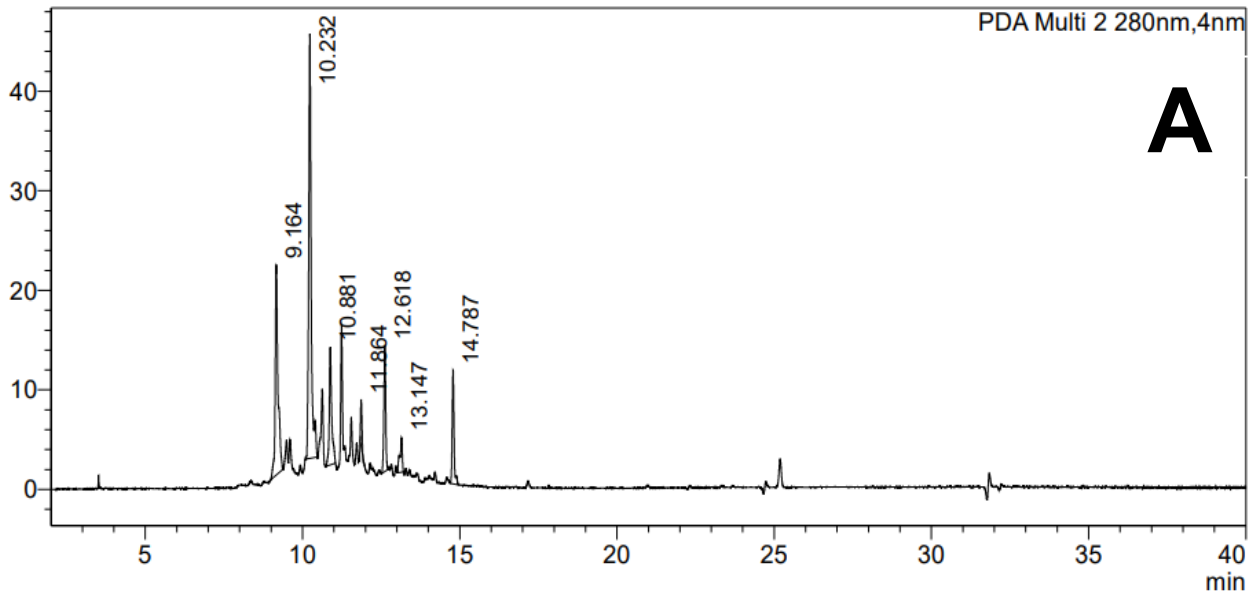
Table 10. Mass % yield of each peptide synthesized.

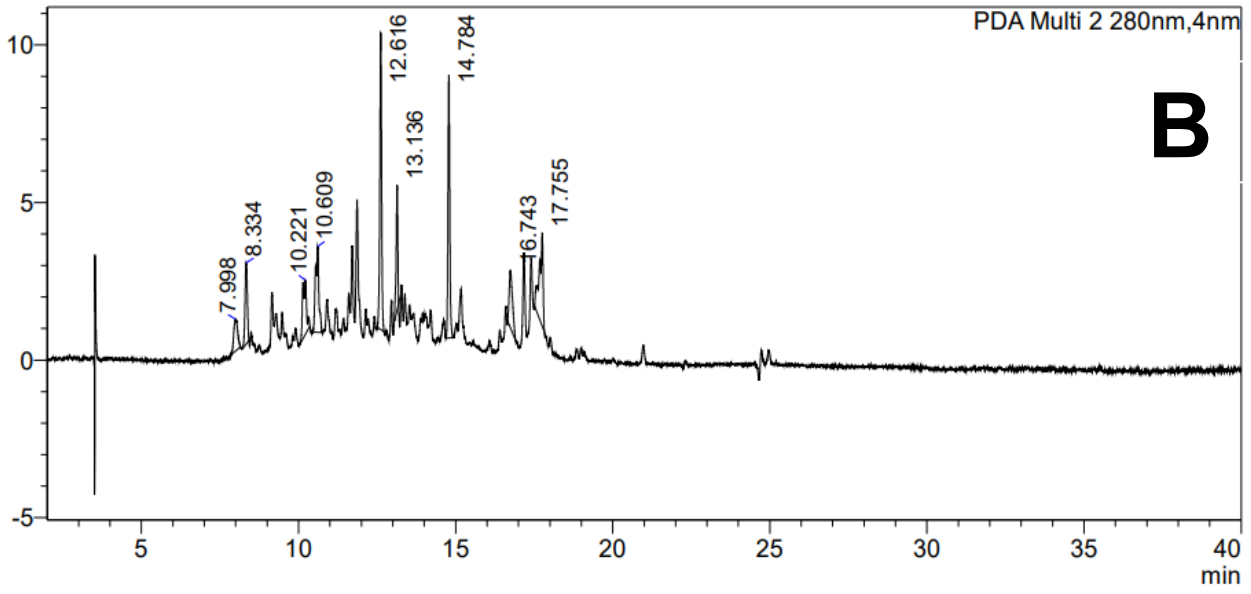
Peptide	FQ15	FQ15MA	FG10	FG10MA
% Yield	19.56%	12.72%	97.35%	52.62%

Equation 2. % purity

$$\% \text{ purity} = \frac{\text{Area under peptide peak}}{\text{Area under all peaks (including peptide peak)}}$$

V. *Analytical HPLC of Purified Peptide Fractions*





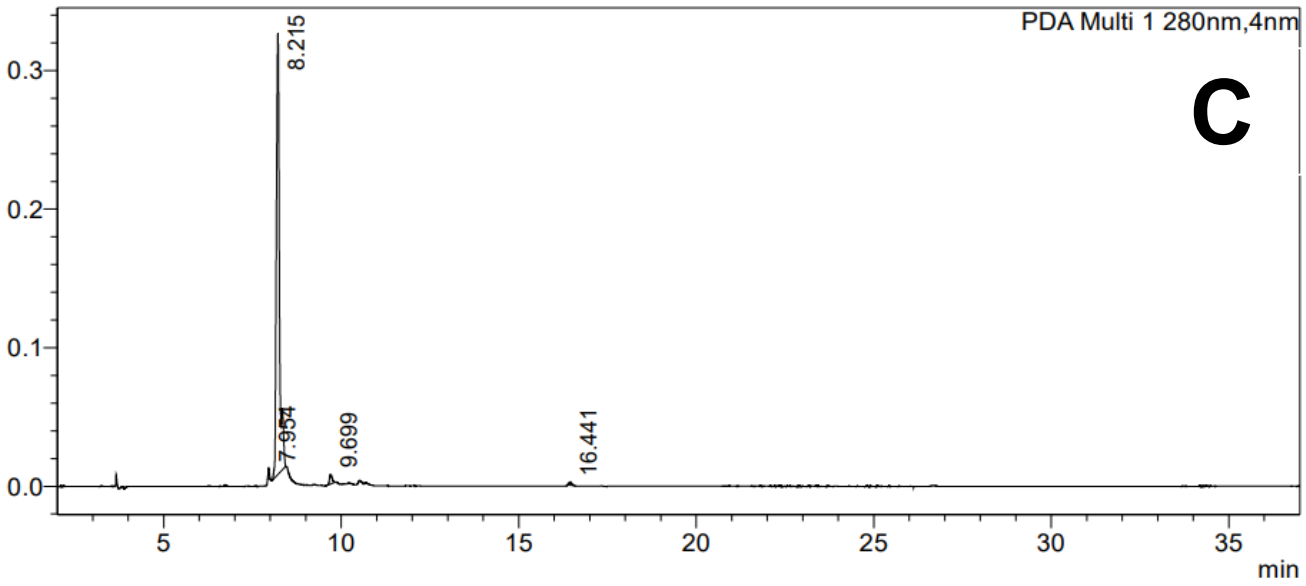


Figure 10. Analytical HPLC data at 280 nm due to tryptophan exhibiting absorbance at this wavelength for the following peptides: FQ15(**A**), FQ15MA (**B**), and FG10MA (**C**). The concentration of each peptide was 1 mg/mL. 30% of ACN + 70% of water solution was used to dissolve the peptides. Using 30/70 was the best option since 50/50 had some solubility problems and the peptides crashed before the solution was vortexed (Galaxy Software).

Table 11. (**A**), (**B**), and (**C**) present the peaks' retention time and area.

Peak #	Retention Time (Minutes)	Area
1	9.16	130490
2	10.23	232426
3	10.88	63168
4	11.86	12764
5	12.82	50770
6	13.15	17605
7	14.79	46083
Total	**A**	553305

Peak #	Retention Time (Minutes)	Area
1	7.99	8639
2	8.33	12617
3	10.22	15044
4	10.61	20159
5	12.62	37958
6	13.14	11473
7	14.78	32377
8	16.74	12941
9	17.76	23439
Total	**B**	174646

Peak #	Retention Time (Minutes)	Area
1	7.95	15989
2	8.22	1936658
3	9.69	35670
4	16.44	10886
Total	**C**	1999203

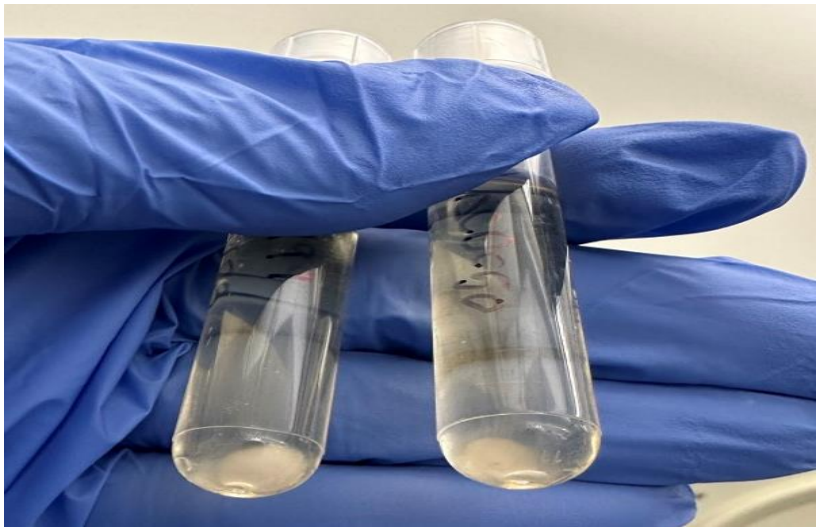
Table 12. The purity (%) of each peptide is presented at 280 nm. Purity was determined by taking the area of the peptide peak divided by the area of all peaks presented.

Peptide	Purity
FQ15	42.00%
FQ15MA	21.73%
FG10MA	96.87%

VI. *Peptides display MIC values against C. neoformans.*

Peptides' effectiveness against the fungal pathogen *C. neoformans* was assessed using MIC assays and checkerboard assays to determine their overall efficiency. New and fresh cells were obtained from the -80 ℃ freezer. Each peptide's effectiveness was examined against *C. neoformans* Δ*CDC50* and *H99* strains passed twice (2nd passage). Each strain was allowed to pass for 2 days at constant temperature, a cloudy spot at the bottom of the tube was observed which confirms a growth as depicted in **Figure 11**.

Figure 11. 2nd Passage of *H99* and Δ*CDC50 C. neoformans* strains.



Plates were streaked out for antifungal testing after vortexing each tube. Plates did not have any growth until 48 hours later. Δ*CDC50* strain grows less than the *H99* strain therefore the MIC assay was only tested for *H99* for peptide FQ15MA. Caspofungin was diluted to 6.4 mg/mL and each peptide was diluted to 256 μg/mL with DMSO. Nonlipidated peptides of FQ15 failed to inhibit *H99* strain at concentrations up to 256 μg/mL and showed negligible impact on enhancing caspofungin activity. Conversely, lipidated peptides displayed promising antifungal activity against *C. neoformans*, with MIC value of 16 μg/ mL and it is a reduced growth compared to the control. Peptides with lipid tails could enhance caspofungin effectiveness against the wild-type strain.

Table 13. MIC values for *H99 C. neoformans* strain. MIC assay was replicated twice to confirm results. Normalized concentrations in parentheses due to the purity being 42% for FQ15 and 21.73% for FQ15MA.

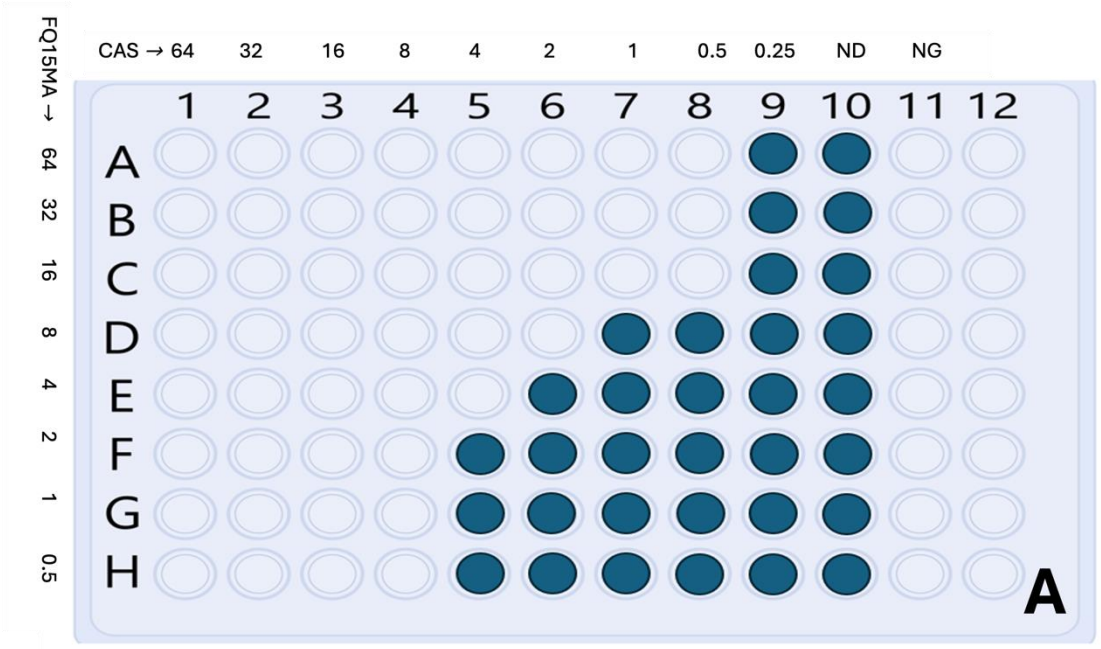
Time (Hours)	Caspofungin	FQ15	FQ15MA
24	No growth	No growth	No growth
48	8 μg/mL	128 (53.76) μg/mL	16 (3.48) μg/mL
72	16 μg/mL	256 (107.52) μg/mL	16 (3.48) μg/mL

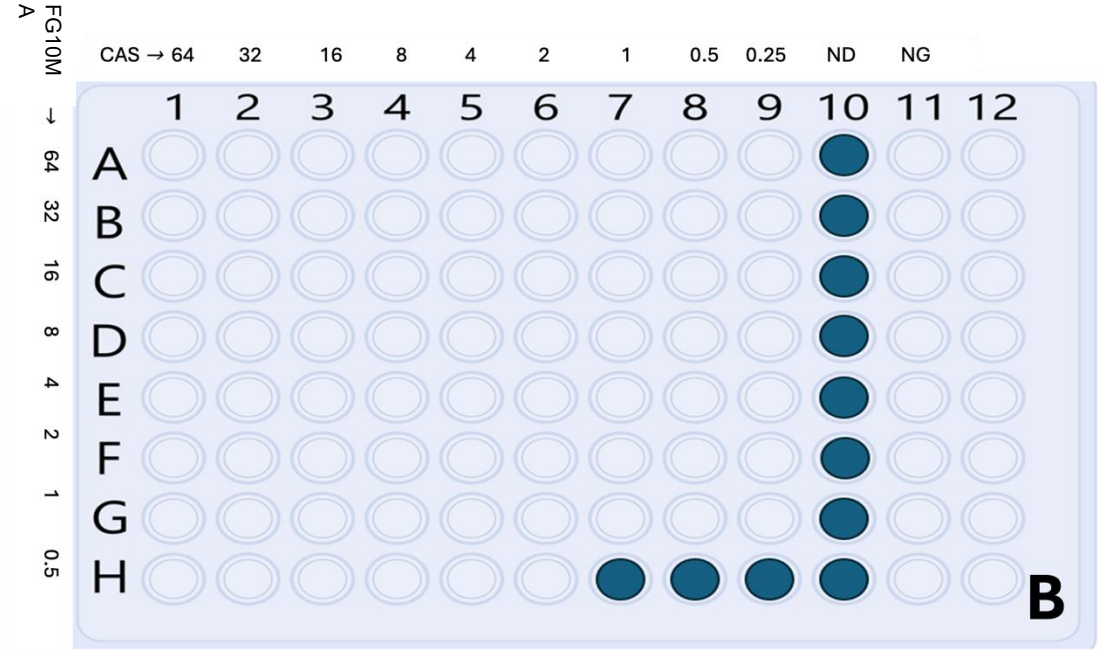
Table 14. MIC values for *H99* and Δ*CDC50 C. neoformans.* MIC assay was replicated three times to confirm results.

Time (Hours)	Caspofungin vs. Δ*CDC50*	Caspofungin vs. *H99*	FG10MA vs. Δ*CDC50*	FG10MA vs. *H99*
24	No growth	No growth	No growth	No growth
48	4 μg/mL	8 μg/mL	64 μg/mL	8 μg/mL
72	4 μg/mL	16 μg/mL	64 μg/mL	8 μg/mL

Moreover, FG10MA exhibited an MIC of 8 μg/mL against the *H99* strain and 64 μg/mL against the strain Δ*CDC50*. FQ15MA and FG10MA were promising candidates due to their effectiveness against Δ*CDC50* and *H99* strains *C. neoformans.*

VII. *Checkerboard Assay*





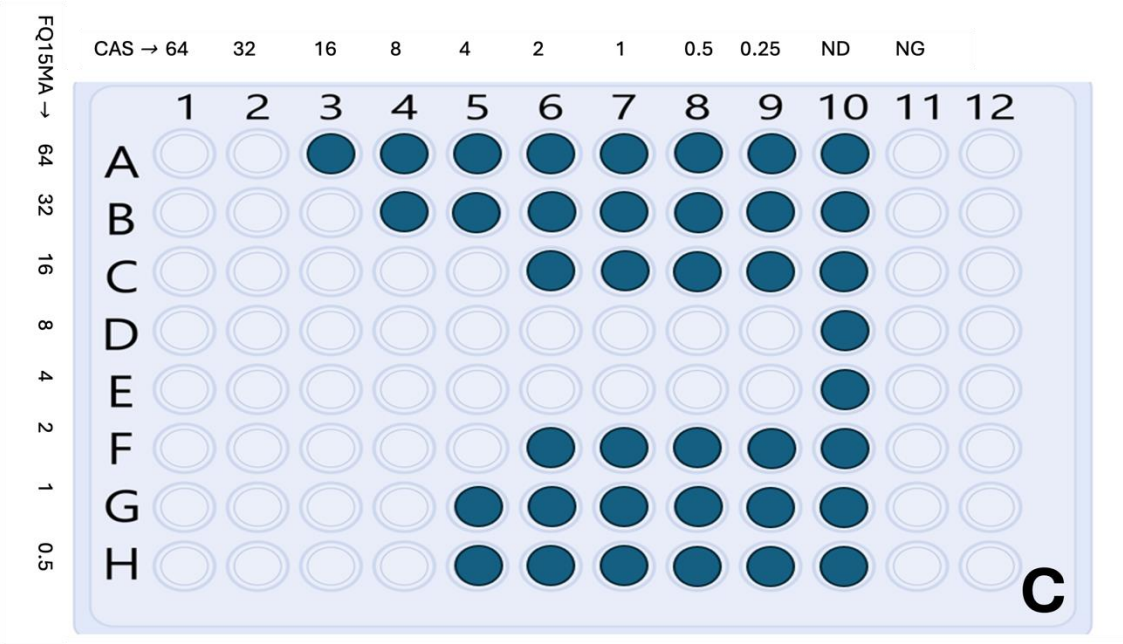


Figure 12. FQ15MA against *H99* (**A**), FG10MA against Δ$CDC50$ (**B**), and FG10MA against *H99* (**C**). Combing FQ15MA and FG10MA with caspofungin demonstrated synergy. These results show FQ15MA's and FG10MA's potential as antifungal peptides capable of boosting caspofungin's efficacy against both wild-type and mutant strains of *C. neoformans* (PowerPoint).

Chapter 5. Discussion

I. FIC Index Calculations

$$FIC\ Index = \frac{FIC_A}{MIC_A} + \frac{FIC_B}{MIC_B}$$

$$FQ15MA\ againt\ H99: FIC\ Index = \frac{4}{16} + \frac{2}{16} = \mathbf{0.375}$$

$$FG10MA\ against\ \Delta CDC50: FIC\ Index = \frac{0.5}{4} + \frac{1}{64} = \mathbf{0.141}$$

$$FG10MA\ againt\ H99: FIC\ Index = \frac{2}{16} + \frac{2}{8} = \mathbf{0.375}$$

These calculations prove that both peptides have synergy with caspofungin since FIC Index <0.5.

II. Shortening the peptide sequence led to improved activity.

The initial peptide length of 15 amino acids was selected based on previous findings utilizing loop-targeting antibodies. The objective was to explore whether shortening peptides to regions shorter than 15 amino acids would impact their activity. Thus, a second peptide was synthesized of the truncated peptide that has only one charged amino acid removed. Alanine's substitution will be done as future work. The results indicated that removing one charged amino acid for FG10 increased the activity of the peptide. Notably, FG10MA demonstrated improved activity, showing a detectable MIC value of 8 μg/mL and 64 μg/mL *against C. neoformans H99* and Δ*CDC50*, respectively. The myristic peptide tail contributes to the activity of the peptides.

Chapter 6. Conclusion

I. Conclusion

The objective of this project was to synthesize peptides that are linked to the CDC50 protein loop region, given their similarities in physical traits in hydrophobicity, net charge, molecular weight, and isoelectric point. The synthesis of diverse peptides and adding fatty acid tails is essential to fully understand their characteristics. FQ15 shared similar physical traits with AS15 peptide, which showed that they share potential antifungal properties as well. Peptides FQ15/FQ15MA and FG10/FG10MA could serve as antifungal flippase inhibitors in the future. A mass yield and purity yield of less than 50% was achieved in the synthesized peptides FQ15 and FQ15MA. The mass % yield for FQ15 was 19.56% and the purity % yield was 42.00%. The mass % yield for FQ15MA was 12.72% and the purity % yield was 21.73%. A more extensive HPLC method will be performed to improve purity. The mass and purity yield percentage were improved, where higher than 50% was achieved in the synthesized FG10 and FG10MA. The mass % yield for FG10 was 97.35%. The mass % yield for FG10 MA was 52.62% and the purity % yield was 96.87%. This study's goal was to synthesize FQ15 and FG10 peptides where they share similar physical traits with AS15 peptide, suggesting potential antifungal properties. This research allowed us to dive deeper into the functionality of these peptides and their possible usage in microbiology.

Drug resistance of microbes has been linked to the activity of flippases. The inhibition of the P4 ATPase flippase with a lipidated segment of the CDC50 protein amplifies the efficacy of caspofungin. These peptides serve as inhibitors. The lipid flippases present in *C. neoformans* and drug- resistant microbes stand out as promising crucial targets for drug development and antimicrobial medications. However, these flippases remain poorly characterized in many organisms and require deeper exploration. Advancing our understanding of the structures and

functions will facilitate the development of peptides, presenting an innovative strategy for enhancing the efficacy of currently available drugs in the market.

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