Synthesis and evaluation of antifungal peptoid derivatives against *Cryptococcus neoformans*

by

Meghan Wassom

A thesis presented to the Honors College of Middle Tennessee State University in partial fulfillment of the requirements for graduation from the University Honors College

Spring 2022

Thesis Committee:

Dr. Kevin L Bicker, Thesis Director

Dr. Scott Handy, Second Reader

Dr. Andrienne C. Friedli, Thesis Committee Chair

Synthesis and evaluation of antifungal peptoid derivatives against *Cryptococcus neoformans*

by Meghan Wassom

APPROVED:

_____

Dr. Kevin L. Bicker, Thesis Director

Associate Professor, Department of Chemistry

_____

Dr. Scott Handy, Second Reader

Professor, Department of Chemistry

_____

Dr. Andrienne C. Friedli, Thesis Committee Chair

Professor, Interim Chair, Department of Chemistry

Acknowledgements

First off, I would like to thank my thesis advisor, Dr. Kevin Bicker, for being such an incredible mentor and teacher. He has been such a role model for me and has always pushed me to strive for success. I would not have been able to complete this project without his patience, guidance, and support. I will always be so thankful that he decided to bring me into his lab group. I would also like to thank my lab members Mary Tran, Maria Clark, Devin Weaver, Erin Pratt, and Cameron Maddux for all the time and thoughtful discussions that helped me to continue my research. I would also like to thank Dr. Madison Green for his guidance and all of the time he took in teaching me lab techniques. Lastly, I would like to thank URECA for the partial funding for my project and the MTSU Chemistry Department.

Abstract

The fungi *Cryptococcus neoformans* is often the cause of cryptococcal meningitis in immunocompromised individuals. High focus has been placed on searching for antimicrobial drugs that have a relatively long half-life *in vivo* while also retaining a low mammalian cytotoxicity. The purpose of this project is to synthesize repeat and multimeric derivatives of a known antifungal peptoid, termed β-5, in an effort to increase the potency against the fungi *Cryptococcus neoformans* without increasing cytotoxicity towards mammalian cells. Repeats and multimeric derivatives of β-5 have successfully been synthesized, following protocols previously reported from our lab. These derivatives have been evaluated by traditional minimum inhibitory concentration (MIC) assays to evaluate antifungal potency. These derivatives have also been tested against HepG2 hepatocytes and erythrocytes to evaluate mammalian cytotoxicity. The future plans for this project are to begin synthesizing cyclic derivatives of β-5.

Table of Contents

List of Tables

List of Figures

Terms and Definitions

1. Minimum Inhibitory Concentration (MIC_{90}): the minimum concentration at which 90% of fungal growth has been inhibited.
2. Toxic Dose (TD_{50}): the concentration of compound that would result in 50% decrease of viable cells
3. HC_{10}: the compound concentration at which there was 10% hemolysis
4. H_{max}: the percent hemolysis at 100 µg/mL
5. Peptide: strings of amino acids condensed together.
6. Peptoids: N-substituted glycines; mimics of peptide structures that incorporate functional "R" groups on the amide nitrogen as opposed to peptides that have the R groups on the alpha carbon.
7. Solid phase synthesis: the peptoid chain is assembled stepwise, one submonomer at a time, while attached to an insoluble resin.
8. Submonomer Method: a method of solid-phase peptoid synthesis that consists of a bromoacylation of a terminal amine on a solid material, followed by a nucleophilic displacement of the added bromide by a primary amine.

Introduction

Cryptococcus neoformans and *Cryptococcus gattii* are the main fungal agents that lead to cryptococcal meningitis[1]. Cryptococcal meningitis is an infection that attacks the central nervous system (CNS)[1]. Generally, primary pulmonary infection happens after inhalation of fungal spores. If there is not a strong enough immune system to produce an effective immune response against the infection, the yeast can expand to the central nervous system (CNS)[2]. One study reported that it was more common for *C. neoformans* caused cryptococcosis to occur in immunocompromised individuals and for *C. gattii* caused cryptococcosis to occur in the elderly, immunocompetent hosts[3]. Immunocompromised individuals include those diagnosed with HIV/AIDS, organ transplant patients, and individuals with autoimmune diseases [3]. In immunocompromised individuals, contracting cryptococcal meningitis could turn fatal[4]. The most recent study found that fungal infections that lead to cryptococcosis are more common worldwide with *C. neoformans* than with *C. gattii*[5]. A 2014 study reported that roughly 223,000 individuals' contract cryptococcal meningitis and about 181,000 (82%) individuals die annually from cryptococcal meningitis[6]. Many known antifungal agents are used to treat *C. neoformans*. The main drugs given to treat these infections include amphotericin B, flucytosine, and fluconazole. Due to long term drug treatment and overuse, many of these treatments exhibit drug resistance and mammalian cytotoxicity[7]. These treatments can also result in gastrointestinal issues, vomiting, and hepatitis because of their high mammalian cytotoxicity[8].

Antimicrobial peptides (AMP) are commonly used by organisms to target specific fungal pathogens over mammalian cells[9]. The mode of action of AMPs is typically known to be that the peptide compromises the fungal membrane's integrity, causing a leak in the cell's content that results in cell death[10]. One reason AMPs are so useful is because they are minimally toxic to mammalian cells. AMPs also have a very high specificity for microorganisms compared to mammalian cells[10]. However, AMPs do degrade easily due to proteases present *in vivo*, giving them poor half-lives and limiting their clinical use[11].

Peptoids, also called N-substituted glycines, are more useful than peptides because they are not recognized by proteases and therefore have longer half-lives *in vivo*[12]. The difference between peptoids and peptides is that peptides have an R group attached to the α carbon and peptoids have an R group attached to the amide nitrogen[13]. This slight change of the location of the R group between peptoids and peptides is what makes peptoids slower to break down *in vivo*[12]. Due to their increased stability *in vivo*, peptoids are a much better candidate to treat fungal infections than AMPs. Peptoids are also cost effective and can be easily synthesized using a solid phase submonomer method[14]. Peptoids maintain many of the properties of peptides, like having low mammalian toxicity[14]. These advances make peptoids a great candidate for antifungal drugs.

A peptoid with similar effectiveness against *C. neoformans* as many mainline treatments has been previously reported, termed AEC5[15]. This peptoid was discovered using a high-throughput screening assay called the Peptoid Library Agar Diffusion (PLAD) assay[16]. The PLAD assay allows for the screening of peptoid libraries with varying R group combinations[16]. An important part of developing new peptoids is to increase the compound's potency against pathogens, while decreasing any mammalian cytotoxicity.

This can be done by changing the identity and position of each submonomer of the peptoid. AEC5 is a tripeptoid that consists of a 13-carbon alkyl tail, a positively charged amine, and a furan ring[15]. After characterization, AEC5 displayed low mammalian cytotoxicity towards a HepG2 cell line and increased potency against *C. neoformans*[15].

In addition, a peptoid, β5, was found to have decreased toxicity toward mammalian cells and improved potency against *C. neoformans* when compared to AEC5[13]. β5 was identified by performing a sarcosine scan on the previously identified peptoid, AEC5 (**Figure 1**)[13]. The purpose of the sarcosine scan was to determine the pharmacological importance of AEC5's three unique monomers that each have a different chemical moiety. The sarcosine scan was done by replacing each submonomer with a sarcosine residue. This was followed with a three-round iterative structure activity relationship (SAR) study that explored different chemical groups in each of AEC5's positions. The first round of SAR evidenced the importance that the alkyl tail length had on potency against *C. neoformans*[13]. It was concluded that the best antimicrobial potency and least amount of cytotoxicity resulted from positioning a 13-carbon alkyl tail in position 1[13]. When the furan heterocycle of AEC5 was replaced with sarcosine, the antifungal potency decreased, and the cytotoxicity stayed very similar to AEC5[13]. It was determined that placing a thiophene ring in position 3 provided the best efficacy against *C. neoformans* while also improving mammalian cytotoxicity[13]. The third round of SAR revealed that a trimethylated amine locked in the cationic state in position 2 provided the best outcome of potency against *C. neoformans* and the lowest mammalian cytotoxicity[13]. Through many rounds of SAR, β5 (**Figure 2.**) was identified as being more potent against *C. neoformans* and less toxic towards mammalian cells than AEC5[13].

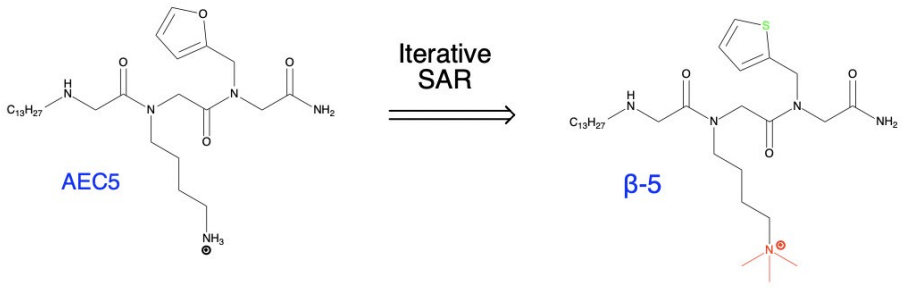

Figure 1. Structure-activity relationship of AEC5 and β5. This figure explains the results from the iterative structure activity relationship (SAR) study[13]. In position 2, the cationic amine (from AEC5) was changed to a trimethylated cationic amine (β5). In position 3, the furan ring (from AEC5) was changed to a thiophene ring (β5).

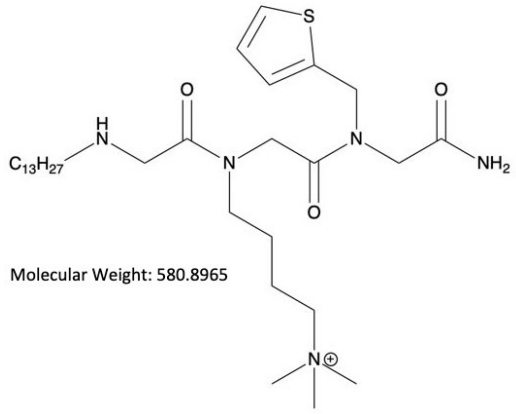

Figure 2. The structure and molecular weight of peptoid β5.

According to previously reported data, the residues on β5 demonstrated the best antifungal and mammalian cytotoxicity properties[13]. In order to explore new derivatives of β5, it was hypothesized that by repeating β5 and also linking the repeats with amino acid linkers, it would be possible to increase the peptoid's ability to selectively target the fungal cell membrane of *C. neoformans*, while also retaining a low cytotoxicity towards HepG2 cells. The main objective of this project was to design, synthesize, and characterize repeat and dimeric version of β5. Amino acid linkers, specifically lysine, are commonly used in cross linking[17,18]. In addition to incorporating amino acids, repeating monomers on a single compound would add more opportunities for multivalent interactions. Multivalent interactions are known for enhancing binding affinity[12]. By repeating the 3 monomers of β5 on a singular compound, it could potentially increase the effective molarity by providing more interactions between the compound and the fungal membrane.

Experimental

I. Synthesis of β52X derivative

The synthesis of the β5 derivative, β52X **(Figure 4.),** was performed using the submonomer method **(Figure 5.)**. The submonomer method is a process of solid phase synthesis where peptoid monomers are individually added to solid phase synthesis resin in a chain-like fashion through an S_N2 reaction[14]. This process involves a series of bromoacylation and amination steps, where a desired amine replaces a bromine on a specific monomer. This process begins by swelling Rink Amide resin (loading capacity: 0.75 mmol/g) with dimethylformamide (DMF) for 20 minutes. The resin was then treated with 20% piperdine in DMF and set to rock for 10 minutes. After repeating this step twice, the resin was washed with 3-5 mL of DMF. This step deprotects the resin by removing Fmoc groups. To confirm the deprotection, a Kaiser test was performed by adding 75 μL of ninhydrin to a small sample of resin in a centrifuge tube. After briefly centrifuging, the tube is then placed in a laboratory oven for 45 seconds. If the resin stayed colorless, the test is negative, indicating no free amines. If the resin sample turned a dark purple color, this indicated a positive test and that free amines were present due to a successful deprotection. The acylation step was performed using 1.5 mL of 2 M bromoacetic acid in anhydrous DMF and 1.5 mL of 3.2 M N,N′-diisopropylcarbodiimide (DIC) in anhydrous DMF. The mixture was added to the resin, shaken, and microwaved for 15 seconds twice at 10% power and then rocked for 8 minutes. The resin was then washed with DMF 3 times

to remove unreacted starting material and by-products. A Kaiser test was then performed to confirm the addition of the bromine to the primary amine, a negative test would be expected which would exhibit no color change. The bromine was subsequently substituted with an amine by adding 3 mL of 2 M 2-thiophenemethylamine in anhydrous DMF to the resin. The resin was subsequently shaken, microwaved, rocked, and washed with DMF as described above. The amination is confirmed by performing a Kaiser test, which at this step would be expected to be positive, providing a purple color change in the presence of free amines. The bromoacylation and amination steps described above were then repeated for the addition of 1,4-diaminobutane. After the addition of 1,4-diaminobutane, 10 equivalents of 2-acetyldimedone (DDE) in 5 mL of DMF was added to the resin and rocked for 30 minutes at room temperature. This reaction step served to add a protecting group (DDE) to the primary amine of the 1,4-diaminobutane, to protect it from succeeding amination steps. The synthesis was checked by adding 500 µL of trifluoroacetic acid (TFA) to a small sample of resin in a 1.5 mL centrifuge tube. The tube was rocked for 30 minutes, and the solution was then bubbled off. After the TFA was bubbled off, 500 µL of water was added to the tube in preparation for the sample to be analyzed using Electrospray Ionization Mass Spectrometry (ESI-MS) on a Waters Synapt ESI-MS instrument. After the sample mass was verified, synthesis would continue. The third monomeric unit, aminotridecane, was installed by repeating the bromoacylation and amination step provided above.

To synthesize the dimer of β5, the bromoacylation and amination steps for the addition of the three monomeric units (thiophene ring, DDE protection, 1,4-diaminobutane, and the tridecyl tail) were repeated.

After the final addition of aminotridecane was completed, a Boc protection was performed to protect the N-terminus before deprotecting the DDE-diaminobutane. A solution of 10 molar equivalents of Boc-anhydride in 3 mL of 5% N-methylmorpholine (NMM) in DMF was added to the resin. To prevent pressure build-up, the reaction vessel was inverted, a flea stir bar was added, and the Luer cap was removed before transferring the reaction vessel to a stir plate for 1 hour at room temperature. Next, the DDE protecting group was removed by treating the resin with 3 mL of 2% hydrazine in DMF. The resin was then rocked for 30 minutes at room temperature. The resin was washed 3 times with DMF and then the DDE deprotection step was repeated. A Kaiser test was then performed to confirm the removal of the DDE group.

The primary amine at position two was then methylated by treating the resin with 10 molar equivalents of methyl iodide and 5 molar equivalents of cesium carbonate in DMF. The resin was then rocked overnight at room temperature. To confirm methylation, a sample of the resin was analyzed using ESI-MS using the procedure described above.

After the desired groups were added and the peptoid chain was complete, the peptoid was cleaved from the resin. This was done by adding a total volume of 5 mL of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% H_2O to the resin and rocked for 1 hour. The solution was drained and collected from the resin and bubbled for 30 minutes to remove TFA. The solution was then resuspended in 1:1 acetonitrile and water to prepare for purification. The solution was purified by reverse-phase High Performance Liquid Chromatography (RP-HPLC) on a Varian Prepstar instrument with a C18 semi-prep column and a gradient of 100% water to 100% acetonitrile containing 0.05% TFA. The samples that were collected were analyzed using ESI-MS to confirm their

identity. The final compound was then frozen with liquid nitrogen and dried down using a lyophilizer.

II. Synthesis of β5D1 derivative

The synthesis of the β5 derivative, β5D1 **(Figure 4)**, was also performed using the submonomer method **(Figure 5)**. This synthesis begins by swelling Rink amide resin with DMF and treating with 20% piperdine in DMF to deprotect the resin by removing Fmoc groups as described in the previous synthesis. The amino acid linker, Fmoc-Lys(Mmt)-OH, was then coupled to the resin using peptide chemistry. To do this, 10 molar equivalents of Fmoc-Lys(Mmt)-OH, 4 molar equivalents of N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), and 5 mL of 5% NMM in DMF were added to a glass vial and allowed to react for 10 minutes. This solution was then added to the resin and rocked for 1 hour at room temperature. The resin was then washed with DMF 3 times, and a Kaiser test was performed. At this point in the synthesis, the Kaiser test should be negative, indicating that the amino acid coupling was successful. Then to remove Mmt groups, the resin was treated with 3 mL of 1% TFA in CH_2Cl_2 for 10 minutes 7 times. The resin was then washed with DMF 3 times, and a Kaiser test was performed. At this point in the synthesis, the Kaiser test should be positive, indicating that the Mmt protecting groups have been removed. The resin was then treated with 20% piperdine in DMF for 10 minutes 2 times to remove the Fmoc groups. After removing Fmoc groups, the resin is then

washed with DMF 3 times. The next step would be to proceed with normal β5 synthesis, cleavage, and purification procedures as previously described.

III. Synthesis of β5D2 and β5D3 derivatives

The synthesis of the β5 derivatives, β5D2 and β5D3 **(Figure 4)**, was also performed using the submonomer method **(Figure 5)**. For both derivatives, the synthesis begins by swelling Rink amide resin with DMF and treating with 20% piperdine in DMF to deprotect the resin by removing Fmoc groups as described in the previous synthesis. The amino acid linker, Fmoc-Lys(Mmt)-OH, was then coupled to the resin using peptide chemistry. To do this, 10 molar equivalents of Fmoc-Lys(Mmt)-OH, 4 molar equivalents of HBTU, and 5 mL of 5% NMM in DMF were added to a glass vial to and allowed to react for 10 minutes. This solution was added to the resin and rocked for 1 hour at room temperature. The resin was then washed with DMF 3 times, and a Kaiser test was performed. At this point in the synthesis, the Kaiser test should be negative, indicating that the amino acid coupling was successful. The resin was treated with 20% piperdine in DMF for 10 minutes 2 times to remove the Fmoc groups. After removing Fmoc groups, the resin was then washed with DMF 3 times. For the synthesis of β5D2, the amino acid linker, Fmoc- β-Ala-OH, was coupled to the resin using peptide chemistry. To do this, 10 molar equivalents of Fmoc- β-Ala-OH, 4 molar equivalents of HBTU, and 5 mL of 5% NMM in DMF were added to a glass vial to and allowed to react for 10 minutes. This solution was added to the resin and rocked for 1 hour at room temperature. The resin was then washed with DMF 3 times. For

the synthesis of β5D3, the amino acid linker, Fmoc-Aca-OH, was coupled to the resin using peptide chemistry. To do this, 10 molar equivalents of Fmoc-Aca-OH, 4 molar equivalents of HBTU, and 5 mL of 5% NMM in DMF were added to a glass vial to and allowed to react for 10 minutes. This solution was added to the resin and rocked for 1 hour at room temperature. The resin was then washed with DMF 3 times. The rest of the synthesis was the same for both β5D2 and β5D3. The next step would be to remove Mmt groups. To do this, the resin was treated with 3 mL of 1% TFA in CH_2Cl_2 for 10 minutes 7 times. The resin was then washed with DMF 3 times, and a Kaiser test was performed. At this point in the synthesis, the Kaiser test should be positive, indicating that the Mmt protecting groups have been removed. The resin was then treated with 20% piperdine in DMF for 10 minutes 2 times to remove the Fmoc groups. After removing Fmoc groups, the resin was washed with DMF 3 times. The next step would be to proceed with normal β5 synthesis, cleavage, and purification procedures as previously described.

IV. Biological Characterization of β5 derivatives

The Minimum Inhibition Concentration assay (MIC) against *Cryptococcus neoformans* was then performed with all β5 derivatives. The MIC_{90} is defined as the minimum compound concentration resulting in greater than 90% inhibition of fungal growth consistently on multiple days. To begin this process a yeast peptoid dextrose (YPD) plate was streaked with frozen stock of *C. neoformans* for isolation and was incubated at 37 ˚C for 96 hours. Two-fold serial dilutions of all peptoids were prepared in water at 100x

the concentration to be tested. The culture was inoculated in a 5 mL solution of 0.85% saline until there was an optical density (OD_{530}). The optical density was adjusted to be within the range of 0.15-0.25. Next, a 1:100 solution of Rosewell Park Memorial Institute (RPMI) media containing 3-morpholinopropane sulfonic acid (MOPS) and cells was prepared by adding 100 µL of the 0.85% saline inoculated with cells solution to 9.9 mL of RMPI-MOPS. A 1:20 solution of RPMI-MOPS and cells was prepared by adding 500 µL of the 1:100 solution to a 9.5 mL of RPMI-MOPS. In a 96-well plate, 198 µL of 1:20 cell solution was added to each well. Next, 2 µL of 100x peptoid stock was added to the 96-well plate in triplicate. Sterile water was used as a negative control and Amphotericin B (AmpB; 2 µg/mL) was used as a positive control. To prevent any evaporation during incubation, 200 µL phosphate-buffered saline (PBS) was added to all surrounding cells. The plate was then incubated at 37 ˚C for 72 hours and manually read to determine the MIC. All MIC assays were collected in biological triplicate.

The cytotoxicity of all β5 derivatives was then evaluated against HepG2 hepatocytes and human red blood cells (hRBCs). For HepG2 hepatocytes, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin, streptomycin, and glutamine (PSG), at 37 ˚C in a 5% CO_2 incubator. Cells were then pipetted into a 96-well plate in phenol red free DMEM containing 10% FBS. After 2-3 hours, the cells were treated with 10x stock solutions of each concentration to be tested. The 10x stock solutions were prepared through two-fold serial dilutions using sterile water. The concentrations ranged from 200-3.125 µg/mL and sterile water was used as a negative control. The plate was then incubated at 37 ˚C for 72 hours. Next, 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) in sterile water was added to each well being tested. The plate was then incubated at 37 ˚C for 3.5 hours. In the effort to not disturb adhered cells, the media was gently removed using an electronic pipettor and glass Pasteur pipette and discarded. To lyse the cells and solubilize metabolized MTT, 100 µL of dimethyl sulfoxide (DMSO) was added and the plate for incubated for an additional 15 minutes. Absorbance was measured and recorded using a SpectraMax M5 Plate Reader set at 570 nm. Percent inhibition was calculated to produce the half-maximal inhibitory concentration (IC_{50}) using GraFit. The IC_{50} represents the concentration of compound at which there is a decrease in at least 50% of viable cells (also known as toxic dose or TD_{50}). This assay was repeated at least three times across different days.

For erythrocyte testing, human red blood cells (hRBCs), red blood cells were washed 3x with a PBS buffer by centrifugation and aliquoted into a 96-well plate. Two-fold serial dilutions of peptoid solutions were added to the wells along with negative control (PBS) and positive (1% Triton X-100) control. The plate was incubated at 37 ˚C for 1 hour, centrifuged, and 5 µL of supernatant was diluted into 95 µL of PBS in a new 96-well plate. Released hemoglobin was measured using a SpectraMax M5 Plate Reader reading the absorbance at 405 nm. Absorbance was collected and used to calculate percent hemolysis (HC_{10}). HC_{10} refers to the hemolytic ability of a compound at which there was 10% hemolysis. This assay was repeated at least three times across different days.

Results and Discussion

I. Compound Synthesis and Verification

The repeat peptoid, β52X, was synthesized using a similar reaction scheme as β5 (**Figure 3**). However, with β52X, the bromoacylation and amination steps for the addition of the three monomeric units (thiophene ring, DDE protection, 1,4-diaminobutane, and the tridecyl tail) were repeated. To complete the synthesis of β52X, a Boc protection was performed on the amine at the N-terminus, DDE deprotection was performed, and the cationic amines were trimethylated using methyl iodide and cesium carbonate. Then the compound was verified using ESI-MS before the compound was then cleaved from the resin and purified (**Appendix 1**). The product yield of β52X was measured to be 4.9 mg (percent yield = 8.9%) (**Table 1**).

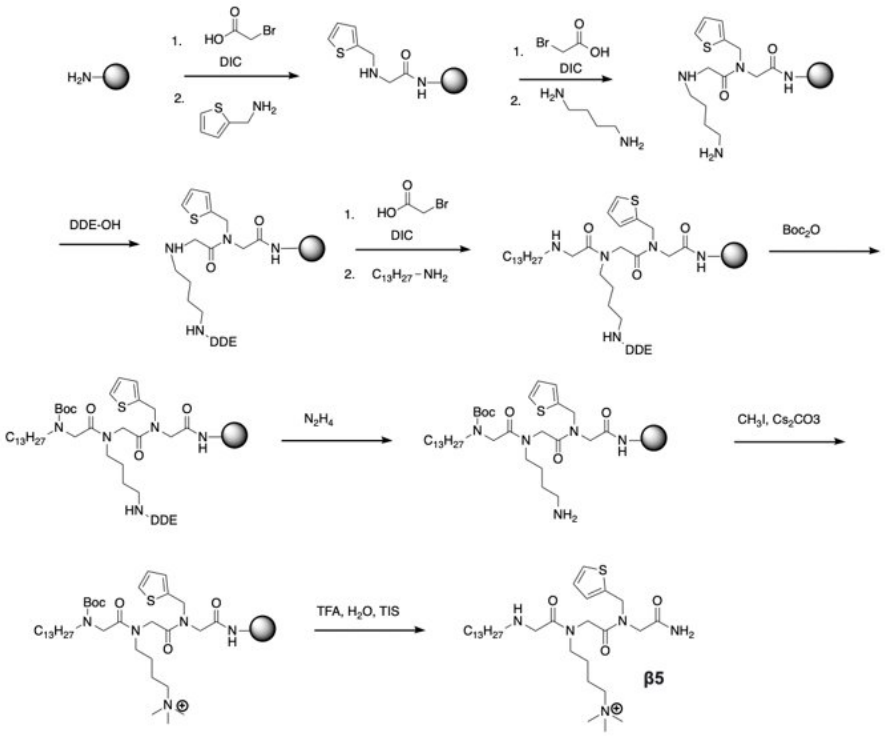

Figure 3. Reaction scheme for the Solid Phase Synthesis of β5. This figure shows the repeated bromoacylation and amination steps required to synthesize β5. This scheme also shows the protecting groups, DDE and Boc, that serve to keep succeeding steps from acylating or methylating any free amines required later in the synthesis. The cationic amine is trimethylated nearing the end of the synthesis. Boc is removed during the cleavage of β5 from resin with TFA, H_2O, and TIS.

A series of β5 dimers were synthesized to explore how dimerization of this peptoid would affect its antifungal efficacy and mammalian cytotoxicity. Three dimers were synthesized, each with a different length linker between the two molecules of β5 to determine if spacing between the dimer would affect biological properties **(Figure 4)**. The

first dimer, β5D1, was synthesized by coupling Fmoc-Lys(Mmt)-OH to the resin using peptide chemistry to serve as a branching point and linker between two molecules of β5. After coupling the amino acid linker and deprotecting Fmoc and Mmt, the synthesis proceeded using the previously described β5 synthesis **(Figure 5)**. Before cleaving β5D1 from the resin, the compound was verified using ESI-MS (**Appendix 2**). The product yield of β5D1 was measured to be 3.3 mg (percent yield = 5.5%) (**Table 1**).

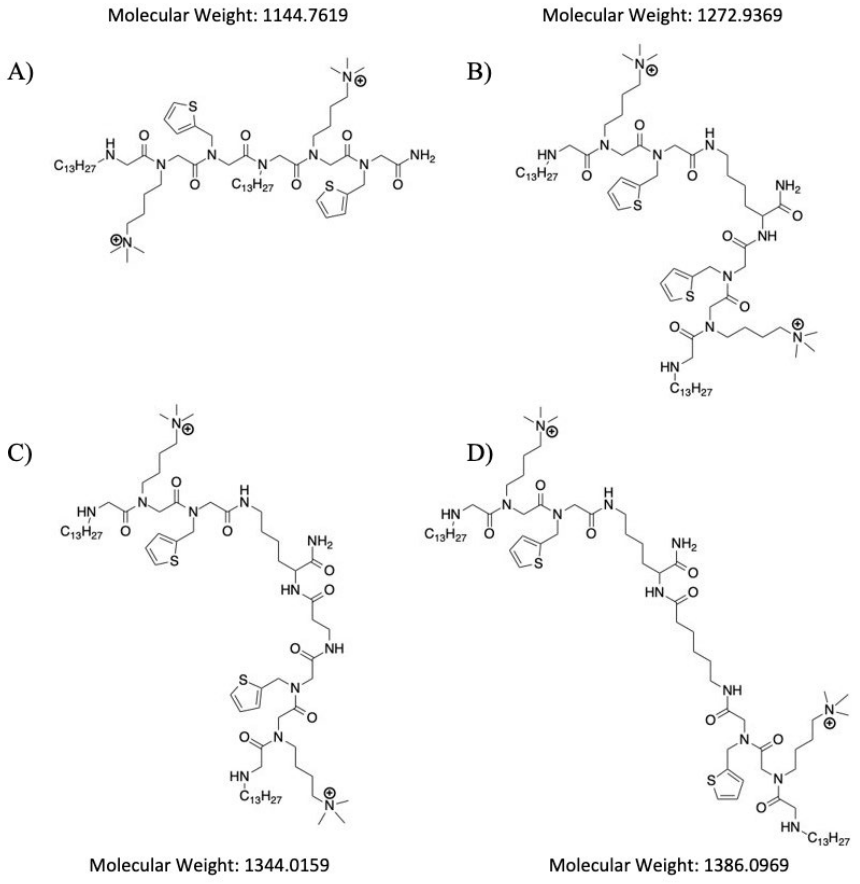

Figure 4. The structures and molecular weights of derivatives of β5. (A) β52X. (B) β5D1. (C) β5D2. (D) β5D3.

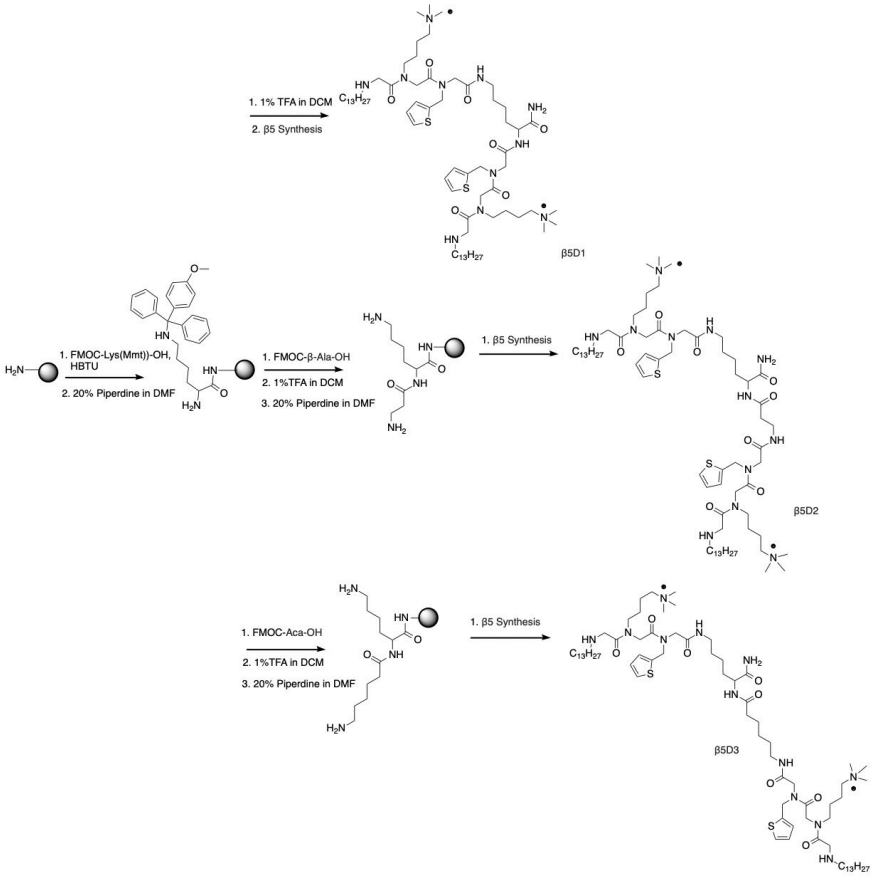

Figure 5. Reaction scheme for the Solid Phase Synthesis of β5D1, β5D2, β5D3. This scheme shows the coupling of Fmoc-Lys(Mmt)-OH as a building block for synthesizing all β5 derivatives. For β5D1, the synthesis proceeds with normal β5 synthesis. For β5D2, Fmoc- β-Ala-OH is coupled to the peptoid and proceeds with normal β5 synthesis. For β5D3, Fmoc- Aca-OH is coupled to the peptoid and then continues normal β5 synthesis.

The second dimer, β5D2, was synthesized by coupling Fmoc-Lys(Mmt)-OH to the resin using peptide chemistry followed by removal of the Fmoc group from the α-amine of lysine. After the addition of the first amino acid linker, Fmoc- β-Ala-OH was also coupled to the resin using peptide chemistry to give a slightly longer linker between the two molecules of β5 than β5D1.The synthesis then proceeded using the previously described β5 synthesis **(Figure 5)**. Before cleaving β5D2 from the resin, the compound was verified using ESI-MS (**Appendix 3**). The product yield of β5D2 was measured to be 4.2 mg (percent yield = 5.1%) (**Table 1**).

The second dimer, β5D3, was synthesized by coupling Fmoc-Lys(Mmt)-OH to the resin using peptide chemistry followed by removal of the Fmoc group from the α-amine of lysine. After the addition of the first amino acid linker, Fmoc-Aca-OH was also coupled to the resin using peptide chemistry to give a slightly longer branch between the two molecules of β5 than β5D2. The synthesis then proceeded using the previously described β5 synthesis **(Figure 5)**. Before cleaving β5D3 from the resin, the compound was verified using ESI-MS (**Appendix 4**). The product yield of β5D3 was measured to be 3.7 mg (percent yield = 5.7%) (**Table 1**).

Table 1. Compound name, molecular weight (g/mol), net yield (mg), and percent yield (%) for all derivatives of β5.

Compound	Molecular Weight (g/mol)	Yield (mg)	Percent Yield (%)
β52X	1144.8	4.9	8.9
β5D1	1272.9	3.3	5.5
β5D2	1344.0	4.2	5.1
β5D3	1386.1	3.7	5.7

II. Minimum Inhibitory Concentration (MIC)

All synthesized β5 derivatives were evaluated for their efficacy against the fungal pathogen, *C. neoformans*. This was done using the broth microdilution method in 96-well plates. The compounds were tested in 2-fold serial dilution at concentrations ranging from 25-0.78 μg/mL. A previously tested and published value of MIC for β5 was reported as 3.13 μg/mL[13]. The value of MIC for β52X and β5D1 was determined to be 6.3 μg/mL (**Table 2**). Additionally, the value of MIC for β5D2 and β5D3 was determined to be 3.13 μg/mL (**Table 2**). When compared to the previously published value of MIC for β5, it suggests that β52 and β53 have similar efficacy against *C. neoformans* as β5. The results also indicate that β52X and β5D1 do not have as strong activity against *C. neoformans*,

though only 2-fold less. Overall, the results from this assay showed that antifungal potency decreased slightly in comparison to β5 with derivatives β52X and β5D1 and that there was no change in antifungal potency when compared to β5 with derivatives β5D2 and β5D3. We anticipated that repeating the β5 sequence or using the dimer could improve antifungal potency, but this was not the case.

III. Mammalian Toxicity Against HepG2 Cells

To determine the toxicity against mammalian cells, all of the newly synthesized β5 derivatives were tested using HepG2 liver cells. All compounds delivered *in vivo* have to first pass through the liver, so these cells serve as a good indicator of initial toxicity. The purpose of this assay was to determine the concentration of compound that would result in 50% decrease of viable cells (TD_{50}). A previously reported value of TD_{50} for β5 is 91.2 μg/mL[13]. The TD_{50} for β5 derivatives was determined to be 23.9 μg/mL for β52X, 31.5 μg/mL for β5D1, 27.3 μg/mL for β5D2, and 30.7 μg/mL for β5D3 (**Table 2**). Overall, in comparison to the previously reported TD_{50} value for β5, all new derivatives were found to be around three-fold more toxic towards HepG2 cells. In general, increased mammalian cytotoxicity usually goes hand-in-hand with increased antifungal efficacy, however, this was not the case for the compounds tested here.

IV. Mammalian Toxicity Against Human Red Blood Cells

In an additional effort to determine the mammalian toxicity, all of the newly synthesized β5 derivatives were evaluated against human red blood cells (hRBCs). Because of the ubiquitous nature of RBCs in the body and their sensitivity towards drug-like molecules, determining the hemolytic activity of a compound is an important part of characterization. The purpose of this assay was to determine the compound concentration at which there was 10% hemolysis (HC_{10}) and the percent hemolysis at 100 μg/mL (H_{max}). Previously reported data on β5 for this assay determined an HC_{10} value of 42.6 μg/mL and an H_{max} value of 46.0%[13]. Because hRBCs are primary cells that come from donors, different donor samples can have dramatically varying sensitivity to hemolysis. Therefore, β5 and all newly synthesized β5 derivatives were tested against hRBCs originating from the same donor. In this trial, it was determined that β5 had an HC_{10} of 81.3 μg/mL and an H_{max} of 27.6% (**Table 2**). β52X was found to have an HC_{10} of 50.1 μg/mL and an H_{max} of 55.9%. β5D1 was found to have an HC_{10} of 43.7 μg/mL and an H_{max} of 45.1%. β5D2 was found to have an HC_{10} of 57.3 μg/mL and an H_{max} of 36.3%%. β5D3 was found to have an HC_{10} of 45.7 μg/mL and an H_{max} of 58.5%. Overall, this assay confirmed that all newly synthesized β5 derivatives tested were more hemolytic than β5. Again, this finding goes against a general trend seen with antifungal peptoids that would suggest that increased toxicity accompanies increased antifungal efficacy. Altogether, these data indicate that sequence repeating or dimerization of β5 does not improve the therapeutic potential of this compound.

Table 2. Minimum inhibitory concentration (MIC_{90}) values of β5 derivatives against *C. neoformans*, toxic dose concentration (TD_{50}) values for β5 derivatives in HepG2 cells, concentration at 10% hemolysis (HC_{10}) values for β5 and β5 derivatives in red blood cells, and percent hemolysis at 100 μg/mL (H_{max}) values for β5 and β5 derivatives in red blood cells.

Compound	**MIC_{90} (μg/mL)**	**TD_{50} (μg/mL)**	**HC_{10} (μg/mL)**	**H_{max} (%)**
β52X	6.3	23.9 ± 2.6	50.1 ± 23.0	55.9 ± 9.3
β5D1	6.3	31.5 ± 3.2	43.7 ± 4.2	45.1 ± 15.6
β5D2	3.13	27.3 ± 2.2	57.3 ± 11.2	36.3 ± 1.6
β5D3	3.13	30.7 ± 2.5	45.7 ± 3.1	58.5 ± 10.0
β5	3.13*	91.2 ± 4.7*	81.3 ± 15.9	27.6 ± 6.2

*Values previously reported and published.[13]

Conclusions and Future Plans

In summary, our goal with this project was to synthesize new derivatives of β5 to further increase their efficacy against *C. neoformans* while retaining low cytotoxicity against mammalian cells. Although we hoped that by synthesizing a repeat compound of β5, and β5 dimers linked with amino acids, we would find increased potency against *C. neoformans* and lower mammalian toxicity, we confirmed no advances with potency or with decreased toxicity.

The future plans for this project are to synthesize cyclic derivatives of β5. Well established methods for cyclizing peptoids, including amide formation and alkyne/azide "click" chemistry, will be explored. The antifungal activity against *C. neoformans,* mammalian cytotoxicity against HepG2 cells, and mammalian cytotoxicity against hRBCs will be determined for all derivatives as described here.

References

1. Li, Y., Zou, M., Yin, J., Liu, Z. & Lu, B. Microbiological, epidemiological, and clinical characteristics of patients with cryptococcal meningitis at a tertiary hospital in China: A 6-year retrospective analysis. *Frontiers in Microbiology* **11,** (2020).
2. Tenforde, M.W., Shapiro, A.E., Rouse, B., Jarvis, J.N., Li, T., Eshun-Wilson, I., & Ford, N. Treatment for HIV-associated cryptococcal meningitis. *Cochrane Database of Systematic Reviews* (2018). doi:10.1002/14651858.cd005647.pub3
3. Chen, J., Varma, A.V., Diaz, M.R., Litvintseva, A.P., Wollenberg K.K., & Kwon-Chung, K.J. Cryptococcus neoformans strains and infection in apparently immunocompetent patients, China. *Emerging Infectious Diseases* **14,** 755–762 (2008).
4. Cogliati, M. Global Molecular Epidemiology Of Cryptococcus neoformans and Cryptococcus gattii: An atlas of the molecular types. *Scientifica* **2013,** 1–23 (2013).
5. Yuchong, C., Fubin, C., Jianghan, C., Fenglian, W., Nan, X., Minghui, Y., Yalin, S., & Zhizhong, Z. Cryptococcosis in China (1985–2010): Review of cases from Chinese database. *Mycopathologia* **173,** 329–335 (2011).
6. Rajasingham, R., Smith, R.M., Park, B.J., Jarvis, J.N., Govender, N.P., Chiller, T.M., Denning, D.W., Loyse, A., & Boulware, D.R. Global burden of disease of HIV-associated cryptococcal meningitis: An updated analysis. *The Lancet Infectious Diseases* **17,** 873–881 (2017).

7. Kim, H., Jung, K., Maeng, S., Chen, Y., Shin, J., Shim, J.E., Hwang, S., Janbon, G., Kim, T., Heitman, J., Bahn, Y., & Lee, I. Network-assisted genetic dissection of pathogenicity and drug resistance in the opportunistic human pathogenic fungus Cryptococcus neoformans. *Scientific Reports* **5,** (2015).
8. Pappas, P.G., Kauffman, C.A., Andes, D., Benjamin, D.K., Calandra, T.F., Edwards, J.E., Filler, S.G., Fisher, J.F., Kullberg, B., Zeichner, L.O., Reboli, A.C., Rex, J.H., Walsh, T.J., & Sobe, J.D. Clinical practice guidelines for the management candidiasis: 2009 update by the Infectious Diseases Society of America. *Clinical Infectious Diseases* **48,** 503–535 (2009).
9. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **415,** 389–395 (2002).
10. Molchanova, N., Hansen, P. & Franzyk, H. Advances in development of antimicrobial peptidomimetics as potential drugs. *Molecules* **22,** 1430 (2017).
11. Latham, P. W. Therapeutic peptides revisited. *Nature Biotechnology* **17,** 755–757 (1999).
12. Miller, S.M., Simon, R.J., Ng, S. Zuckermann, R., Kerr, J., & Moos, W. Proteolytic studies of homologous peptide and N-substituted glycine peptoid oligomers. *Bioorganic & Medicinal Chemistry Letters* **4,** 2657–2662 (1994).
13. Middleton, M. P., Armstrong, S. A. & Bicker, K. L. Improved potency and reduced toxicity of the antifungal peptoid AEC5 through submonomer modification. *Bioorganic & Medicinal Chemistry Letters* **28,** 3514–3519 (2018).
14. Zuckermann, R. N., Kerr, J. M., Kent, S. B. & Moos, W. H. Efficient method for the preparation of peptoids [oligo(n-substituted glycines)] by submonomer solid-

phase synthesis. *Journal of the American Chemical Society* **114,** 10646–10647 (1992).

15. Corson, A. E., Armstrong, S. A., Wright, M. E., McClelland, E. E. & Bicker, K. L. Discovery and characterization of a peptoid with antifungal activity against Cryptococcus neoformans. *ACS Medicinal Chemistry Letters* **7,** 1139–1144 (2016).
16. Fisher, K. J., Turkett, J. A., Corson, A. E. & Bicker, K. L. Peptoid library agar diffusion (PLAD) assay for the high-throughput identification of antimicrobial peptoids. *ACS Combinatorial Science* **18,** 287–291 (2016).
17. Chen, X., Zaro, J. L. & Shen, W.-C. Fusion protein linkers: Property, design and functionality. *Advanced Drug Delivery Reviews* **65,** 1357–1369 (2013).
18. Brubaker, C. E. & Messersmith, P. B. Biological adhesion. *Polymer Science: A Comprehensive Reference* 211–229 (2012). doi:10.1016/b978-0-444-53349-4.00235-1.

Appendix

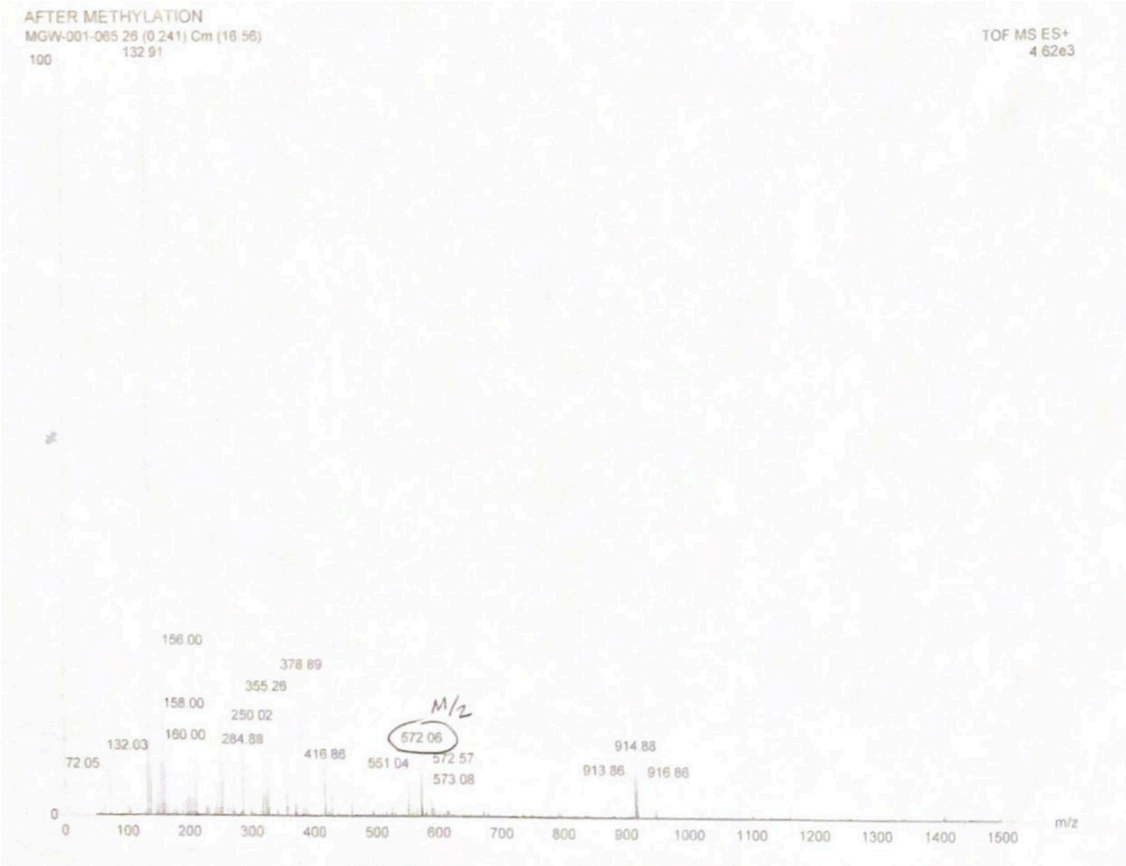

Figure A1. Mass spectra of β52X after cleavage from resin. The molecular weight of β52X is 1144.8 g/mol, and is represented by the base peak at 572.06 m/z.

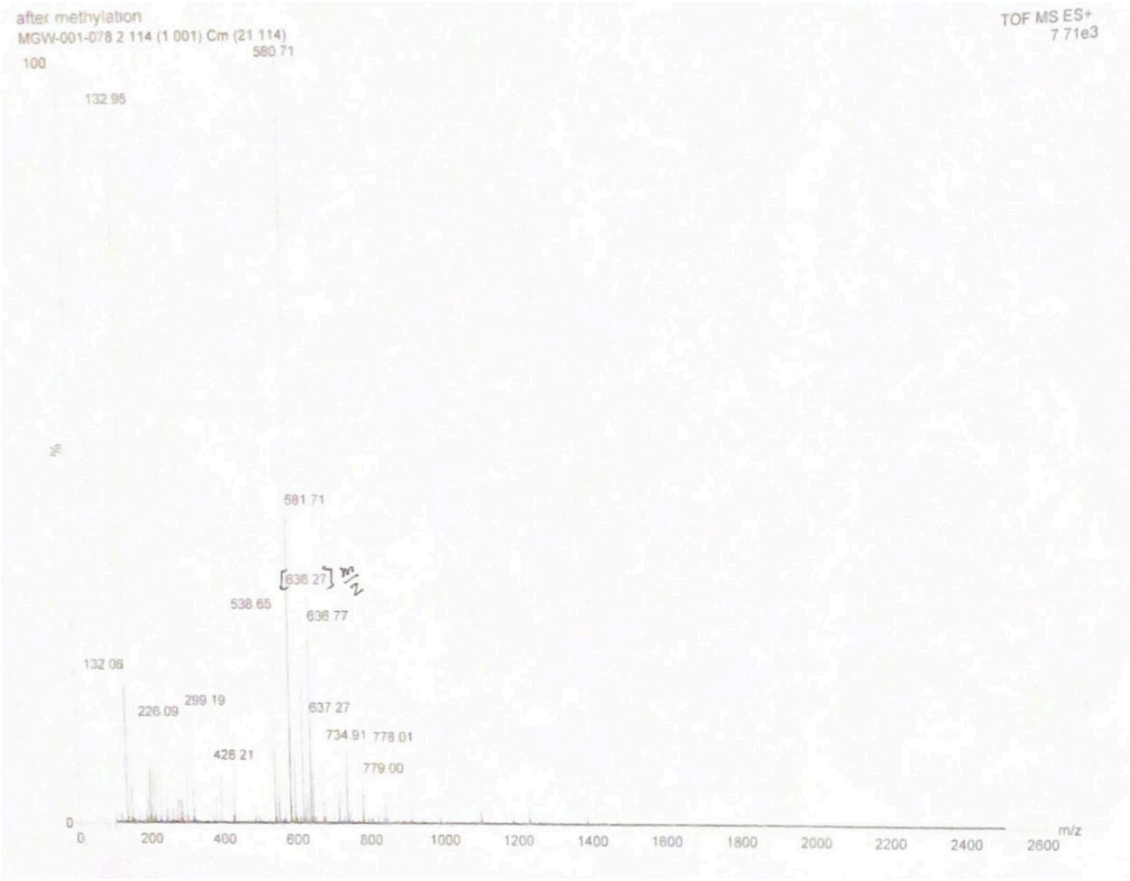

Figure A2. Mass spectra of β5D1 after cleavage from resin. The molecular weight of β5D1 is 1272.9 g/mol, and is represented by the base peak at 636.27 m/z.

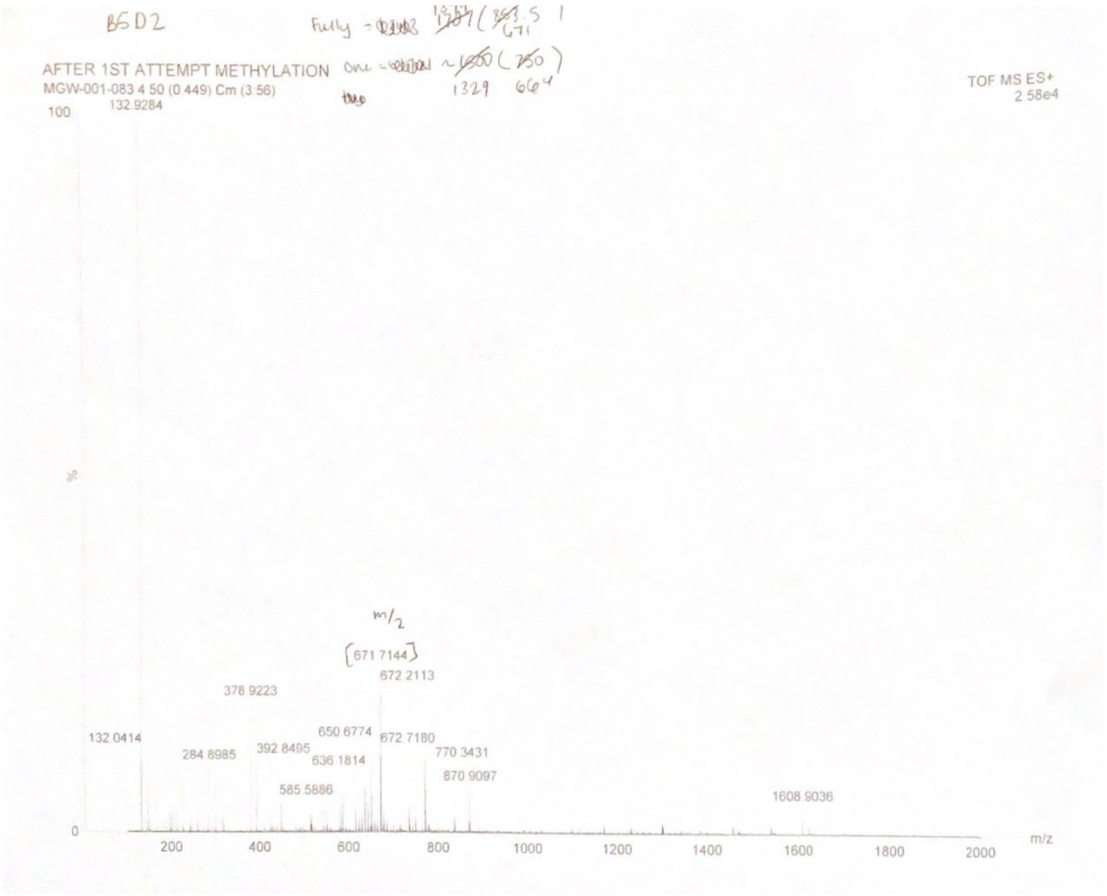

Figure A3. Mass spectra of β5D2 after cleavage from resin. The molecular weight of β5D2 is 1344.0 g/mol, and is represented by the base peak at 671.7144 m/z

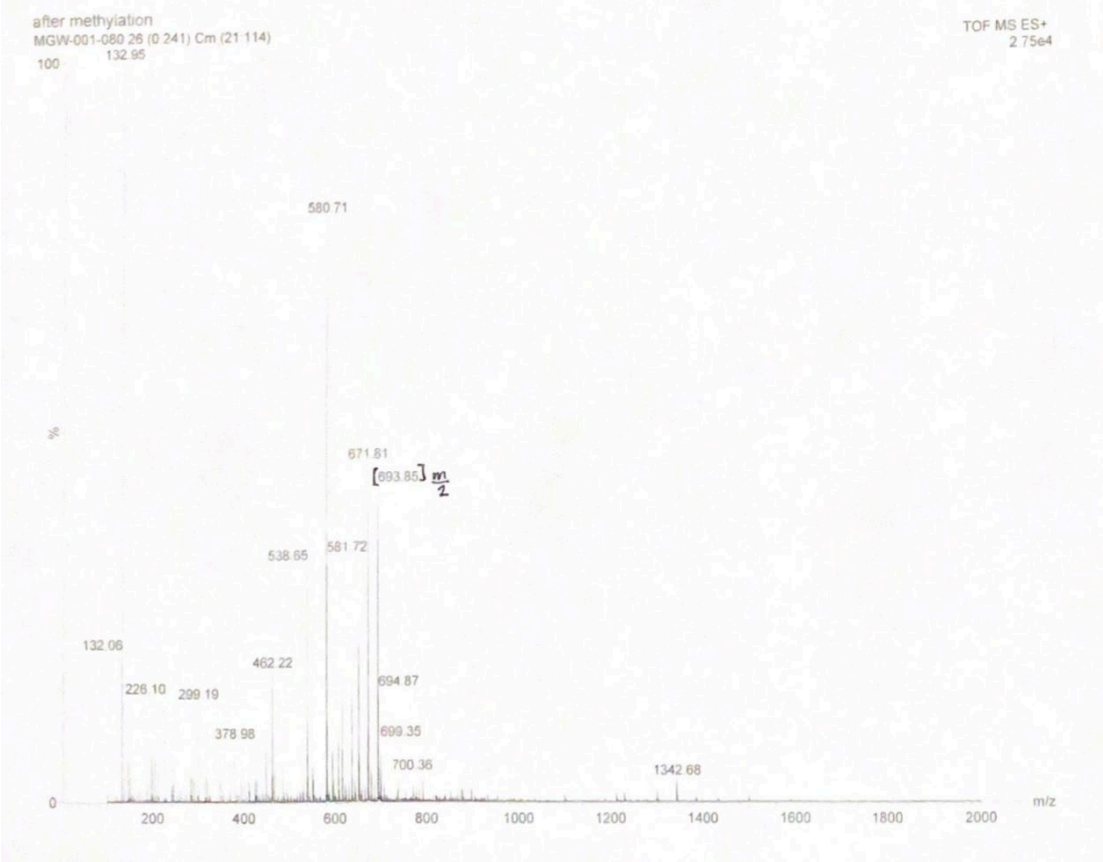

Figure A4. Mass spectra of β5D3 after cleavage from resin. The molecular weight of β5D3 is 1386.1 g/mol, and is represented by the base peak at 693.85 m/z.