Synthesis and Screening of Antimicrobial Peptoid Combinatorial Libraries Against the Fungi *Aspergillus, Candida,* and *Cryptococcus*

by
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Abstract

Studies show there is an increasing rate of fungal infections; a theory for the shift seen in infection patterns may be due to the escalation of patients with immune-compromising diseases. Treatments for fungal genera, such as *Aspergillus*, *Candida*, and *Cryptococcus*, carry many risks, including high toxicity levels that result in gastrointestinal complications, and hepatitis, as well as, a chance of developing resistance to anti-fungal medicine. Antimicrobial peptides (AMPs) offer alternative therapies for these infections due to little to no toxicity to mammalian cells, as well as their ability to singularly target fungal cells. AMPs have proven to be very effective for antibacterial activity, and this research has expanded to use peptide compounds against other organisms, such as fungi and viruses. However, peptides are also easily degraded within the human body. This allows for them to easily be targeted and degraded by proteases before the AMPs can reach their targeted organisms. A likely alternative to this problem is the employment of peptide mimics called peptoids.

Peptoids have a slight structural difference compared to peptides that allow for increased stability and an extended half-life while having the same or very similar actions to peptides. This research consists of alternative therapeutic treatments for combating antimicrobial resistance by synthesizing a diverse combinatorial library of peptoids. The purpose of this research is to optimize the techniques of synthesis, screening, and sequencing of viable antimicrobial peptoids from a combinatorial library. These methods will give a one-bead-one-compound library that can be successfully screened against fungal genera *Aspergillus, Candida,* and *Cryptococcus*. The one-bead-one-compound combinatorial library's design will allow for hundreds

of thousands of unique compounds to be synthesized and screened for anti-fungal properties in a few days. This will be an efficient way to determine other therapeutic options in dealing with anti-fungal drug resistant infections and high toxicity levels to mammalian cells.

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I. Introduction

Studies show an increase in fungal infections compared to previously seen data (4). For example, diseases due to *Candida* species are now the fourth most common nosocomial infection (4). Theories about the rise of fungal infections include the increase of these illnesses in patients with immune- compromising diseases, the growth in international travel, changes in land use, and incorporation of anti-fungal drugs in agriculture (2, 4).

Currently, nearly 75% of fungal infections isolated from patients in hospitals are of the *Candida* spp. Of the remaining 13% and 6% of fungal infections are due to *Aspergillus* spp. and *Cryptococcus* spp., respectively (7). Treatment for infections due to organisms such as *Aspergillus*, *Candida*, and *Cryptococcus* vary depending upon the patient and species of the infectious organism. Treatments for these organisms carry their own risks, including high toxicity to patients that results in gastrointestinal complications, vomiting, QT prolongation (heart problems), and hepatitis, as well as a chance of developing resistance to anti-fungal medication (9, 10,15). The Infectious Disease Society of America has established treatment guidelines for these organisms that consist of a cocktail of drugs and require close monitoring of patients due to high toxicity of these treatments (types of toxicity including hematologic, hepatic, and renal) (1, 9, 10, 15). Current therapeutics, such as amphotericin B and fluconazole, used today, share similar structures in their composition, such as azoles, polyenes, and hydroxyl groups. These structures can be exploited as the basis of other alternative therapeutic treatments. The organisms of this study include *Cryptococcus, Aspergillus,* and *Candida.*

Cryptococcus neoformans is a yeast-like fungus with a capsule that causes mortal infections located in the pulmonary system and central nervous system (8). *C. neoformans* is the main cause of fungal meningitis, and a common infectious agent of transplant and HIV-infected patients (11). Infections are caused by the inhalation of fungal spores due to the polysaccharide capsule surrounding the spore and the products the capsule sheds interacting with host cells (8, 11). The capsule consists of glucuronoxylomannan, galactoxylomannan and mannoproteins, which all contribute to the lowering of the host immune system (7). Current treatments include a cocktail of drugs with high toxicity to the hosts and possible surgery if there is no response to medication (10).

Aspergillus is a mold that, like *Cryptococcus*, forms spores that are found abundantly in the air. Also similar to *Cryptococcus*, *Aspergillus* is an opportunistic pathogen where the host's immune system prevents the growth of the pathogen's spores; however, if the immune system of the host is compromised in any way then infection via the spores will develop (13). *A. fumigatus* and *A. flavus and A. niger* are the most common transmittable agents of this species to infect humans (11). *Aspergillus* infections are the second most common fungal infections for organ and stem cell transplant patients due to the nature of treatment of these patients with suppression of the immune system (11). Once infection starts in the respiratory tract, it spreads to the central nervous system. *Aspergillus* infections are also treated with a cocktail of highly toxic drugs (11).

Candida spp. infections are the most common fungal infections for immune-

compromised patients, and cause the most fatalities out of all other systemic mycosis (11). An increase of infections has been observed and is likely due to an increase in patient population, in part due to the rise in improved patient survival rates of certain operations (11). Similar to the previously stated organisms, *Candida* is a yeast-like fungus that is very commonly found in the environment. Notably, *C. glabrata*, *C. lusitaniae*, *C. guilliermondii*, and *C. krusei* are species that are drug resistant to commonly used fungal treatments (11). This genus has well-known virulence factors, such as ability to bind to the host cells. When the immune system is affected, the fungus can access the blood stream. Unlike the other organisms, *Candida* rarely infects the host through the respiratory tract, instead mainly colonizing in the gastrointestinal tract (11).

One alternative to combat high toxicity levels and drug resistance is the use of antimicrobial peptides (AMPs);(5). Peptides can display minimal toxicity to mammalian cells, as well as their ability to target fungal cells only. An example of an AMP is pexiganan (**Figure 1**).

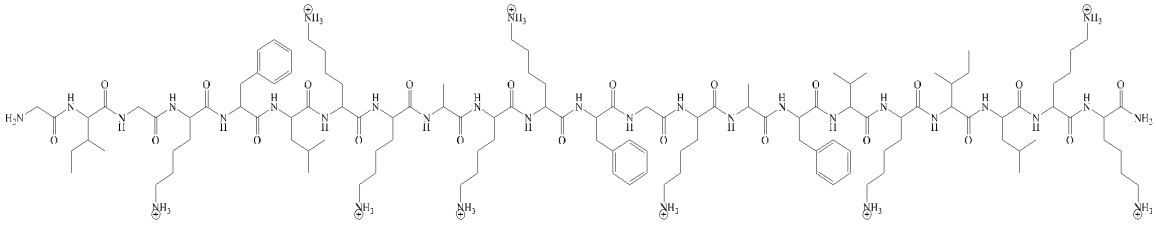


Figure 1: Pexiganan is an example of an antimicrobial peptide. These peptides are naturally occurring antimicrobial compounds that can display minimal toxicity toward mammalian cells, as well as an ability to target fungal cells only. Pexiganan was developed based on a natural product, magainin-2, isolated from a tropical tree frog.

Pexiganan is a synthesized peptide that was developed based on a natural

product, magainin-2, isolated from a tropical tree frog (6). Since peptides have proven to be very effective for antibacterial activity, this research has expanded to use peptide compounds against organisms such as *Candida krusei*, a fungal pathogen. The AMP synthesized to be screened against *C. krusei* is shown in **Figure 2**, which consists of three amino acids bonded together via peptide bonds (5).

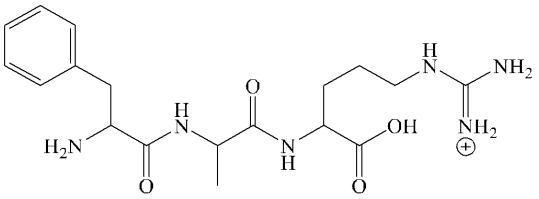


Figure 2: The tripeptide shown has proven to contain anti-fungal properties. The side chains represent a specific amino acid sequence that that has been successfully screened against *C. krusei*.

Peptides used against fungi are characterized by their ability to either disrupt the cell structure or form pores that causes intracellular compounds to leak out of the cell, leading to cellular death (14). While fungal and mammalian cells share many similar features, AMPs are able to target key differences. Ergosterol is a principle sterol only present in fungal cell membranes (15). The peptides that target ergosterol are cationic and hydrophobic in nature, which would make them an excellent selection for therapeutic treatment, if not for certain limitations, as discussed below (14).

Antimicrobial peptides, while having great potential as a treatment option, are also easily degraded within the human body. Peptides are naturally synthesized and largely abundant in the body, serving a variety of purposes. This allows for peptides to easily be targeted and degraded by proteases in the body before the AMPs can reach their targeted organism, signifying a limited half-life (14).

A likely alternative to this problem is the employment of peptide mimics called peptoids (**Figure 3)**.

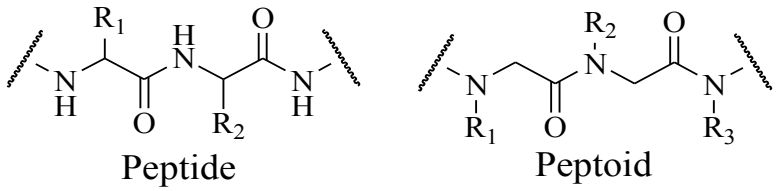


Figure 3: A peptide has side chains branching from the α-carbon and hydrogen attached to the nitrogen. A peptoid has the side chains attached to the α-nitrogen. The absence of the hydrogen on the nitrogen allows for an increase in half-life of the peptoids.

Peptoids have a slight structural difference compared to peptides that allow for increased stability and an extended half-life while having the same or very similar mode of actions to peptides (3). A peptide has side chains branching off of the α-carbon. A peptoid has side chains that are shifted to the α-nitrogen with the absence of the hydrogen as seen on peptides. Little research has been performed using peptoids as a therapeutic agent against fungi. Nonetheless, studies have successfully demonstrated that peptoids may be used against some fungal pathogens such as *Candida albicans* (14).

Further studies can be performed to identify antimicrobial peptoids through combinatorial library screenings. Combinatorial libraries allow for a large amount of unique compounds to be synthesized and screened in a matter of days (3). The compounds are synthesized on TentaGel beads. These beads do not have any chemical functions for synthesis; they are used as anchor points and for physical manipulation of the compounds. The sub-monomer building blocks that were incorporated into the combinatorial library were chosen based on structural elements of known anti-fungal

drugs, as well as peptoids with cationic and hydrophobic characteristics (9, 10, 14, 15). Sub-monomers are the individual compounds that are used to construct a combinatorial library. The goal of combinatorial libraries is to achieve one-bead-one compound, where each bead contains thousands of copies of a unique peptoid that is different from any other bead.

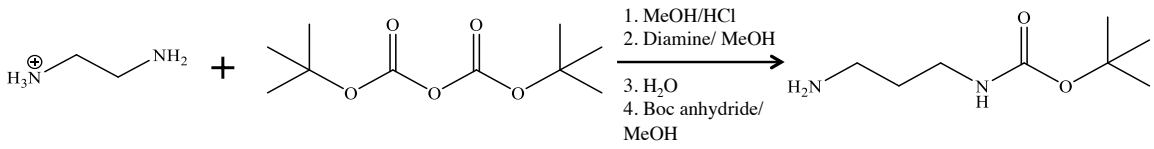
The purpose of this research is to synthesize a one-bead-one-compound combinatorial library of peptoids and develop library-screening methods to quickly identify peptoids with anti-fungal properties against *Aspergillus* spp., *Candida* spp., and *Cryptococcus* spp. Once these compounds are identified, they will be resynthesized and tested further against other species within these genera. The ultimate goal is to design compounds that can be used universally with these species. Sub-monomer synthesis, library synthesis via split-and-pool techniques, and screening and sequencing optimization process against the targeted organisms will be discussed herein.

II. Methods and Materials

All mass specctrometry spectra were collected on a Waters Synapt HDMS QToF with ion mobility Mass Spectrometer. All ^{1}H NMR spectra were collected on a Joel ECA 500 mHz NMR Spectrometer. YPD inoculated plates with H99S strain *C. neoformans* and synthesized library were analyzed on a Leica M165 FC microscope and processed with Adobe Photoshop. All chemicals were obtained from commercial chemical companies, such as Sigma-Aldrich, Fischer, etc.

Sub-monomer synthesis:

N-Boc-Ethylenediamine

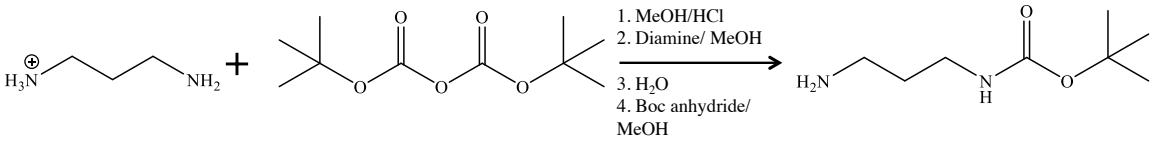


Concentrated hydrochloric acid (HCl);(0.777 mL, 0.00932 mol, 1 eq.) was added dropwise to 5 mL reagent grade methanol (MeOH) and stirred for 15 minutes at room temperature (RT). Ethylenediamine (0.56 g, 0.00932 mol, 1 eq.) was placed on ice to cool. After 15 minutes for HCl/MeOH to mix, ethylenediamine was added dropwise to the solution. The mixture was stirred for 15 minutes at RT. Deionized water (H_2O);(1.6 mL) was added drop wise to the solution at RT and stirred for 30 minutes. Di-tert-butyl-dicarbonate (Boc anhydride);(2.33 mL, 0.140 mol, 1.5 eq.) was mixed with 6.70 mL MeOH and placed on ice to cool. After 30 minutes, the Boc anhydride/MeOH solution was added dropwise over 10 minutes and then stirred at RT for 1 hour. The reaction mix was concentrated under vacuum to remove excess

MeOH and H_2O. Diethyl ether was added to the mixture and the organic layer was collected two times. Approximately 20 mL 2 M sodium hydroxide (NaOH) and 30 mL dichloromethane (CH_2Cl_2) was added to the organic layer.

The newly separated organic layer was collected and CH_2Cl_2 was added two more times to the aqueous layer to extract all organic material. Brine was added to the organic layer and the organic layer collected again. Calcium chloride ($CaCl_2$) pellets were added to remove any excess water and the solution filtered. CH_2Cl_2 was then removed under vacuum overnight. The yield was determined to be 76%. Mass spectrometry was performed to confirm synthesis of the compound by coupling the compound to four methoxyethylamine sub-monomers, followed by another methoxyethylamine sub-monomer after ethylenediamine. The predicted molecular weight was 692 da.

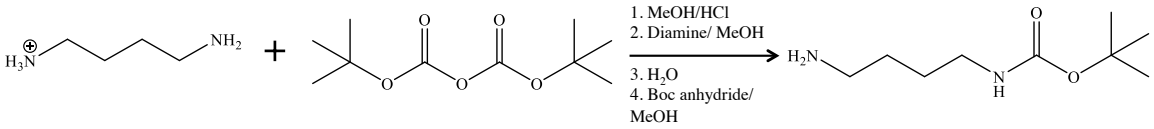
N-Boc-Diaminopropane



HCl (1.91 mL, 0.0229 mol, 1 eq.) was added dropwise to 30 mL MeOH and stirred for 15 minutes at RT. 1,3-diaminopropane (2.30 mL, 0.0229 mol, 1 eq.) was placed on ice to cool. After 15 minutes for HCl/MeOH to mix, 1, 3-diaminopropane was added dropwise to solution. The mixture was stirred for 15 minutes at RT. Deionized H_2O (10 mL) was added drop wise to the solution at RT and stirred for 30 minutes. Boc anhydride (7.90 mL, 0.344 mol, 1.5 eq.) was mixed with 40 mL MeOH

and placed on ice to cool. After 30 minutes was completed, the Boc anhydride/MeOH solution was added drop wise over 10 minutes and then stirred at RT for 1 hour. After 1 hour, the solution was concentrated under vacuum to remove excess MeOH and H_2O. Diethyl ether was added to the mixture and organic layer collected two times. Approximately 50 mL 2 M NaOH and 75 mL CH_2Cl_2 was added to the organic layer. The newly separated organic layer was collected, and CH_2Cl_2 was added two more times to the aqueous layer to extract all organic material. Brine was added to the organic layer and the organic layer collected again. $CaCl_2$ pellets were added to remove any excess water and the solution filtered. CH_2Cl_2 was removed under vacuum overnight. The yield was 95%.

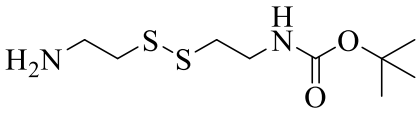
N-Boc-Diaminobutane



HCl (1.91 mL, 0.0229 mol, 1 eq.) was added dropwise to 30 mL MeOH and stirred for 15 minutes at RT. 1,4-diaminobutane (2.30 mL, 0.0229 mol, 1 eq.) was placed on ice to cool. After 15 minutes for HCl/MeOH to mix, 1,4-diaminobutane was added drop wise to solution. The mixture was stirred for 15 minutes at RT. Deionized H_2O (10 mL) was added drop wise to the solution at RT and stirred for 30 minutes. Boc anhydride (7.90 mL, 0.344 mol, 1.5 eq.) was mixed with 40 mL MeOH and placed on ice to cool. After 30 minutes was completed, the Boc anhydride/MeOH solution was added dropwise over 10 minutes and then stirred at RT for 1 hour.

After 1 hour, the solution was concentrated under vacuum to remove excess MeOH and H_2O. Diethyl ether was added to the mixture and organic layer collected two times. Approximately 50 mL 2 M NaOH and 75 mL CH_2Cl_2 was added to the organic layer. The newly separated organic layer was collected and CH_2Cl_2 was added two more times to aqueous layer to extract all organic material. Brine was added to the organic layer and organic layer collected again. $CaCl_2$ pellets were added to remove any excess water and the solution filtered. CH_2Cl_2 was removed under vacuum overnight. The yield was 67%. Mass spectrometry was performed to confirm synthesis of the compound by coupling the compound to four methoxyethylamine sub-monomers, followed by another methoxyethylamine sub-monomer after ethylenediamine. The predicted molecular weight was 720 da. ^1H NMR was also performed to confirm synthesis of the compound: ^1H NMR ($CDCl_3$) δ 1.45 (s, 9H), δ 1.94 (s, 2H), δ 2.74 (s, 2H), δ 3.13 (s, 2H).

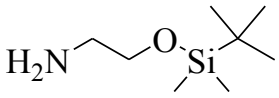
N-Boc-Cystamine



Cystamine hydrochloride salt (5 g, .0222 mol, 1 eq.) and triethylamine (Et_3N);(9.29 mL, .0666 mol, 3 eq.) were dissolved in 250 mL MeOH on ice for 25 minutes. Boc anhydride (5.09 mL, .0222 mol, 1 eq.) was slowly added over 10 minutes and then stirred at RT for 1 hour. The solution was concentrated under vacuum. Diethylether was then added twice to separate the organic and aqueous layer. The

compound was placed on the vacuum line for approximately 3 minutes to insure all ether had been removed. A 1 M NaOH (100 mL) and 100 mL CH_2Cl_2 were added to dried compound and the organic layer collected. The aqueous layer was rinsed twice more times with CH_2Cl_2. The organic layer was washed five times with H_2O. CH_2Cl_2 was removed under vacuum overnight. The yield was 52%. Mass spectrometry was performed to confirm synthesis of the compound by coupling the compound to four methoxyethylamine sub-monomers, followed by another methoxyethylamine sub-monomer after ethylenediamine. The predicted molecular weight was 783 da. ^{1}H NMR was also performed to confirm synthesis of the compound: ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9H), δ 2.77 (q, 4H, J=6.19 Hz), δ 3.02 (t, 4H, J=6.19 Hz), δ 3.45 (m, 2H), δ 5.02 (s, 1 H).

N-TBDMS-Ethanolamine



Ethanolamine (1.21 mL, 0.021 mol, 1 eq.) and imidazole (2.72 g, 0.040 mol, 2 eq.) were dissolved in 20 mL CH_2Cl_2. Tert-butyldimethylchlorosilane (TBDMS);(3.15 g, 0.021 mol, 1 eq.) was dissolved in 10 mL CH_2Cl_2. The TBDMS/CH_2Cl_2 solution was slowly added to the ethanolamine/imidazole mixture for 5 minutes. The reaction proceeded for 1 hour. Deionized H_2O (20 mL) was added and the organic layer collected. CH_2Cl_2 was added two more times and the organic layer collected each time. $CaCl_2$ pellets were added to remove excess water and the solution filtered. The solution was then concentrated under vacuum and then

transferred to vacuum line overnight. The yield was 85%. Mass spectrometry was performed to confirm synthesis of the compound by coupling the compound to four methoxyethylamine sub-monomers, followed by another methoxyethylamine sub-monomer after ethylenediamine. The predicted molecular weight was 693 da. ^{1}H NMR was also performed to confirm synthesis of the compound: ^{1}H NMR ($CDCl_3$) δ 0 (s, 6H), δ 0.834 (s, 9H), δ 2.74 (t, 2H, J=5.16 Hz), δ 3.59 (t, 2H, J=5.5), δ 53.96 (s, 2H).

Tag Synthesis

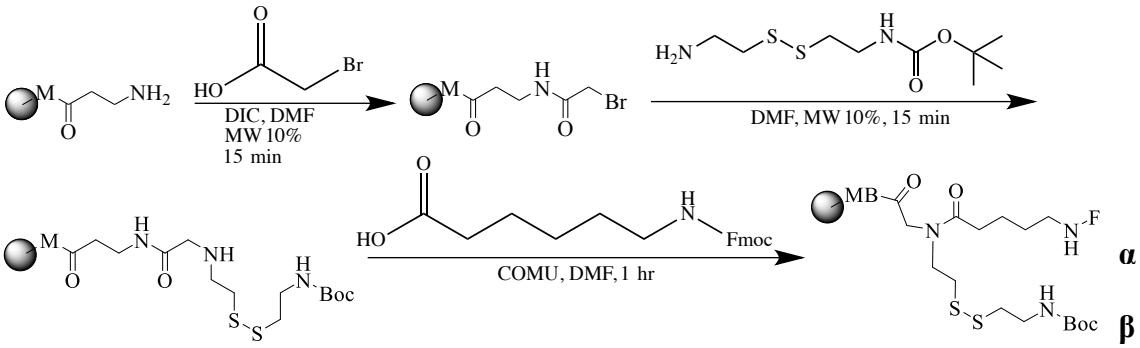


Figure 4: Synthesis of the initial linker system for all beads (represented by dark circles). The library tag consisted of methionine (M) followed by β-alanine (B). The disulfide linker (S-S) was attached before any sub-monomers were added to the library so that the strands could be chemically manipulated individually. The disulfide linker was followed by 6-aminohexanoic acid (AHA). The peptoid strand that will be built on the tag and AHA is called "α-strand". The peptoid strand built on the cystamine and deployed into the environment is called "β-strand."

To begin library synthesis, the initial tag was synthesized for each bead (**Figure 4**). TentaGel beads (600 mg) were soaked in reagent grade dimethylformamide (DMF) for 20 minutes to swell the beads. Fmoc-methionine-OH (M) (0.2259 g, $6x10^{-4}$ mol, 4 eq.) and COMU (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexaflurorphosphate);(0.260 g, $6x10^{-4}$ mol, 4 eq.) was mixed with 7 mL 5% N-methlymorpholine (NMM) solution for 10 minutes. The solution was

then added to the beads and the reaction rocked for 1 hour at RT. The beads and solution were then filtered and the beads rinsed with DMF three times to remove remaining amounts of methionine and COMU. A Kaiser test was performed to confirm that coupling occurred by testing for the presence of a free terminal amine. Once confirmed, the beads were soaked in 20% piperidine solution to remove Fmoc protecting group for 20 minutes total. The beads were filtered and washed 3 times with DMF.

Fmoc-β-alanine-OH (B);(0.1894 g, $6x10^{-4}$ mol, 4 eq) and COMU (0.260 g, $6x10^{-4}$ mol, 4 eq.) were mixed with 7 mL 5% NMM solution for 10 minutes. The solution was then mixed with the beads and the reaction proceeded for 1 hour. The beads were filtered and washed with DMF three times again. A Kaiser test was performed to confirm coupling success. The beads were soaked in 20% piperidine solution for a total of 20 minutes and the beads were washed with DMF 3 times again.

For the coupling of N-Boc-cystamine, peptoid synthesis was performed (**Figure 5**).

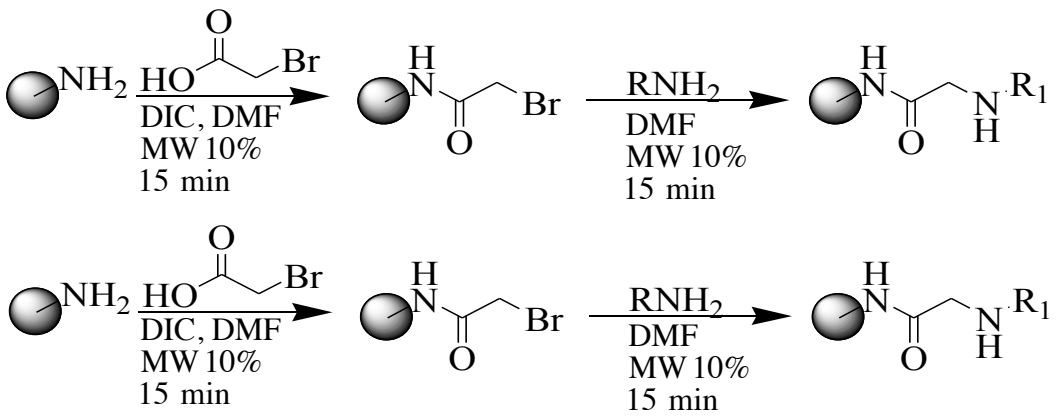


Figure 5: After the library tag was synthesized, the resin was treated with 2 M bromoacetic acid and 3.2 M diisopropylcarbodiimide (DIC). It was then microwaved for 30 seconds at reduced power to form the first amide bond. After 15 minutes, the resin was washed and treated with the desired amine. The beads were once again microwaved and reacted for 15 minutes.

A 2 M solution of bromoacetic acid (BrAcOH) (0.832 g, 0.006 mol, 1 eq.) in 3 mL anhydrous DMF and 3.2 M solution of N-N'-diisopropylcarbodiimide (DIC); (1.5 mL, 0.0096 mol, 0.625 eq.) in 1.5 mL anhydrous DMF were added to the beads. The solution was microwaved (MW) for 30 seconds at 10% power (100kW). The beads and solution proceeded to react for 15 minutes. The resin was then filtered, washed with DMF three times, and a Kaiser test performed to confirm coupling.

A 2 M solution of N-Boc-cystamine (3 g, 0.0119 mol, 1 eq.) in 6 mL anhydrous DMF was coupled with the bromoacylated MB tag, as seen in Figure 4. The compound was MW at 10% for a total of 30 seconds and then allowed to react for 15 minutes total. The resin was filtered and washed three times with DMF and a Kaiser test performed to confirm coupling.

Fmoc-6-aminohexanoic acid-OH (AHA);(0.2137 g, $6x10^{-4}$ mol, 4 eq.) with COMU (0.260 g, $6x10^{-4}$ mol, 4 eq.) were mixed with 7 mL 5% NMM and rocked for

10 minutes. The solution was then added to the resin and the reaction rocked for 1 hour. The resin was then filtered and washed three times with DMF. A Kaiser test was performed to confirm coupling. A sample of the resin was prepared and mass spectrometry (MS) was performed to confirm that no miscouplings had occurred.

Deprotection of Boc and Fmoc protecting groups followed confirmation. A 7 mL solution of 95% trifluoroacetic acid (TFA);(6.65 mL) and 2.5% triisopropylsilane (TIS);(17.5µL) and 2.5% (17.5µL) water was added to the beads for 1 hour to remove Boc groups. The resin was washed three times with CH_2Cl_2 and DMF. Next, 10 mL of 20% piperidine was added for a total of 20 minutes to remove Fmoc groups. A Kaiser test was performed to confirm deprotection of these groups. MS was performed once more to confirm synthesis of library tag.

Test Peptoid Synthesis

TentaGel (500 mg) was swollen in 20 mL reagent grade DMF and tag synthesis proceeded, as previously described. After the coupling of all 5 sub-monomer via peptoid chemistry, a "5-mer" was created, an accurate representation of a compound synthesized in the library (**Figure 6**). A "5-mer" means that five sub-monomers were used to make a peptoid. Approximately beads were treated with 10 mM solution of dithiothreotol (DTT) for 30 minutes and CNBr for 4 hours to confirm coupling.

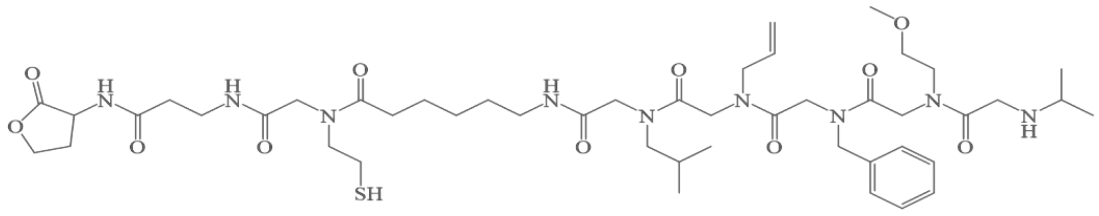


Figure 6: Test peptoid α-strand sequence. The molecular weight was 974 da.

With the test peptoid, single bead analysis with tandem MS was optimized. First, DTT concentrations were varied to determine the best concentration to remove the disulfide linker. Next, several ratios and volumes of 0.05% TFA in water and acetonitrile were added to vials that contained a single bead (50 μL, 100 μL, 150 μL) to determine at what resuspension volume for MS analysis.

Library Synthesis

For library synthesis, all 600 mg of library tag resin was used. Once the protecting groups were removed, synthesis proceeded via peptoid chemistry and split- and-pool synthesis (**Figure 7**).

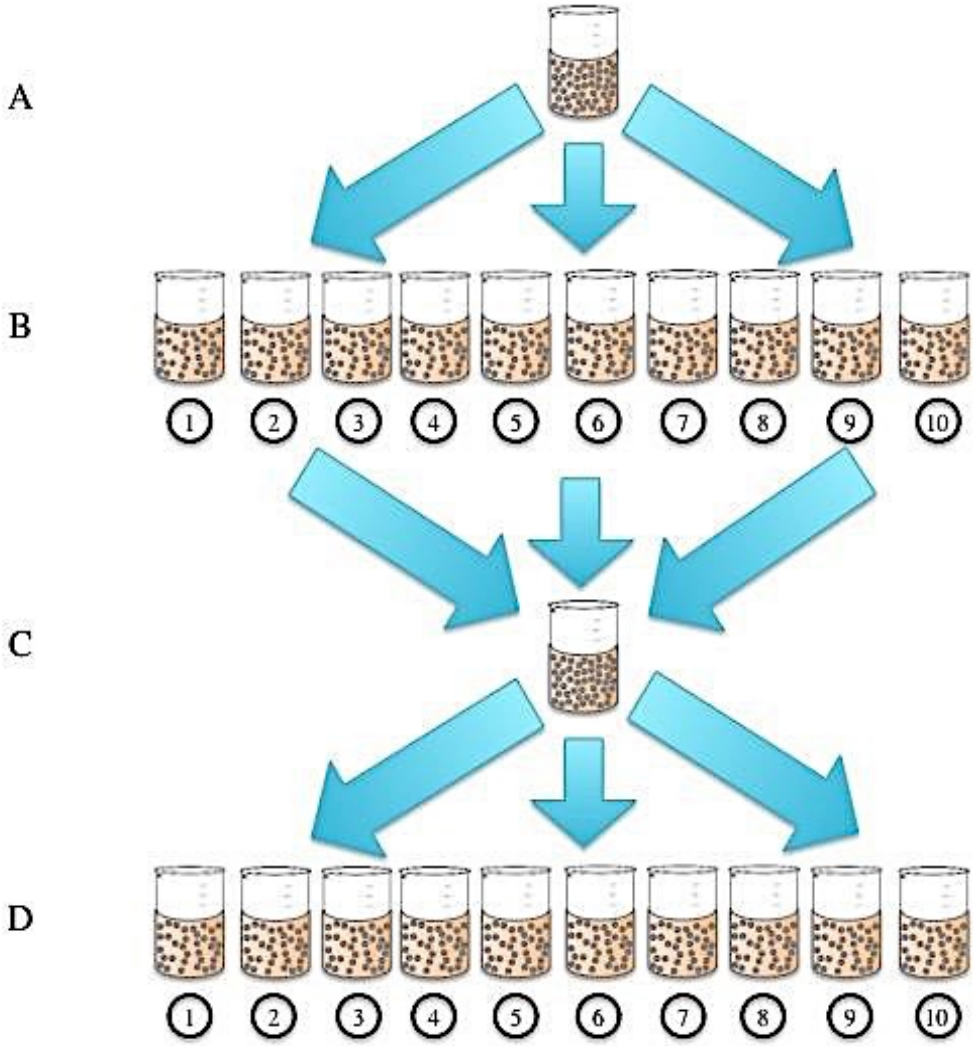


Figure 7: Split and pool synthesis is a technique that gives a one-bead-one-compound library. **A.** The beads, anchor points, started pooled together and initial tag was added to each bead. **B.** The beads were randomly separated into ten vials, and one sub-monomer was added to one vial. **C.** The beads were pooled back together for the next step or peptoid coupling. **D.** The beads were again separated randomly into each vial and one sub-monomer was added to one vial. This procedure was repeated until the desired length was reached.

Ten sub-monomers were measured into separate vials with appropriate amounts of anhydrous DMF (**Table 1**).

Table 1: The molecular weight and density of the ten sub-monomers were used to calculate the exact mass or volume needed to create 2 mL of 2 M solution of each sub-monomer.

Compound	Molecular weight (g/mol)	Density (g/mL)	Mass (g)
N-Dab(Boc) (s)	160.12	------	0.642
N-Orn(Boc) (s)	174.34	------	0.697
N-Lys(Boc) (s)	188.27	------	0.753
N-Fur (l)	97.12	1.05	0.388
N-Mea (l)	75.11	0.874	0.300
N-Dio (l)	131.2	1.012	0.525
N-Leu (l)	73.14	0.736	0.290
N-Val (l)	59.11	0.691	0.236
N-Phe (l)	107.15	0.982	0.429
N-Eth(TBDMS) (s)	175.34	-----	0.700

The resin was equilibrated in anhydrous DMF for approximately 5 min. 2 M of BrAcOH (0.432 g, $6x10^{-4}$ mol, 1 eq.) and 1.5 mL anhydrous DMF with DIC (0.75 mL, $6x10^{-4}$ mol, 0.625 eq.) and 0.75 mL anhydrous DMF were added to the resin. The resin was MW for 30 seconds at 10% power and proceeded to react for 15 minutes. The resin was washed with DMF three times and a Kaiser test performed to confirm coupling.

Approximately 60 mg of resin was added to each vial that contained a sub-monomer. Each vial was MW at 10% for 30 seconds and the reaction rocked for 15

minutes total. After the reaction was complete, each vial was transferred back to the common vial, filtered, and washed with DMF several times. A Kaiser test was used to confirm coupling of sub-monomers. The process was repeated four more times for a total of 5 random sub-monomers after the tag (**Figure 8**).

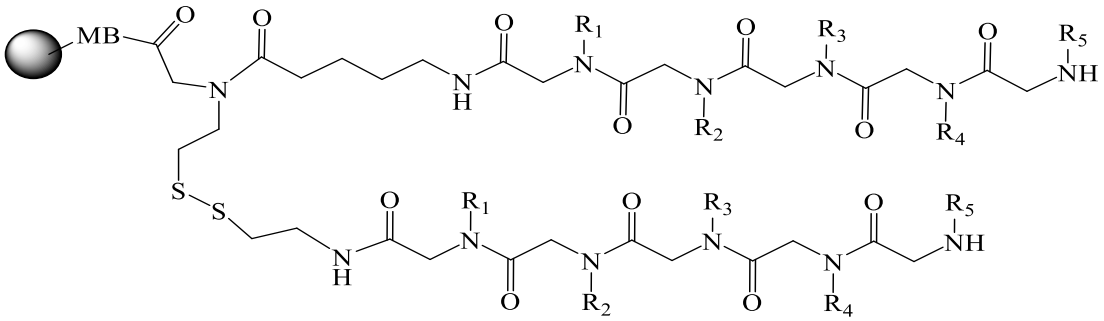


Figure 8: A complete peptoid with the disulfide linker designated by S-S.

To confirm library synthesis and disulfide linker function, a single bead was isolated, 10 mM DTT added, and the reaction proceeded for 30 minutes to reduce the S-S bond. The bead was rinsed and equilibrated with water. Cyanogen bromide (CNBr) with 0.1 M hydrochloric acid (HCl) was added for 24-hours to cleave the compound from the bead. The bead was then resuspended in 100 μL of 75% acetonitrile (ACN) with 0.05% TFA and analyzed via linear and tandem MS.

Screening

The library was rinsed with CH_2Cl_2 for approximately 20 minutes to rid the resin of any remaining DMF and aspirated to dry the resin. For screening, a plate of hard yeast extract-peptone-dextrose (YPD) supported a mixture of 3 mL YPD soft agar, 6 mg library resin, 10 mM DTT, and $1x10^6$ cells mL^{-1} of *C. neoformans* H99S strain.

To accomplish this soft agar was melted and separated into 3 mL quantities before maintaining the temperature at 56°C.

Library resin (6 mg) was measured and equilibrated in water 18 hours. The water was removed and 500 μL of phosphate buffer saline (PBS; pH 7.4) was added to the resin approximately 30 minutes before introduction to the organism. A stock solution of 100 mM DTT was made and the appropriate amount was added to the 3 mL of soft agar to give a 10 mM final DTT concentration. The cells were grown overnight and then concentrated and washed with PBS the day of plating. Cells were diluted 1:200 with PBS to count the cell density with a hemocytometer. The average cell count was multiplied by the dilution factor and volume of the hemocytometer tray (10^4). This concentration was used to determine the appropriate amount of concentrated cells to add to the soft agar.

Each component was added to the soft agar under sterile conditions and then to a plate of hard YPD agar. The plates were incubated at 37°C for 24-hours and analyzed. Analysis of plates involved observation and imaging under a Leica stereoscope and subsequent determination of zones of clearance. A zone of clearance is defined as a uniform area around a bead with no growth. Images were analyzed and zones of clearance measured using Adobe Photoshop. The bead was then removed from the plate for further analysis by MS.

Sequencing

Beads were removed from plates that exhibited zones of clearance and were washed individually with 500 μL of 1% sodium dodecylsulfate (SDS) for 1 hour at

approximately 120°C. SDS was removed from the beads and the beads were washed with water five times. The beads were then placed in 500 μL of 10 mM solution of DTT in 75% ACN for 30 minutes. Once again, the beads were washed five times with water. A 75 μL of 40 mg mL^{-1} solution of CNBr with 0.1 M HCl in 75% ACN reacted for 24 hours. The CNBr solution was removed with a speed vacuum and then the material resuspended in 100 μL 0.05% TFA in 75% ACN. The bead was removed from the solution.

The unknown sequence was analyzed via linear and tandem MS. Sub-monomer sequences were determined by comparing MS spectra to known fragment masses (**Table 2**). The sequence determined was resynthesized by previously stated techniques for further analysis.

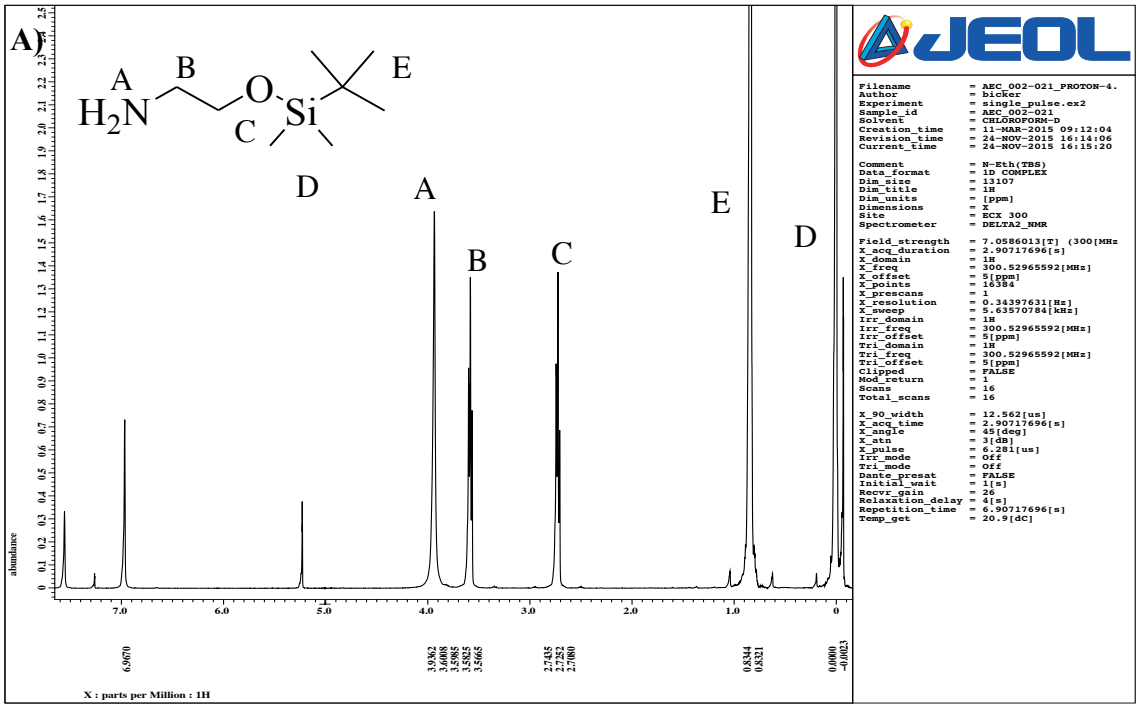
Table 2: Fragment masses are the molecular weights of the sub-monomers when they are fragmented in MS. Using the fragment masses found in tandem MS, an unknown sequence can be determined.

Compound	Fragment mass (g)
N-Dab	104
N-Orn	114
N-Lys	128
N-Fur	137
N-Mea	115
N-Dio	104
N-Leu	113
N-Val	99
N-Phe	147
N-Eth	101

III. Results and Discussion:

Sub-monomer synthesis

^{1}H NMR and/or MS confirmed the synthesis of all sub-monomers. The compound was removed from the vacuum and analyzed for confirmation of protecting group coupling. Due to the symmetry of the compound, it was necessary to analyze the compound by MS and not solely by NMR. A 2 M solution of the amine was prepared and coupled by peptoid chemistry onto a known sequence of sub-monomers. Generally, a sequence of four residues of methoxyethylamine (N-Mea) was used on Rink amide resin. Rink amide is a different type of resin that requires a strong acid to cleave the peptoid strand. The mono-protected diamine was then coupled and analyzed. To insure that no unprotected amine remained, a subsequent sub-monomer was coupled to confirm no branching (**Figure 9**). All sub-monomer protection syntheses were verified by the same method with ^{1}H NMR (**See Appendix, Figure A1-A7)**.



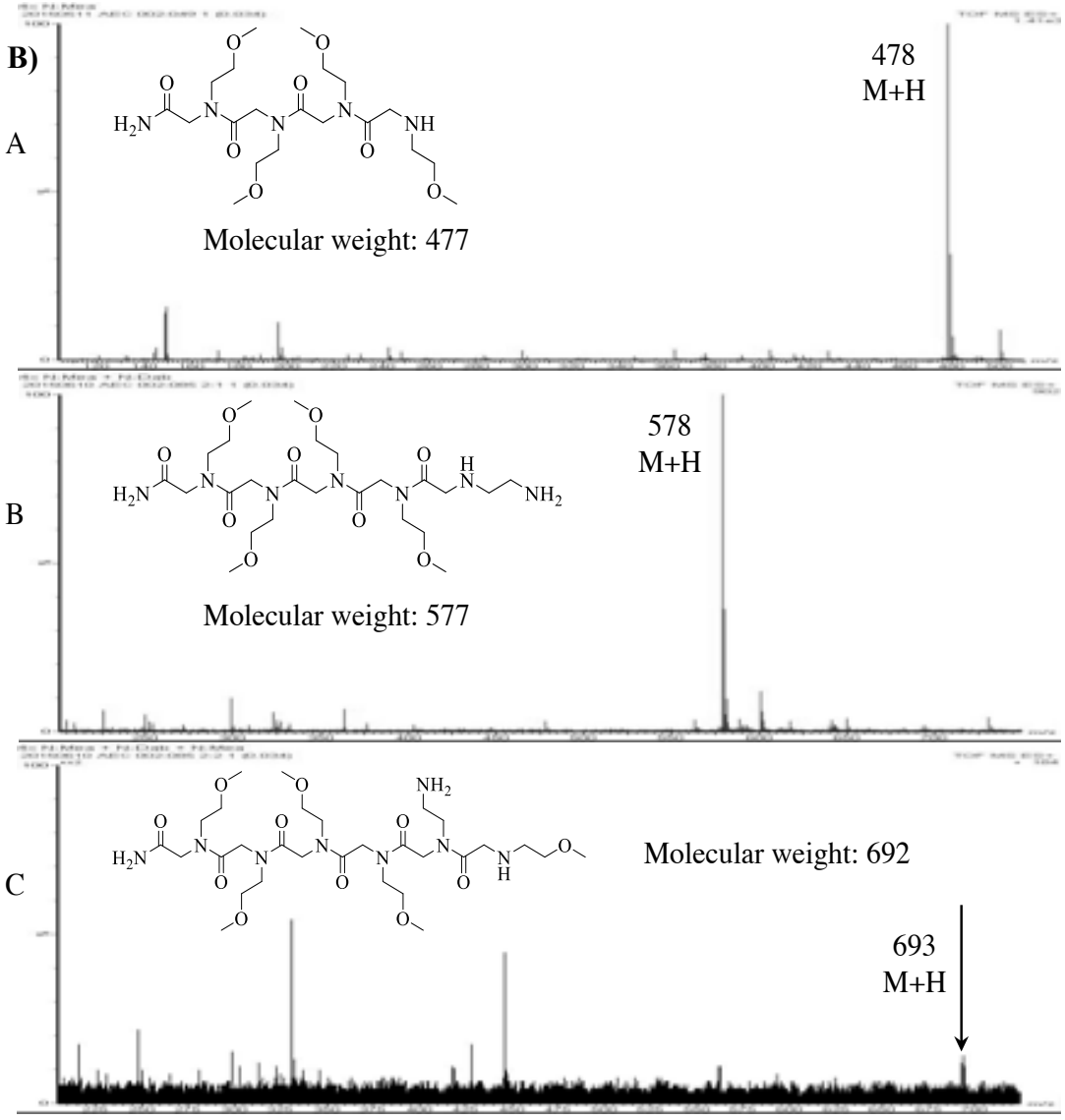


Figure 9: NMR (**9A**) and MS (**9B**) were used to verify the synthesis of TBDMS protected ethanolamine. Due to the symmetry of the compound, NMR analysis could not be the only process used for verification of synthesis. Peptoid chemistry was used to also confirm that no unprotected ethanolamine remained. **A.** A known sequence (four residues of methoxyethylamine) was used as the tag on Rink amide. The molecular weight of this sequence is known. **B.** The sub-monomer of interest is coupled to the sequence. TFA is used to cleave the peptoid from the Rink resin; however, TFA also removes TBDMS protecting groups. **C.** Another sub-monomer of known coupling abilities is coupled afterwards to verify that no unprotected ethanolamine remains. If any unprotected remained, there would be branching and a higher molecular weight than expected. This method was used for all sub-monomers synthesized in lab.

Five sub-monomers were removed from the original library due to miscouplings when using the previously stated technique. The synthesis of N-Arg synthesis proved to be inefficient and yielded an impure compound. High Performance Liquid Chromatography (HPLC) confirmed this. The coupling of the azoles, aminothiazole and histamine, proved to be inefficient. It would appear that Aminothiazole (N-Tha) is not nucleophilic enough to couple with the BrAcOH and would not be used in the library. Histamine coupling was efficient, but resulted in undetermined side reactions upon reactions with subsequent sub-monomers. This can be also seen when testing the coupling of morpholine. Coupling of allylamine (N-All) proved unsuccessful during synthesis of the test peptoid and was subsequently removed because it also was not showing complete coupling with BrAcOH.

Tag synthesis

Tag synthesis was confirmed by analyzing the compound via MS after every subsequent coupling following the coupling of β-Alanine (**A**6). Once the initial sequence was confirmed, the protecting groups were removed. This removal was confirmed with Kaiser tests.

Proof-of-Concept Test Peptoid

A proof-of-concept test peptoid was synthesized to confirm that the synthesis, including peptide and peptoid chemistry, with 5 sub-monomers from the library could be synthesized. It was determined that allylamine did not couple correctly and was subsequently removed from the library of sub-monomers after synthesis of the test peptoid. The inefficiency of allylamine coupling resulted in both a “4-mer” and a “5-

mer" peptoid. During analysis of sub-monomer coupling, these 2 compounds were found in the spectra (**Figure 10**). It was with the test peptoid that the optimal conditions for peptoid cleavage and MS analysis were defined. It was determined that the optimal conditions were to use 100 μL 0.05% TFA in 75% ACN for resuspension of the dried peptoid after cleavage from the bead. The ACN produces a more hydrophobic environment for the hydrophobic peptoid and allows for better analysis of the peptoid with MS.

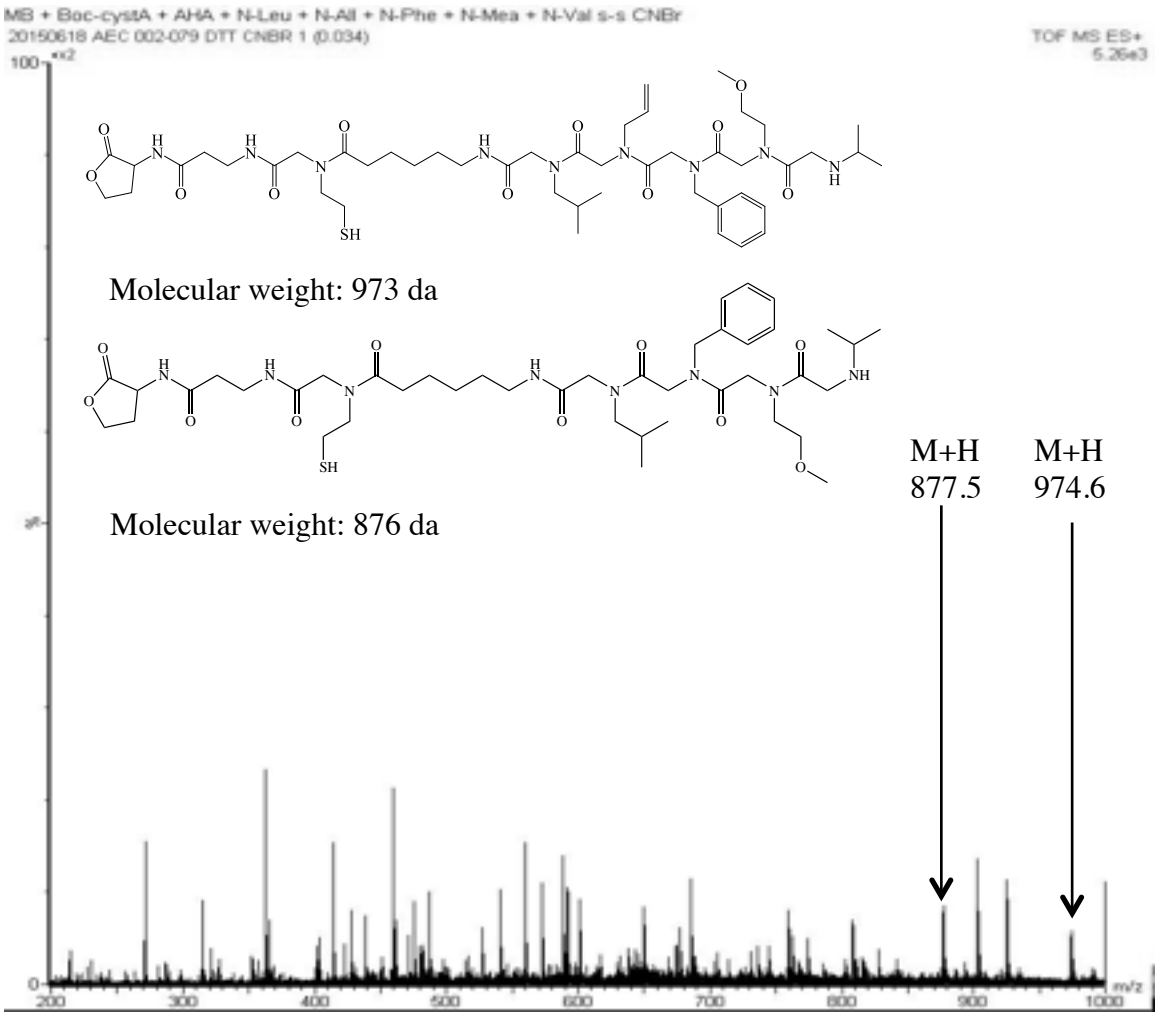


Figure 10: Proof-of-concept peptoid synthesized before synthesis of the library. It was during this synthesis that allylamine was removed from the library sub-monomer list. The molecular weight of the "5-mer" is 974 da. The peak at 877.5 da is the molecular weight of the "4-mer," which is the test peptoid without allylamine.

Library

Synthesis of the tag proceeded as established previously. The library was synthesized with sub-monomers that had been confirmed to accurately couple during peptoid chemistry (**Figure 11)**.

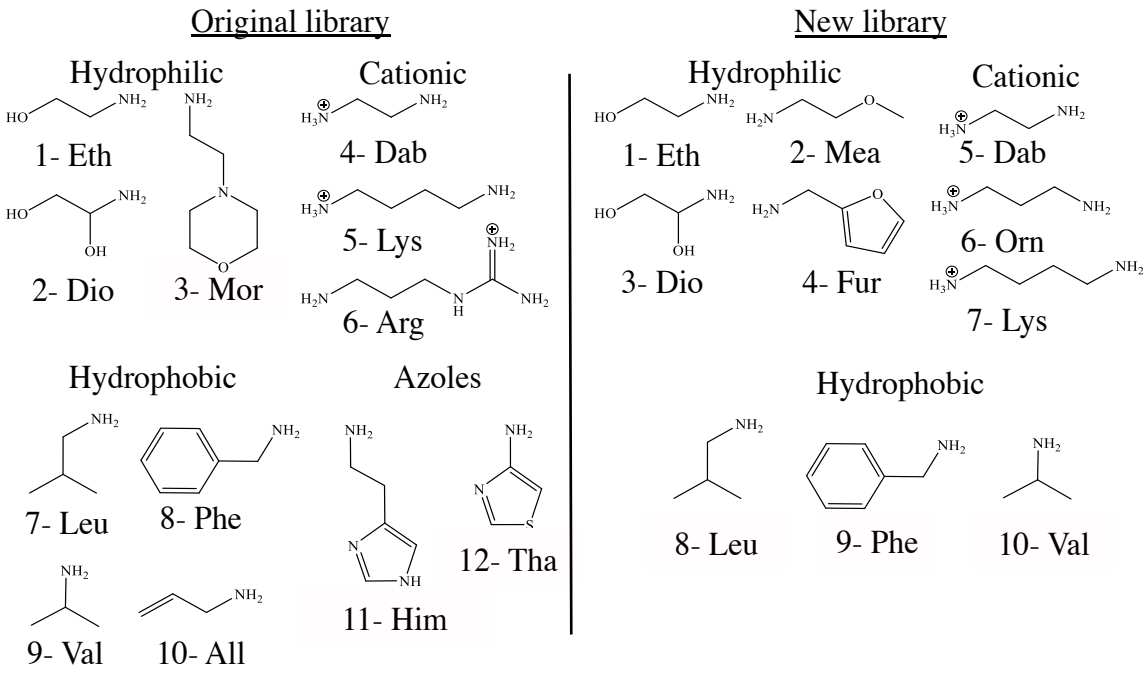


Figure 11: The proposed library consisted of twelve sub-monomers chosen based on hydrophilic, hydrophobic, cationic, and azole structures seen in current fungal therapeutics. The actual library consists of ten sub-monomers that also have hydrophilic, hydrophobic, and cationic properties. Certain sub-monomers from the proposed library were removed due to problematic coupling conditions. The sub-monomers that replaced the sub-monomers removed in the actual library were chosen based on the original properties stipulated in choosing sub-monomers.

Once split-and-pool synthesis began, the peptoids could not be analyzed with MS. Kaiser tests were performed after each coupling to confirm each sub-monomer. After library synthesis was complete, 20 random beads were selected from the library for linear and tandem MS analysis as a way to evaluate library quality and MS sequencing techniques. This quality control analysis resulted in a success rate of 9/20 successful sequences (**Figure 12**).

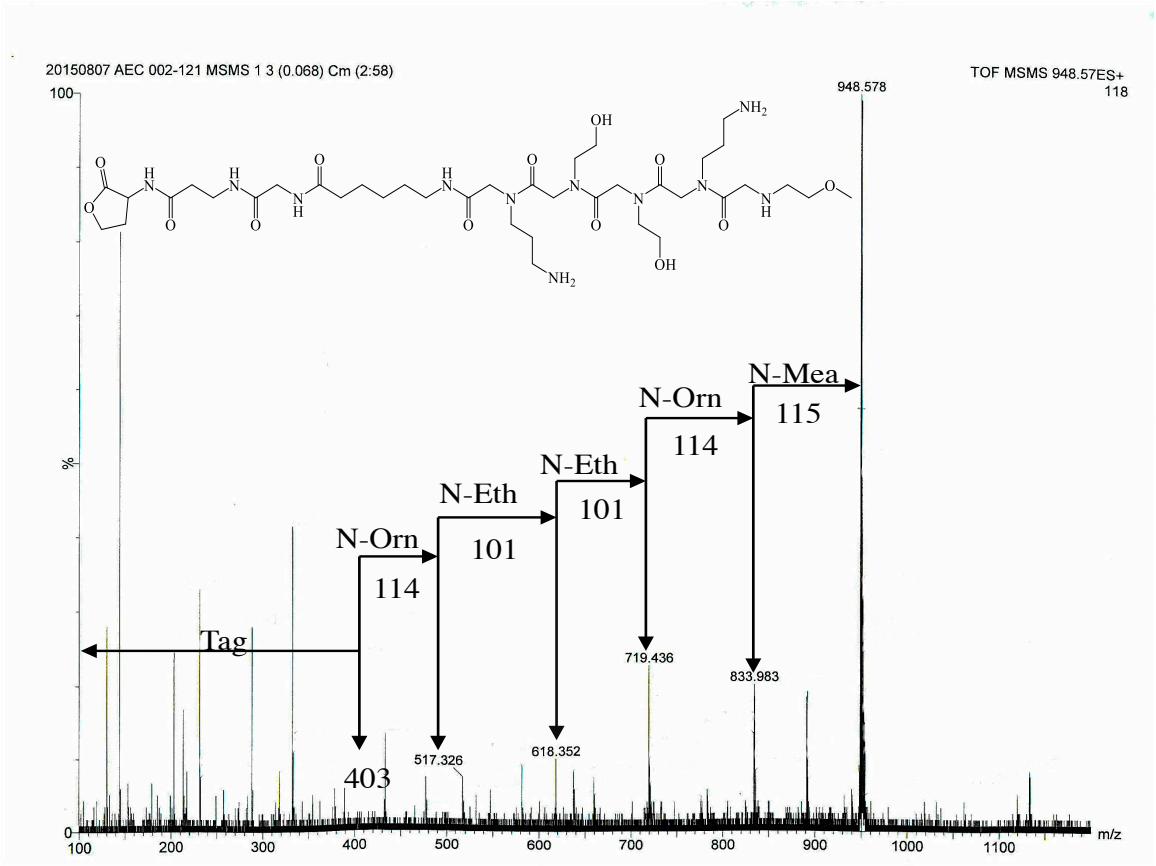


Figure 12: The sequence of an unknown test peptoid randomly synthesized in the combinatorial library and verified via tandem mass spectrometry. The sequence was determined with y ions, which result from N-terminal fragmentation of the peptoid.

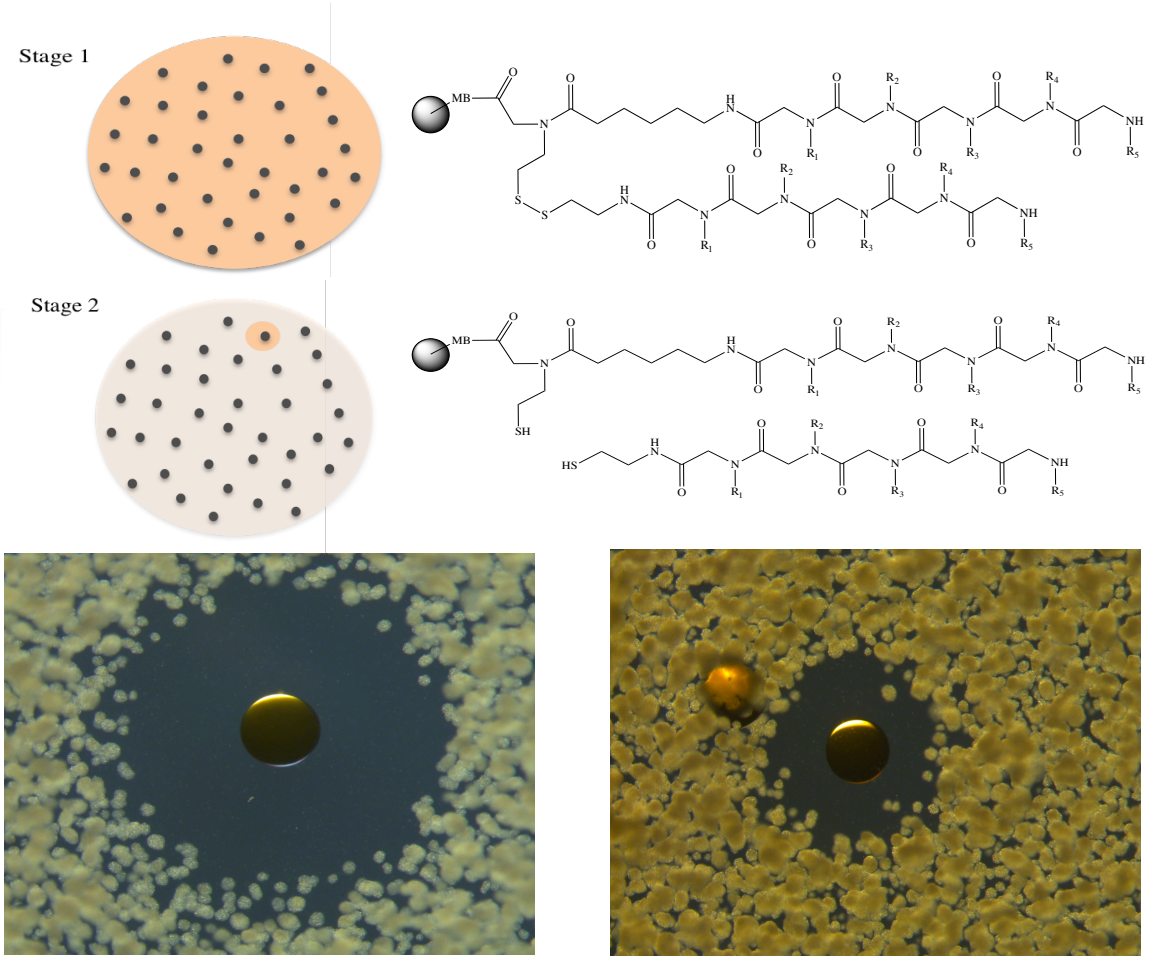
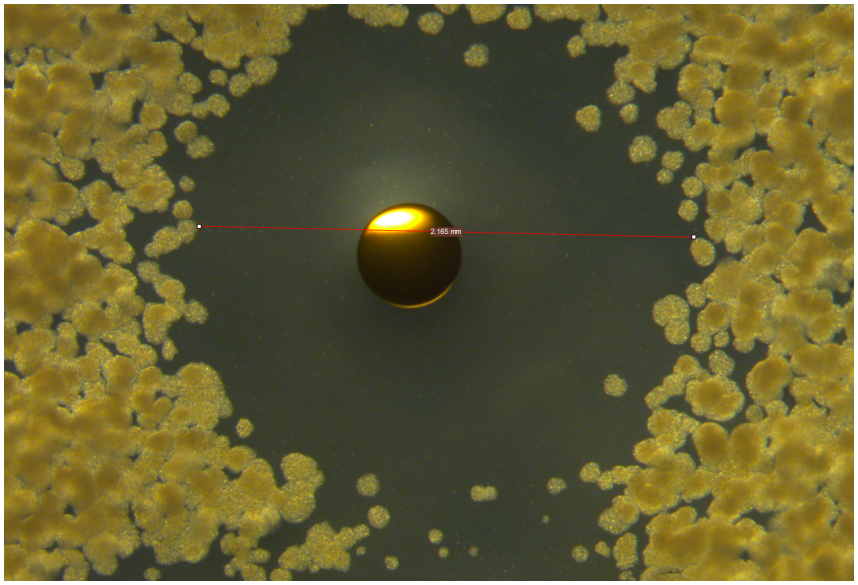


Figure 13: The compound-screening phase was separated into two stages. The first stage was the introduction of the combinatorial library peptoids. After incubation, the second stage was the detachment of the second strand at the S-S linker and identification of peptoids with anti-fungal properties was seen in the zone of clearance via the strand attached to the bead. The photo is a microscopic image of a zone of clearance after detachment of the second strand.

The concentration of cells and DTT was optimized for plating purposes. It is important to have a concentration of cells that will yield a dense enough lawn to show zones of clearance (inhibition) with a 24-hour incubation time without overwhelming the beads (**Figure 13**). It was determined that 1×10^{6} cells mL^{-1} produced an acceptable lawn. Next, DTT concentration was tested to determine a concentration that would allow

for adequate disulfide cleavage without harming fungal cells. DTT (10 mM) was experimentally determined to be a concentration that allowed for the lawn to grow unharmed while effectively dispersing the beta strand from the bead by performing assays with various concentrations of DTT. With conditions established for screening, the process was repeated 4 times. Unfortunately, there were no detectable zones of inhibition. To insure that conditions produced an environment that would allow for the peptoids to be deployed from the bead, a second library synthesized by a colleague in the laboratory was implemented. This library had different characteristics, including a higher amount of hydrophobicity. A sample of the more hydrophobic library (6 mg) was compared to the same amount of the original library. During the screening of the more hydrophobic library, there were 44 zones of clearance observed meaning this library demonstrates antifungal properties (**Figure 14**). The largest zone of clearance measured was over 2 mm in diameter.



Sequence

Beads from the screened second library were physically removed from the plate using surgical tweezers. They were washed as previously described and prepared for analysis with MS. Due to the higher hydrophobicity of the second library in comparison to this library, it was observed that higher collision energy was needed to produce a readable spectrum from MS. For many sequences, y and b ions were used to determine the structures of different beads. When a peptoid fragments due to collision in the MS, different ions are observed. Y ions are fragments that result from N-terminal fragmentation of the peptoid (the end of the sequence). The fragment masses observed on tandem by y ions are in sequential order of the overall sequence of the peptoid. B ions, on the other hand, result from fragmentation of the C-terminus of the peptoid (sub- monomers closest to tag). The fragments are also in sequential order; however, the opposite way of y ions.

IV. Conclusions

The overall goal of this work, synthesis, screening, and sequencing of combinatorial library peptoids, was achieved. The synthesis of the antimicrobial peptoids via split-and-pool synthesis yielded a one-bead-one-compound library, this meant that each bead in the library was unique and not very much time was spent in making the actual library. This library yielded 40,000 compounds in one week. This is important because the screening process of the library determines which compounds have antimicrobial properties. The screening of the combinatorial library was determined successful once the second library was implemented. This conclusion can be drawn due to the high number of zones of clearance seen after a 24-hour incubation period. Methods of preparing the peptoid of interest, once identified by a zone of clearance, proved to be effective. Sequences were determined for unknown compounds after the screening phase. However, due to the results found when comparing the two libraries tested, it is important that a follow-up antifungal peptoid library be synthesized. Given that the synthesis, screening, and sequencing of this proof-of-concept library was successful, the follow-up library can be can be synthesized with the same techniques with confidence that the methodology of synthesis and screening of the library are sufficient.

Future Direction

Goals for proceeding with this research include the synthesis of another library with more hydrophobic residues and optimizing conditions for coupling of previously removed sub-monomers. This follow-up library will include the excluded sub-

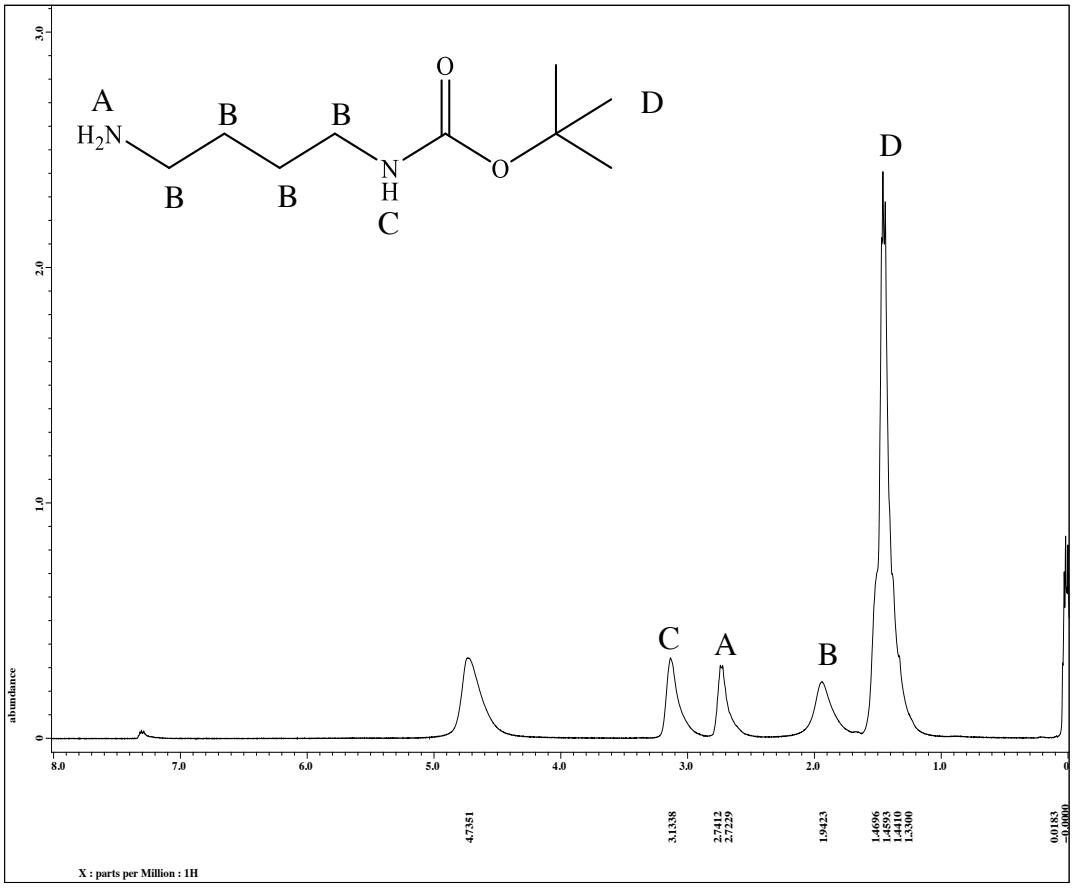
monomers to show whether exploiting functional groups of current antifungal treatments is effective. Histamine, aminothiazole, and allylamine contain two functional groups found in current treatment (azoles and polyenes). Implementing those into the library will help narrow the focus on which characteristics are necessary for a peptoid library used against fungi. Lastly, the peptoid sequences found during the screening and sequencing phase will be resynthesized. Resynthesizing these compounds will allow them to be tested against other fungal organisms. The compounds will be analyzed to determine the minimum inhibitory concentration (MIC) against fungal organisms and the cytotoxicity against mammalian cells.

References

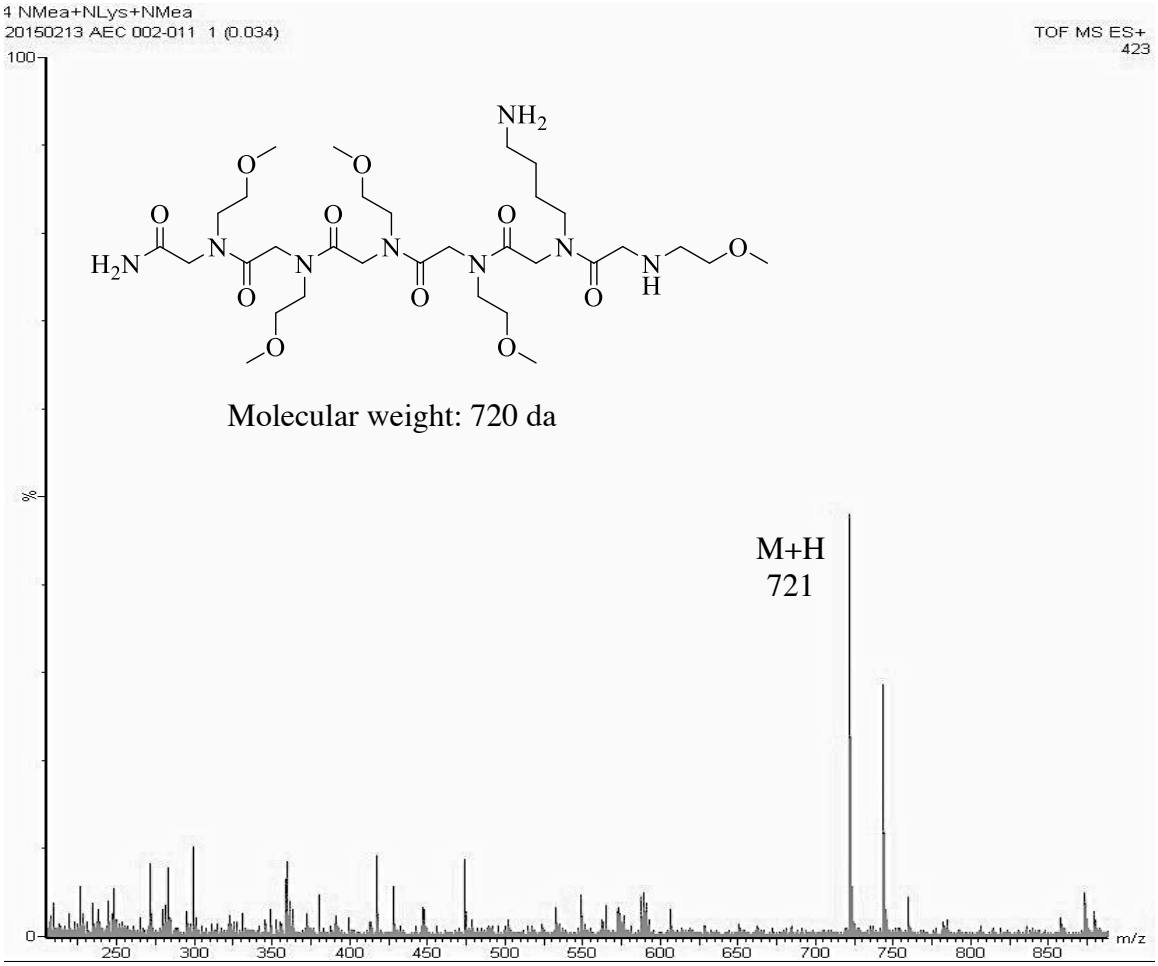
1. Ashley, E. S. D.; Lewis, R.; Lewis, J. S.; Martin, C.; Andes, D. Pharmacology of Systemic Antifungal Agents. *Clinical Infectious Diseases*. **2006**, *43*, S28-S39. Clinical Infectious Disease. http://cid.oxfordjournals.org/content/43/supplement_1/s28.full (accessed March 20, 2015).
2. Brandt, M. E.; Park, B. J. Think Fungus—Prevention and Control of Fungal Infections. *Emerging Infectious Diseases*. **2013**, *19*, 1688-1689. Centers of Disease Control and Prevention. http://wwwnc.cdc.gov/eid/article/19/10/13-1092_article (accessed March 20, 2015).
3. Culf, A. S.; Ouellette, R. J. Solid-Phase Synthesis of N-Substituted Glycine Oligomers (a-Peptoids) and Derivatives. National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/pubmed/20714299 (accessed Feb 1, 2015).
4. Eschenauer, G.; Depestel, D. D.; Carver, P. L. Comparison of Echinocandin Antifungals. *Therapeutics and Clinical Risk Management*. **2007**, *3*, 71-97.
5. Gill, K.; Kumar, S.; Xess, I.; Dey, S. Novel synthetic anti-fungal tripeptide effective against *Candida krusei*. National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/pubmed/25560012 (accessed Feb 1, 2015).
6. Gottler, L. M.; Ramamoorthy, A. Structure, membrane orientation, mechanism, and function of pexiganan — A highly potent antimicrobial peptide designed from magainin. Science Direct, http://www.sciencedirect.com/science/article/pii/s0005273608003362 (accessed Mar 20, 2015).
7. Karkowska-Kuleta, Justyna, and Andrzej Kozik. "Cell Wall Proteome of Pathogenic Fungi." *Acta Biochim Pol Acta Biochimica Polonica* 62.3 (2015): 339-51. *NIH*. Web. 1 Sept. 2015.
8. Movahed, Elaheh, Komathy Munusamy, Grace Min Yi Tan, Chung Yeng Looi, Sun Tee Tay, and Won Fen Wong. "Genome-Wide Transcription Study of Cryptococcus Neoformans H99 Clinical Strain versus Environmental Strains." *PLOS ONE PLoS ONE* 10.9 (2015): n. pag. Web. 1 Sept. 2-15.
9. Pappas, P.; Kauffman, C.; Andes, D.; Benjamin, J. D.; Calandra, T.; Edwards, J. J.; Filler, S.; Fisher, J.; Kullberg, B.; Ostrosky-Zeichner, L.; Reboli, A.; Rex, J.; Walsh, T.; Sobel, J. Clinical Practice Guidelines for the Management of Candidiasis: 2009 Update by the Infectious Diseases Society of America. *Clinical Infectious Diseases*. **2009**, *48*, 503-535.
10. Perfect, J.; Dismukes, W.; Dromer, F.; Goldman, D.; Graybill, J.; Hamill, R.; Harrison, T.; Larsen, R.; Lortholary, O.; Nguyen, M.; Pappas, P.; Powderly, W.; Singh, N.; Sobel, J.; Sorrell, T. Clinical Practice Guidelines for the Management of Cryptococcal Disease: 2010 Update by the Infectious Diseases Society of America. *Clinical Infectious Diseases*. **2010**, *50*, 291-322.

11. Sobel, Jack D., and José A. Vázquez. *Contemporary Diagnosis and Management of Fungal Infections*. Newtown, PA: Hand in Health Care, 2003. Print.
12. Stawikowski, M.; Fields, G. B. Introduction to Peptide Synthesis. *PubMed Center*. **2013**. National Institute of Health. http://www.ncbi.nlm.nih.gov/pmc/articles/pmc3564544/?report=classic (accessed March 20, 2015).
13. Tanaka, Reiko J., Neville J. Boon, Katarina Vrcelj, Anita Nguyen, Carmelina Vinci, Darius Armstrong-James, and Elaine Bignell. "In Silico Modeling of Spore Inhalation Reveals Fungal Persistence following Low Dose Exposure." *Sci. Rep. Scientific Reports* 5 (2015): 13958. *Nature*. Web. 1 Sept. 2015.
14. Uchida, M.; Mcdermott, G.; Wetzler, M.; Gros, M. A. L.; Myllys, M.; Knoechel, C.; Barron, A. E.; Larabell, C. A. Soft X-ray Tomography of Phenotypic Switching and the Cellular Response to Antifungal Peptoids in *Candida Albicans*. *Proceedings of the National Academy of Sciences*. **2009**, *106*, 19375-19380.
15. Walsh, T.; Anaissie, E.; Denning, D.; Herbrecht, R.; Kontoyiannis, D.; Marr, K.; Morrison, V.; Segal, B.; Steinbach, W.; Stevens, D.; Burik, J.; Wingard, J.; Patterson, T. Treatment of Aspergillosis: Clinical Practice Guidelines of the Infectious Diseases Society of America. *Clinical Infectious Diseases*. **2008**, *46*, 327-360.

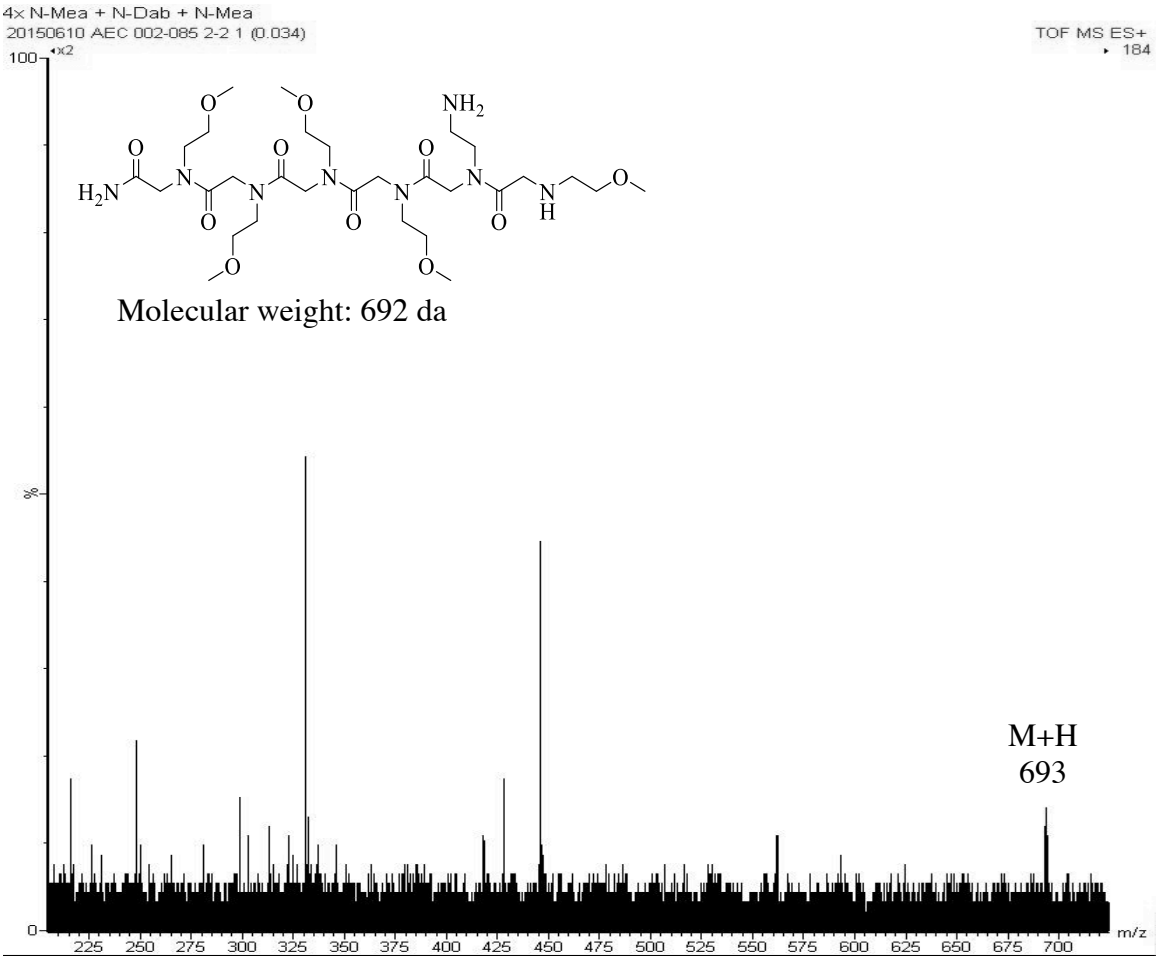
Appendix



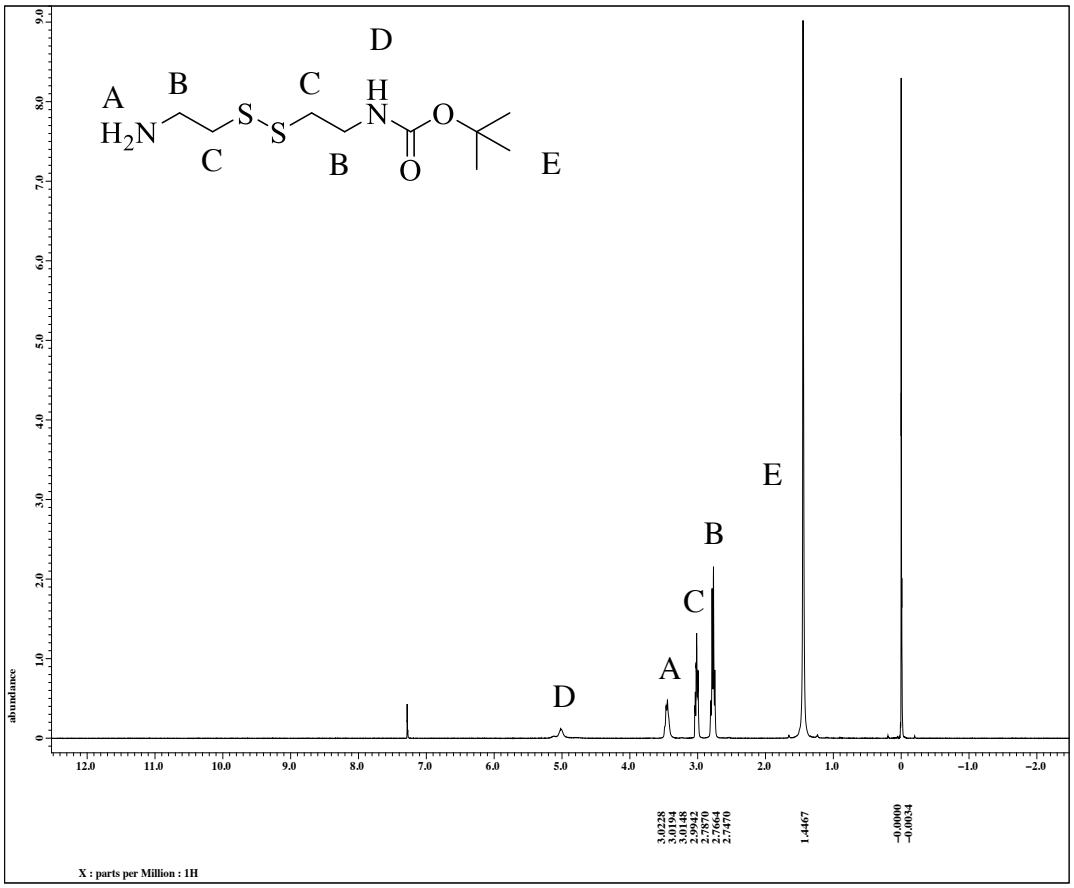
A1: ^{1}H NMR spectrum of N-Diaminobutane (Boc)



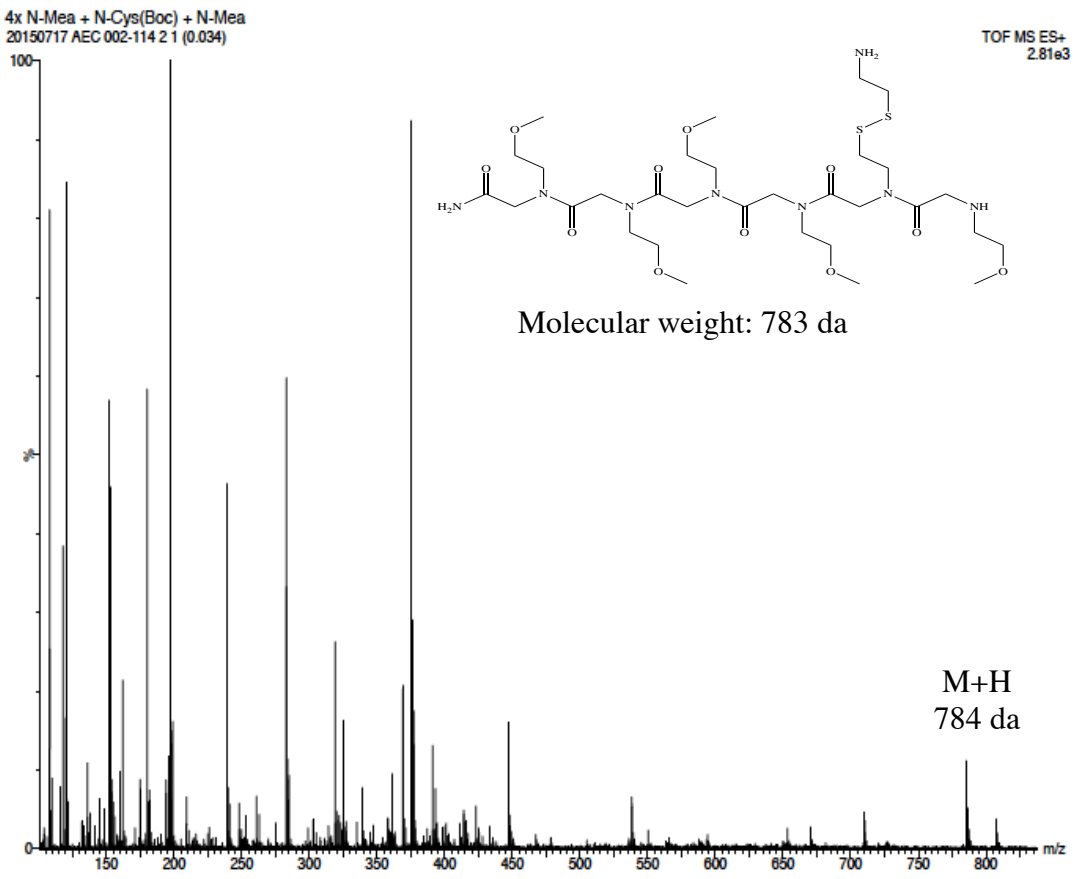
A2: Synthesis confirmation of N-Lys(Boc) by previously discussed methods. The MS confirms that the sub-monomer is pure due to lack of branching due to the symmetry of the compound. MS spectra supplement results of NMR.



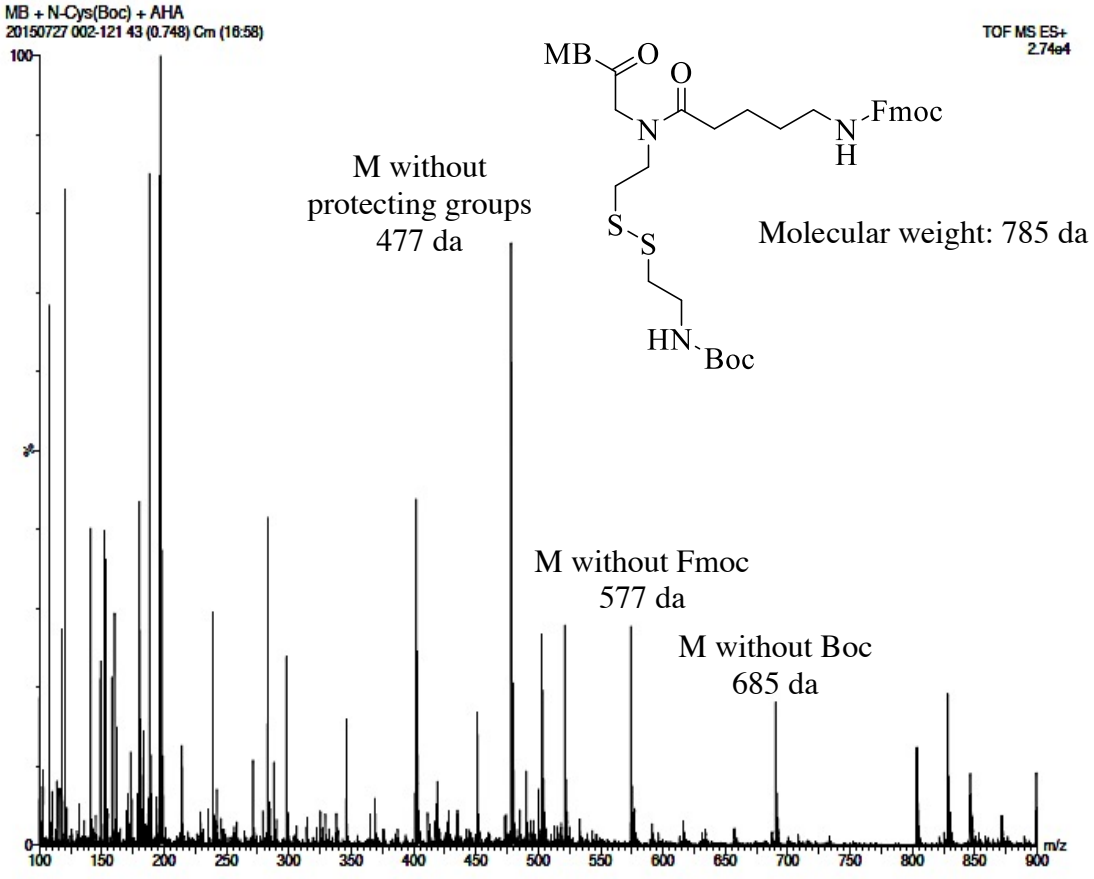
A3: MS confirmation of synthesis of N-Dab(Boc)



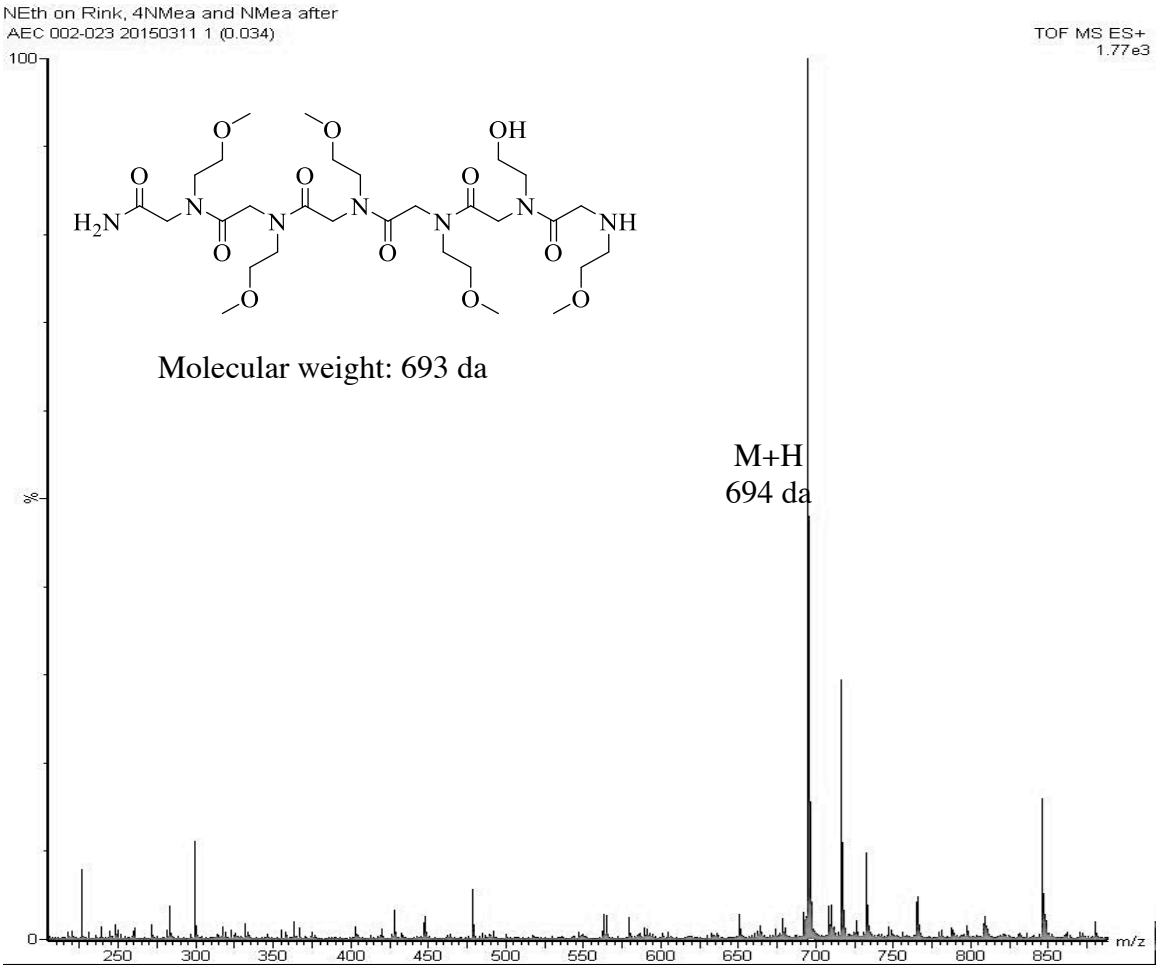
A4: ^{1}H NMR spectrum synthesis confirmation of N-Cys(Boc).



A5: MS confirmation of synthesis of N-Cys(Boc)



A6: Synthesis confirmation of tag by MS. Due to protecting group fragmentation, peaks that correlate to the molecular weight of the compound with the attachment of only one or neither of the protecting groups were found.



A7: MS confirmation of synthesis of N-Eth(TBDMS)