Synthesis and Characterization of Cyclic Peptoids Against *Cryptococcus neoformans* and *Candida albicans*

by
Ross Harrison Sibley

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

Fall 2023

Thesis Committee:

Dr. Kevin Bicker, Thesis Director

Dr. Keying Ding, Second Reader

Dr. Amy Phelps, Thesis Committee Chair

Synthesis and Characterization of Cyclic Peptoids Against *Cryptococcus neoformans* and *Candida albicans*

by Ross Harrison Sibley

APPROVED:

Dr. Kevin Bicker, Thesis Director
Professor, Department of Chemistry

Dr. Keying Ding, Second Reader
Professor, Department of Chemistry

Dr. Amy Phelps, Thesis Committee Chair
Interim Chair, Department of Chemistry

Dedication

I dedicate this thesis to my guardian angels, Omi and Grandpa Bill, who had front row seats for the entire journey.

Acknowledgements

I would like to thank Dr. Kevin Bicker for providing endless support as my research mentor throughout the duration of my undergraduate research experience. I have a deep admiration for his patience, knowledge, and kindness which I hope to reflect as a future researcher and educator. I also want to thank both my past and present Bicker Lab family members-Devin Weaver, Elena Mancera Andrade, Janna Abou-Rahma, Maria Clark, Mary Tran, and Matthew Johnson. The lab environment they created fostered personal growth, merit, and lots of game nights. Thank you to the Honors College and the Undergraduate Research Center for providing the opportunity to complete this project and receive funding to present my finding across the globe. I would like to thank my mom, dad, and sister – Pam, Tim, and Emily – for their unconditional love and support in everything I do. Finally, I would like to thank God for blessing me with the ability to complete this work and guiding me through all things.

Abstract

Antimicrobial resistant strains of fungal pathogens such as *Cryptococcus neoformans* and *Candida albicans* have risen to concerning heights, necessitating the discovery of a safe and effective novel antimicrobial agent. Peptoids, N-substituted peptide mimics, are one class of compounds with increasing interest due to improved stability and bioavailability compared to their peptide counterparts. RMG8-8 and RMG9-11 are two peptoids recently discovered in the Bicker Lab with observed antifungal activity against *Cryptococcus neoformans* and *Candida albicans*, respectively. This study attempted to optimize antifungal activity of RMG8-8 and RMG9-11 through cyclization. Following synthesis, cyclic RMG8-8 (RHS3) and cyclic RMG9-11 (RHS6) were characterized by minimum inhibitory concentration (MIC_{90}) against the two fungal pathogens, mammalian cytotoxicity (MTT) against HepG2 liver cells, and hemolytic activity against human red blood cells. Despite neither of the cyclic peptoids significantly outperforming their linear parent compound, future optimization efforts may be investigated.

Table of Contents:

Chapters

Methodology

Results & Discussion

List of Tables:

List of Figures:

Terms and Definitions:

1. **Peptide:** a molecule with a backbone consisting of a chain of amino acids, each with distinctive side chains branching off an alpha carbon
2. **Peptoid:** peptide-like compound, with side chains branching off the amide nitrogen rather than the alpha carbon
3. **Solid-Phase Synthesis:** a synthetic technique in which a compound is synthesized off a resin (polystyrene) bead with little to no intermediate purification
4. **Minimum Inhibitory Concentration (MIC_{90}):** The lowest concentration of compound required to inhibit 90% growth of a microbial pathogen
5. **Toxic Dose (TD_{50}):** the concentration of compound at which there is a 50% reduction of mammalian cell growth compared to a negative water control
6. **10% Hemolytic Activity (HC_{10}):** the concentration of compound at which 10% of human red blood cells were lysed
7. **Selectivity Ratio (SR):** represents the probability of a peptoid to kill the fungal pathogen cell over a human cell

Abbreviations of Chemical Names:

1. **4-DMAP:** 4-dimethylaminopyridine
2. **ACN:** acetonitrile
3. **DCM:** dichloromethane
4. **DDC:** sodium diethyldithiocarbamate
5. **DIPEA:** diisopropylethylamine
6. **DMEM:** Dulbecco Modified Eagle Media
7. **DMF:** dimethylformamide
8. **DMSO:** dimethyl sulfoxide
9. **FBS:** fetal bovine serum
10. **HBTU:** N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate
11. **HOBt-Cl:** 6-chloro-1-hydroxybenzotriazole
12. **MTT:** thiazolyl blue tetrazolium bromide
13. **NMM:** N-methylmorpholine
14. **PSG:** 1% penicillin, streptomycin, and glutamine
15. **TFA:** trifluoracetic acid
16. **THF:** tetrahydrofuran
17. **TIS:** triisopropylsilane

Introduction:

Whether consciously or not, humans come in contact and interact with fungal pathogens on a daily basis. While this is not inherently a cause for concern due to the body's innate ability to fight of minor fungal infections on its own, fungal pathogens such as *Cryptococcus neoformans* and *Candida albicans*, which are responsible for infections such as cryptococcal meningitis[1] and candidemia[2] respectively, can pose severe threats to immunocompromised individuals. Currently, there is a limited number of antifungal medications are the market that are able to successfully eradicate infections caused by *Cryptococcus neoformans* and *Candida albicans*, mainly due to difficulties developing nontoxic and effective therapeutics. Two current antifungal medications, amphotericin B and fluconazole, can be prescribed to treat *Cryptococcus neoformans* and *Candida albicans*[3], but concern for long-term nephrotic (kidney) and hepatic (liver) toxicity[4] in addition to the emergence of antifungal resistant strains of *Cryptococcus* and *Candida* species[5,6] necessitates investigations into other classes of antifungal therapeutics.

As far as novel therapeutics are concerned, peptoids, or N-substituted glycines, are a class of compounds with significant promise with regards to treating fungal infections as a result of improved stability and bioavailability relative to their peptide counterparts.[7–9] The relatively greater stability exhibited by peptoids can be attributed to the side chains, or R-groups, on amide-nitrogens as opposed to the *alpha*-carbon as is the case for peptides **(Figure 1).** Furthermore, the shift in side chain location in combination with the loss of the amide proton results in an more hydrophobic, achiral backbone, promoting a greater degree of flexibility within the structure as a whole.[10] Equally as beneficial for success as a potential therapeutic agent, the shift in side chain allows peptoids to evade recognition by protease enzymes which otherwise rapidly digest peptides, increasing both half-life and cell permeability for peptoids *in vivo*.[11]

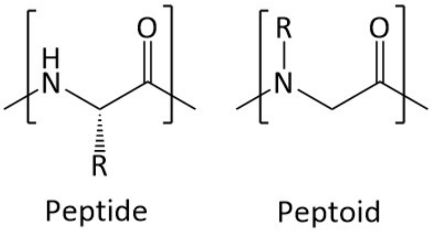


Figure 1. Structural comparison of a peptide and peptoid backbone.

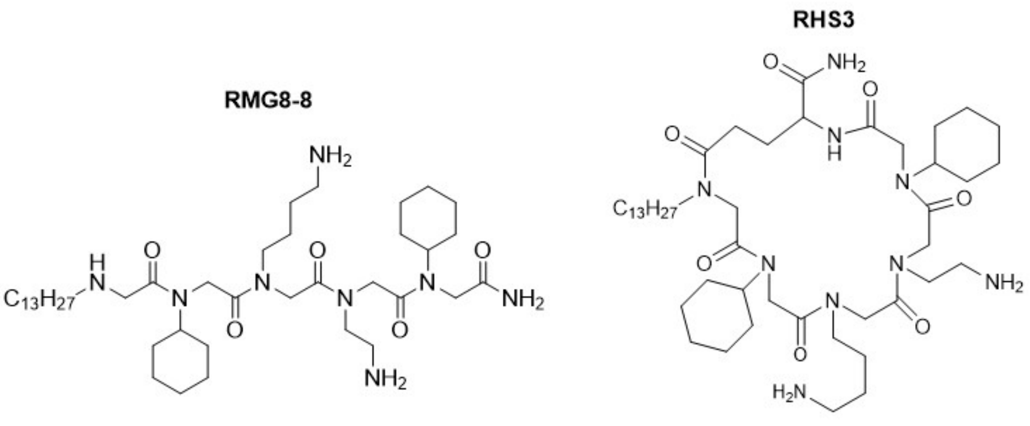
In addition to their biological advantages over peptides, peptoids are relatively simple and inexpensive to synthesize. Peptoids are synthesized via solid-phase synthesis, a stepwise method in which the peptoid is built submonomer by submonomer off a resin (polystyrene) bead, similar to building blocks.[12] Solid phase synthesis also eliminates the need for isolation and purification of chemical intermediates. In this way, different combinations of monomers can be synthesized, purified, and tested against target pathogens or human cell lines in a relatively short period of time.

Under Dr. Kevin Bicker, the Bicker Lab has previously discovered RMG8-8 and RMG9-11, two linear peptoids that have demonstrated promising antifungal activity against *Cryptococcus neoformans* and *Candida albicans*, respectively.[11,13] Baseline efficacy is evaluated using the minimum inhibitory concentration (MIC_{90}), or the minimum concentration of compound at which 90% of the fungal pathogen's growth is inhibited. RMG8-8's *in vitro* MIC of 1.56 μg/mL improved upon the MICs of clinical antifungals fluconazole and flucytosine (2 μg/mL) against *Cryptococcus neoformans*, though amphotericin B's MIC_{90} is consistently lower at a concentration of 0.25 μg/mL.[11] Similarly, RMG9-11 exhibits promise as a potential treatment for *Candida albicans* as its *in vitro* MIC_{90} (6.25 μg/mL) is on par with that of other antifungal peptoids[8] as well as fluconazole and flucytosine.[14] Both RMG8-8 and RMG9-11 demonstrate great potential to treat fungal pathogens and may benefit from optimization efforts.

Cyclization, or the conversion of linear peptoids into cyclic conformations, is one potential method of optimization. As mentioned earlier, linear peptoids have a large degree of flexibility due to the nature of the N-substituted backbone and lack of chiral α-carbon as seen in peptides; however, a greater degree of flexibility is generally regarded a disadvantage in the field of medicinal chemistry as increased rigidity is often accompanied by increased efficacy.[15] Compared to linear peptoids such as RMG8-8 and RMG9-11, cyclic peptoids exhibit greater rigidity due to their inability to change conformations, a feature that is also beneficial in the protein binding process without being entropically intrusive.[16] Additionally, it is unlikely that the permeability of a linear peptoid is affected in the cyclization process as the lack of proton on the amide nitrogen of the backbone is a conserved feature.[10] Furthermore, cyclic peptoids have been posited to have greater potential for fungal cell permeability via the formation of a pore within the cell membrane of fungal pathogens.[17] For these reasons, a cyclization-based method of optimization garnered investigation into the synthesis and characterization of previously discovered linear peptoids.

Thesis Statement:

Cyclic RMG8-8 (RHS3) and cyclic RMG9-11 (RHS6) (**Figures 2** and **3**) will have improved efficacy against *Cryptococcus neoformans* and *Candida albicans*, respectively, when compared to the linear peptoid conformations due to increased rigidity and potential pore formation. Furthermore, the conservation of permeability between linear and cyclic conformations suggests that the cyclic derivatives will have comparable mammalian cytotoxicity and hemolytic activity relative to the parent compounds.



Molecular Weight: 763.1260
$clogD_{7.4} = -2.38$

Molecular Weight: 874.2260
$clogD_{7.4} = -2.05$

Figure 2. Structures of antifungal peptoids RMG8-8 and RHS3. The expected molecular weight and theoretical clogD$_{7.4}$, a measure of lipophilicity, are included.

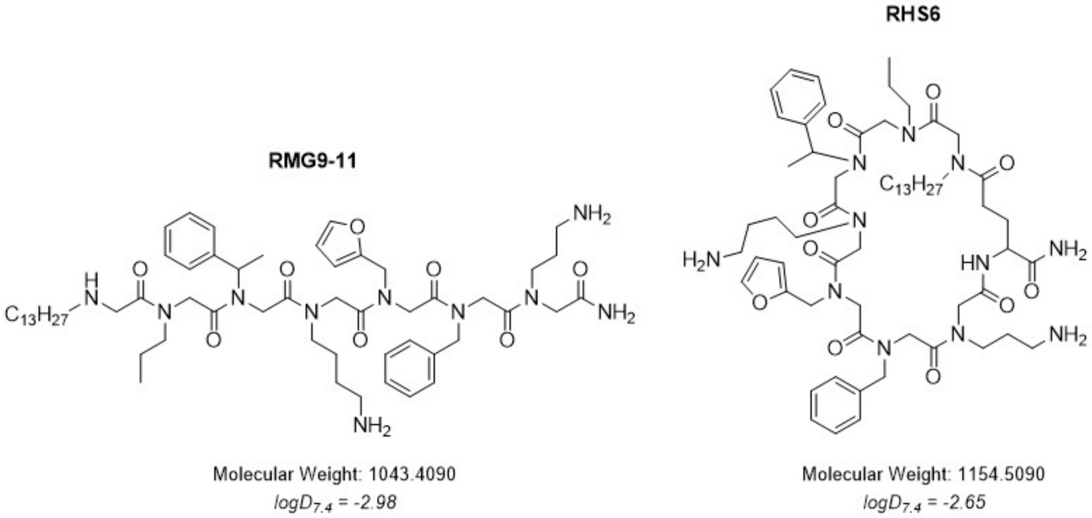


Figure 3. Structures of antifungal peptoids RMG9-11 and RHS6. The expected molecular weight and theoretical $clogD_{7.4}$, a measure of lipophilicity, are included.

Methodology:

Aim I: Cyclic Peptoid Synthesis

In general, peptoids were synthesized submonomer by submonomer off resin (polystyrene) beads by means of solid-phase synthesis. As mentioned before, solid-phase synthesis eliminated intermediate purification between submonomers as excess solution was filtered out, expediting the overall synthesis process. **Figure 4** and **Figure 5** outline the overall synthetic scheme of RHS3 and RHS6 respectively.

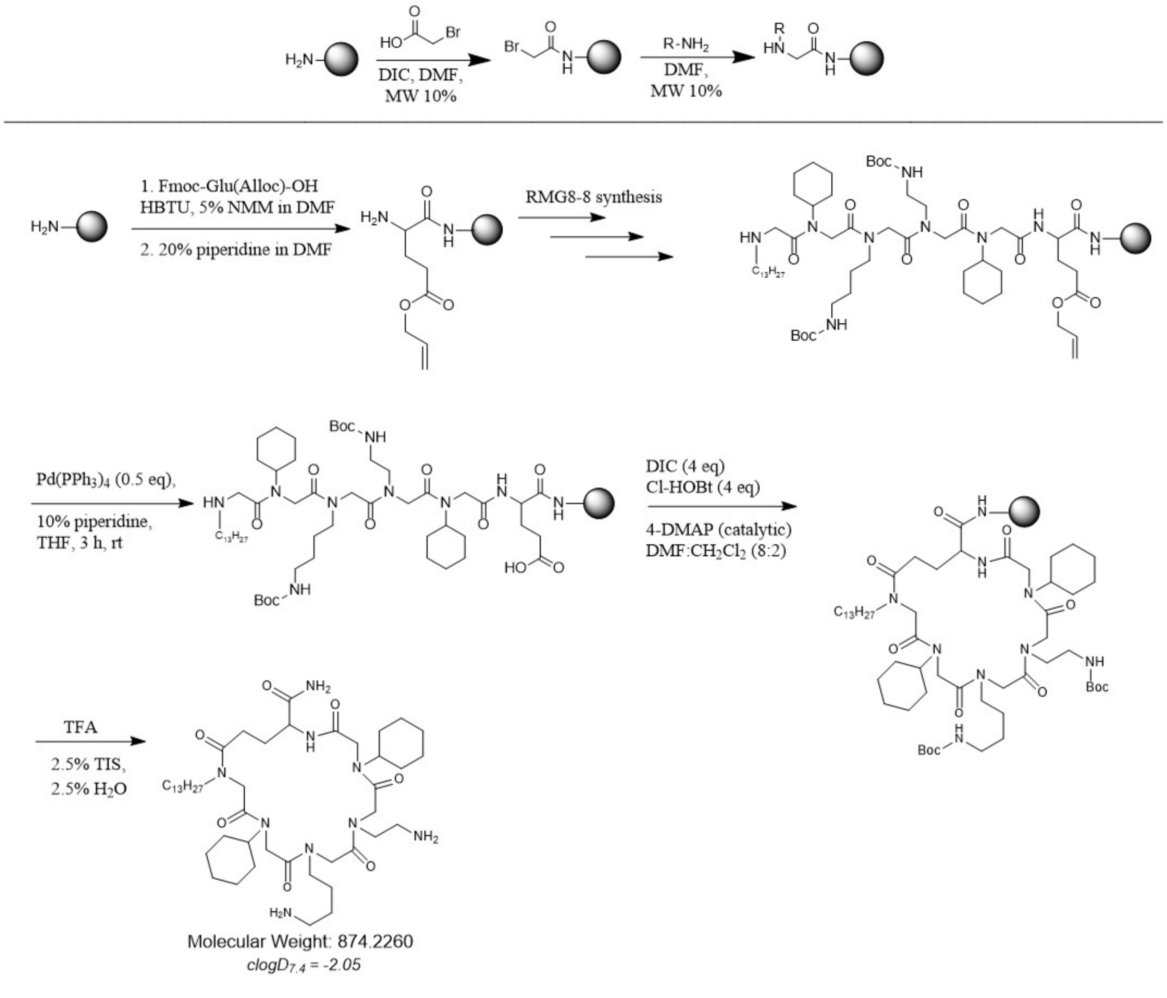


Figure 4. Solid-phase synthetic scheme of RHS3.

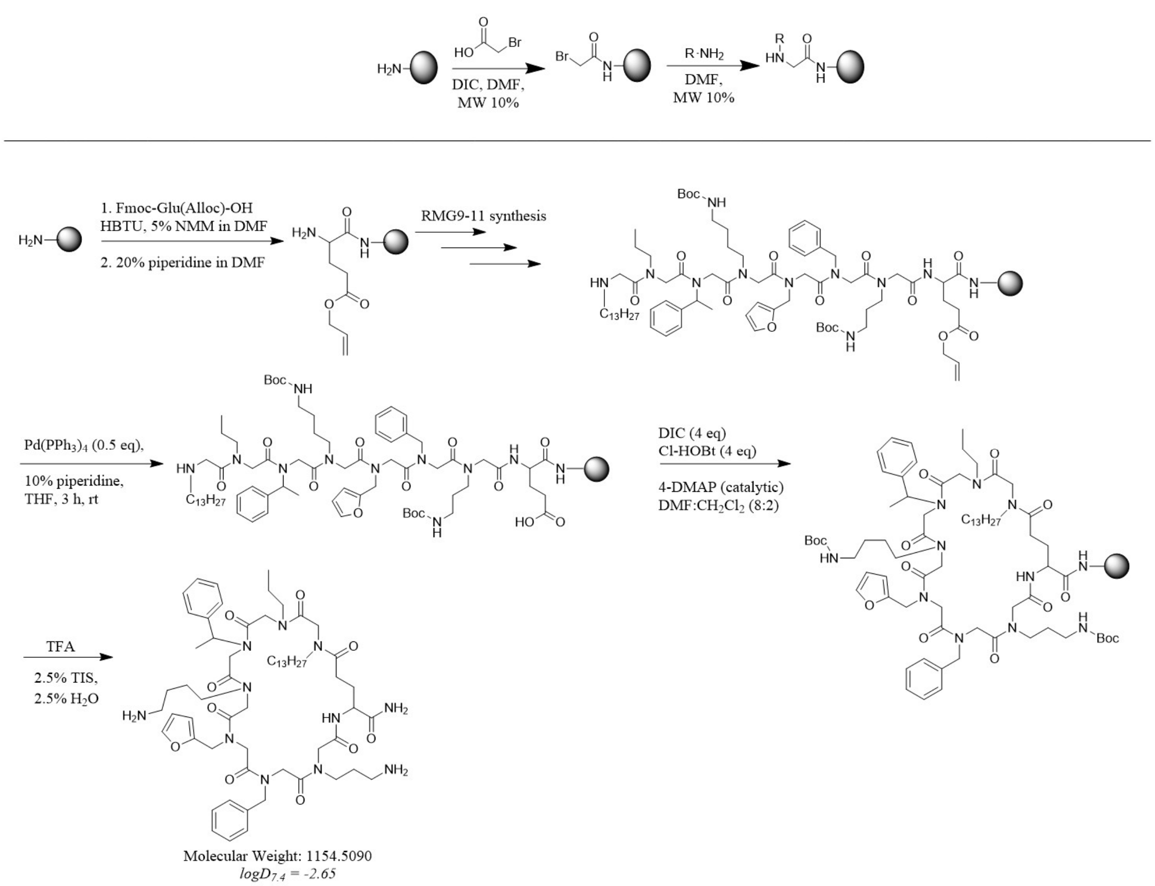


Figure 5. Solid-phase synthetic scheme of RHS6.

Prior to the execution of any reactions, Rink amide resin with a loading level of 0.65 mmol/g was swelled and deprotected using a dimethylformamide (DMF) wash followed by a 20% piperdine/DMF solution. This reaction, known as an Fmoc deprotection, exposed a free amine onto which each monomer was added in a succession of subsequent bromoacylation and amination reactions. Following Fmoc deprotection, the resin-bound amine was coupled for 60 minutes with Fmoc-Glu(Alloc)-OH which was activated with N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU) in DMF containing 5% N-methylmorpholine (NMM). Following the coupling of the amino acid and washing of the resin with DMF to remove residual starting material and byproducts, a bromoacylation reaction was carried out by adding 1.5 mL of a 2M bromoacetic acid solution and 1.5 mL of a 3.2M diisopropylcarbodiimide (DIC) solution in anhydrous DMF to the resin. After the addition of these solutions, the reaction tube was microwaved for 30 seconds at 10% power, pausing at 15 seconds to briefly shake the reaction tube before the final 15 seconds. The reaction tube was placed on a rocker for 15 minutes as the bromoacylation reaction took place. After rocking, the reaction solvent was drained into an aspirator and the resin was washed with DMF to remove residual solvent. A subsequent amination reaction was carried out by adding 3 mL of a 2M solution of the desired amine in anhydrous DMF to the resin. Similar to bromoacylation, the reaction tube was microwaved for 30 seconds on 10% power before rocking for 30 minutes. After rocking, the solvent was drained into an aspirator and the resin was once again washed with DMF to remove residual solvent. **Table 1** summarizes the amines used in RHS3 and RHS6 synthesis. Preceding each bromoacylation and amination reaction, a Kaiser test was performed and indicated the presence (positive) or absence (negative) of a free amine, providing some degree of expected intermediate confirmation. Generally, Kaiser tests were positive after aminations given the addition of a free amine and negative after bromoacylations. However, there were exceptions for some bulky amines such as cyclohexylamine which yielded a negative Kaiser test following amination.

Table 1. Amine submonomer positions in RHS3 and RHS6 synthesis.

Position	***Peptoid***	
	RHS3/RMG8-8	RHS6/RMG9-11
1	Aminotridecane	Aminotridecane
2	Cyclohexylamine	Propylamine
3	N-Boc-1,4diaminobutane	(±)-Phenylethylamine
4	N-Boc-1,2-diaminoethane	N-Boc-1,4-diaminobutane
5	Cyclohexylamine	Furfurylamine
6		Benzylamine
7		N-Boc-1,3-diaminopropane

Once all the desired monomers were added to the resin, 7 mL of 0.5 eq tetrakis (triphenylphosphine) palladium (0) ($Pd(PPh_3)_4$) in 10% piperdine/tetrahydrofuran (THF) was added to the resin and rocked for three hours to deprotect the alloc group from the C-terminal glutamic acid, a crucial step for cyclization later on. Following palladium recapture using 5% weight/volume sodium diethyldithiocarbamate (DDC) in 5% diisopropylethylamine (DIPEA)/DMF, the peptoid was cyclized overnight with 4 eq of DIC and 4 eq of 6-chloro-1-hydroxybenzotriazole (HOBt-Cl) in excess DMF and CH_2Cl_2, a process facilitated by catalytic 4-dimethylaminopyridine (4-DMAP). The resin was washed with DMF, dichloromethane (DCM), and DMF once again. Then, the cyclic peptoids were cleaved from the resin using a solution of 2.5% triisopropylsilane (TIS) 2.5%

water in trifluoracetic acid (TFA). Once cleaved, the TFA solution was bubbled off and the peptoids were resuspended in 10 mL of a 1:1 water/acetonitrile (ACN) solution. The peptoids were purified by reverse-phase high performance liquid chromatography (RP-HPLC) using a Varian Prepstar SD-1 with a Supelco Ascentis C18 column and a gradient of water to ACN containing 0.1% TFA. Following structural confirmation by mass spectrometry (MS), ACN was removed using rotary evaporation for 15 minutes, and the peptoid solution was frozen using liquid nitrogen and dried down by lyophilization overnight.

The syntheses of RHS3 and RHS6 were both successful. Both RHS3 and RHS6 were eluted at ~75% ACN (~31 minutes) from RT-HPLC. RHS3 had an expected molecular weight of 874.2260 da and the observed m/1 peak was 874.2983 da. RHS3 had a 2.75% yield (3.60 mg). RHS6 had an expected molecular weight of 1154.5090 da and the observed m/1 peak was 1154.6038 da. RHS6 had a 4.81% yield (8.1 mg). 20 mg/mL stock solutions of each peptoid were prepared for future assays.

Aim II: Characterization of Cyclic Peptoids

To characterize and develop a better understanding of the therapeutic potential of RHS3 and RHS6, three assays were conducted: minimum inhibitory concentration (MIC_{90}) to evaluate antifungal activity, MTT to evaluate mammalian cytotoxicity, and a hemolysis assay to evaluate hemolytic activity. To ensure reliability, each assay was conducted at least three times in triplicate trials over three different days. The protocols for these assays were well established in the Bicker Lab.[11,13,18]

Antifungal Activity

The antifungal activity of RHS3 and RHS6 was determined by MIC_{90}, the minimum concentration of peptoid required to inhibit at least 90% of a particular fungal pathogen's growth. *Cryptococcus neoformans* or *Candida albicans* colonies were collected and transferred from a YPD plate to 0.85% saline solutions to reach an OD_{530} between 0.18 and 0.25. These colonies were then diluted in RPMI-MOPS, first 1:100, then again 1:20 before their addition to 96-well plates. Then two-fold serial dilutions from 12.5 to 0.39 μg/mL of RHS3 and RHS6 were added and incubated at 37 °C for either 72 hours (*Cryptococcus neoformans*) or 24 hours (*Candida albicans)* (**Figures 6** and **7**). Due to the lengthy incubation period for *Cryptococcus neoformans*, a layer of wells containing PBS surrounded the serial dilutions and controls to prevent edge effect, or evaporation of the outermost wells. Following incubation, the MIC was determined to be the lowest concentration that inhibited any visual fungal growth when compared to amphotericin B (positive) and water (negative) controls. A vehicle control of RPMI-MOPS was included to check for media contamination.

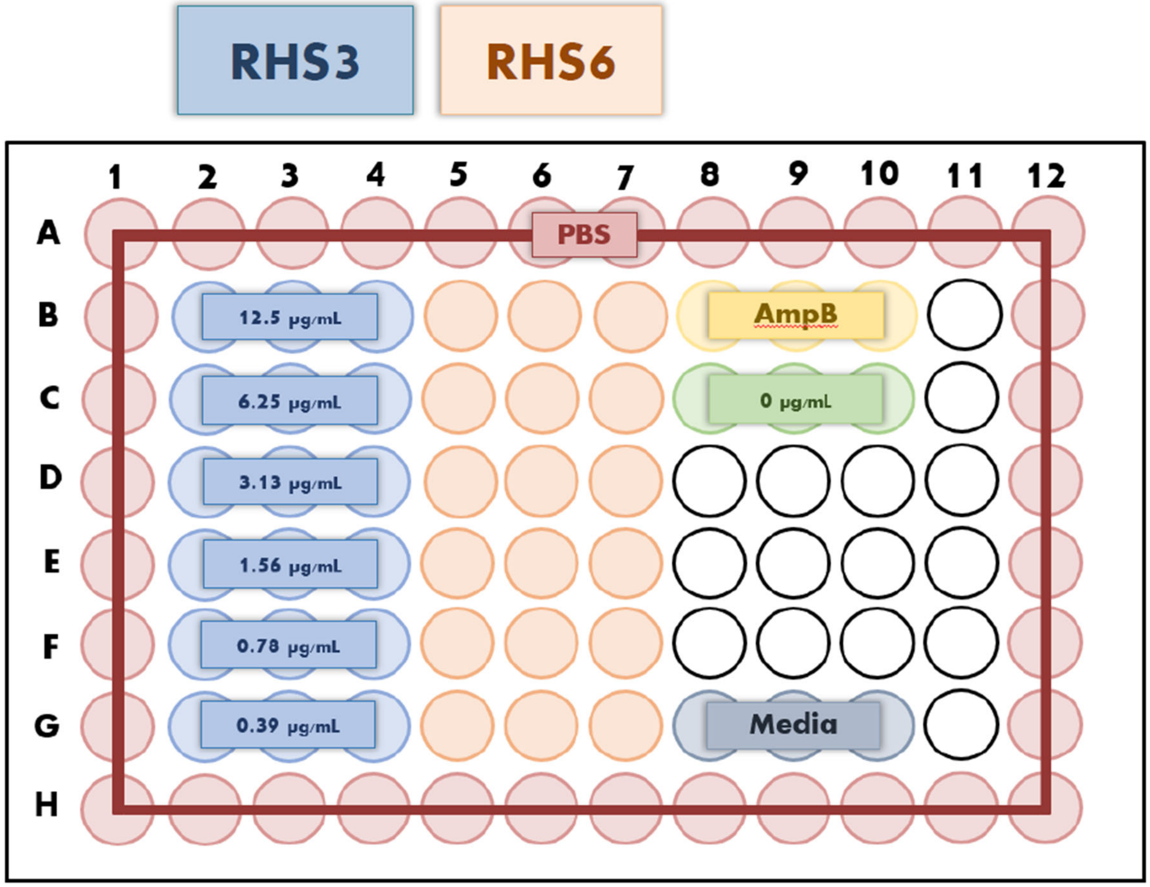


Figure 6. 96-well MIC$_{90}$ plate for *Cryptococcus neoformans*. PBS surrounded the two-fold serial dilutions and controls to prevent edge effect, or evaporation of the outermost wells.

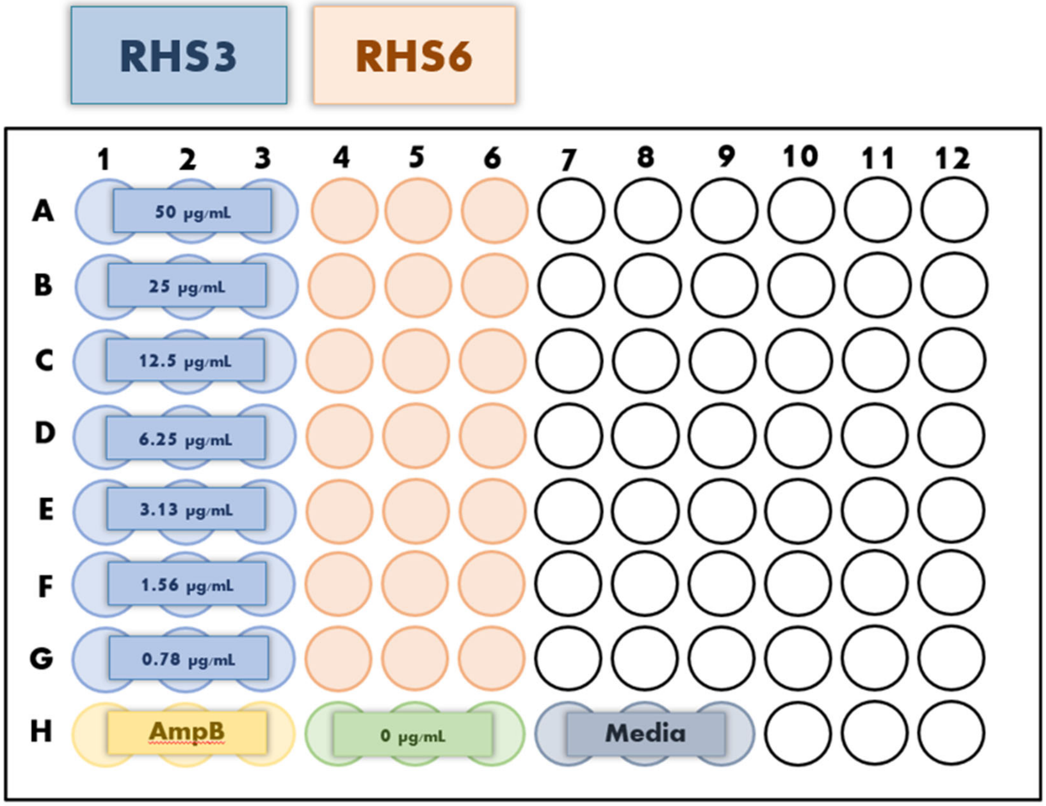


Figure 7. 96-well MIC_{90} plate for *Candida albicans*.

Mammalian Cytotoxicity

Mammalian cytotoxicity was evaluated against Hepatocellular carcinoma (HepG2) cells that populate the mammalian liver and human single donor human red blood cells (hRBCs). The HepG2 cells were cultured in Dulbeco modified eagle media (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin, streptomycin, and glutamine (PSG). Cells were collected in 7.5 mL of DMEM, and a cell concentration between 1×10^5 and 4×10^5 cells/mL was confirmed using a hemacytometer. 100 μL of the cell solution were then added to a 96-well plate and allowed to adhere during a 3-hour incubation period at 37 °C. 11.1 μL of 300, 250, 200, 150, 100, and 50 μg/mL solutions of RHS3 and RHS6 were added to the 96-well plate (**Figure 8**). After a 72-hour incubation at 37 °C, 20 μL of 5 mg/mL thiazolyl blue tetrazolium bromide (MTT) were added to the plates before another 3-hour incubation at 37 °C. Following incubation, the media was removed and 100 μL of dimethyl sulfoxide (DMSO) was added to each well. The plates were then analyzed on a SpectraMaxM5 plate reader at an absorbance of 570 nm. The toxic dose (TD_{50}), the concentration at which there was a 50% reduction of growth compared to a negative water control, was determined using GraFit.

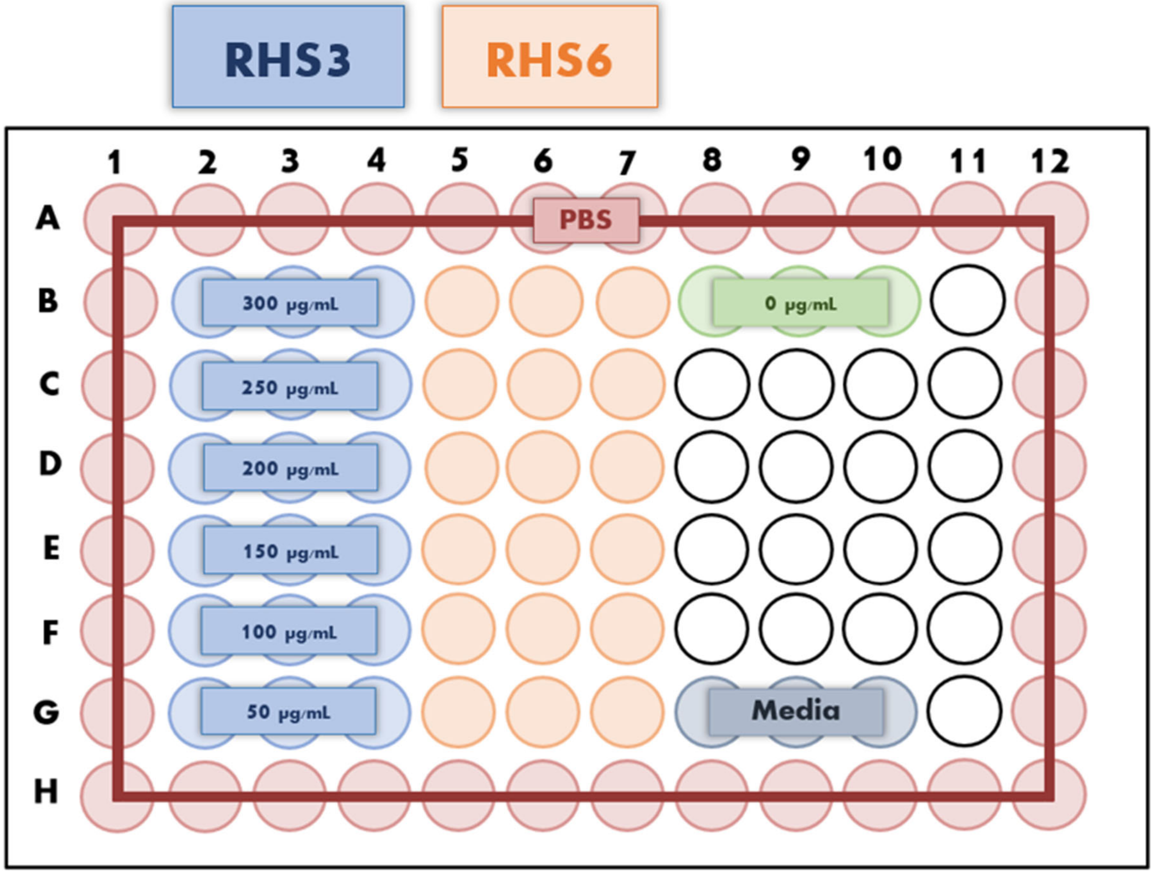


Figure 8. 96-well MTT plate using HepG2 liver cells.

The hemolytic activity of the cyclic peptoids was evaluated using hRBCs from Innovative Research. 7.5 mL of hRBCs were washed with 10 mL of PBS and centrifuged three times for 10 minutes before they were resuspended in 7.5 mL of PBS and aliquoted into a 96-well plate. Two-fold serial dilutions 120-7.5 µg/mL of RHS3, RHS6, RMG8-8, and RMG9-11 were added before the plates were incubated for one hour (**Figure 9**). Peptoid dilutions were compared to a positive control of 1% Triton X-100 and a negative control of phosphate buffer solution (PBS). The plates were then centrifuged before 5 µL of supernatant was collected and diluted in 95 µL of PBS in a new 96-well plate.

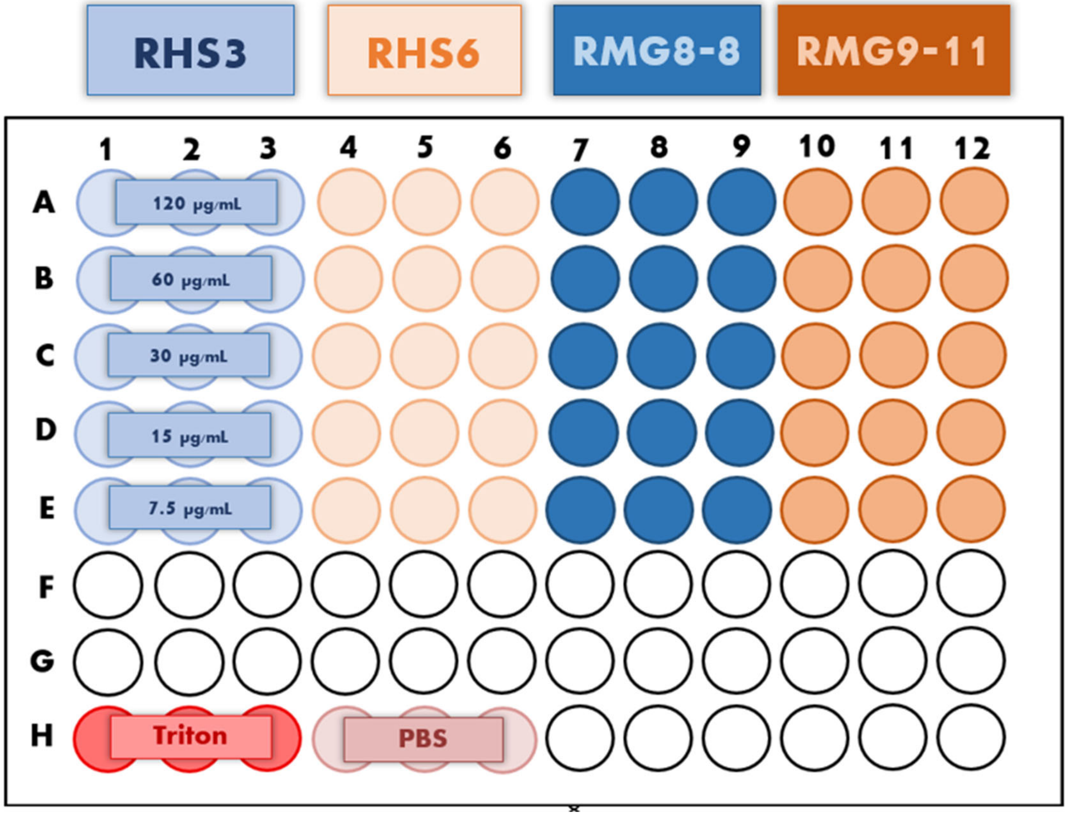


Figure 9. 96-well hemolytic activity plate using hRBCs.

The plates were evaluated on a CLARIOstar plate reader at an absorbance of 405 nm. Percent hemolysis was evaluated using **Equation 1**. Using GraFit, the concentration at 50% hemolytic activity (HC_{50}) and 10% hemolytic activity (HC_{10}) was calculated. HC_{10} was interpreted as the concentration of peptoid at which 10% of hRBCs were lysed.

$$\% \text{ hemolysis } = \frac{(\text{OD}_{405\text{nm}}\,\text{sample} - \text{OD}_{405\text{nm}}\,\text{neg. control})}{(\text{OD}_{405\text{nm}}\,\text{pos. control} - \text{OD}_{405\text{nm}}\,\text{sample})}\; x\ 100$$

Equation 1. Determining percent hemolysis.

Results & Discussion:

I. Peptoid Synthesis and Confirmation

Using solid-phase synthesis, RHS3 and RHS6 were successfully synthesized. Solid-phase synthesis was advantageous because it allowed for the cyclization of the linear peptoid on the Rink amide resin and eliminated intermediate product purification. The initial addition of the C-terminal glutamate with an alloc protecting group was crucial and prevented amination of the glutamate during the synthesis of the linear peptoid. The penultimate alloc deprotection step provided a cyclization point for the N-terminal aminotridecane. Cyclization on the resin allowed for the controlled cyclization of the linear peptoid. The peptoid was then cleaved from the resin.

Both RHS3 and RHS6 were eluted at ~75% ACN (~31 minutes) from RT-HPLC. With an expected molecular weight of 874.2260 da, MS corroborated the structure of RHS3, displaying an m/1 peak of 874.2983 da and an m/2 peak of 437.6507 da (**Figure 10**). RHS3 had a 2.75% yield (3.60 mg). Similarly, with an expected molecular weight of 1154.5090 da, the MS spectra of RHS6 displayed an m/1 peak of 1154.6038 da and an m/2 peak of 577.7944 da, confirming successful synthesis (**Figure 11**). RHS6 had a 4.81% yield (8.1 mg).

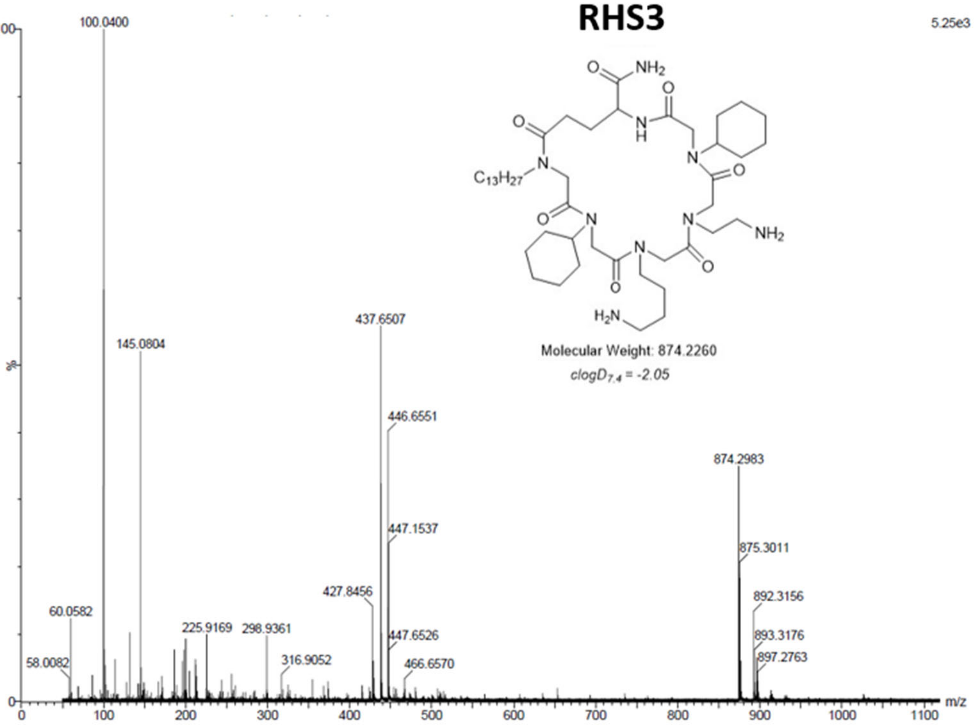


Figure 10. Mass spectra of RHS3. The spectra displays the expected m/1 peak of 874 da and m/2 peak of 437 da.

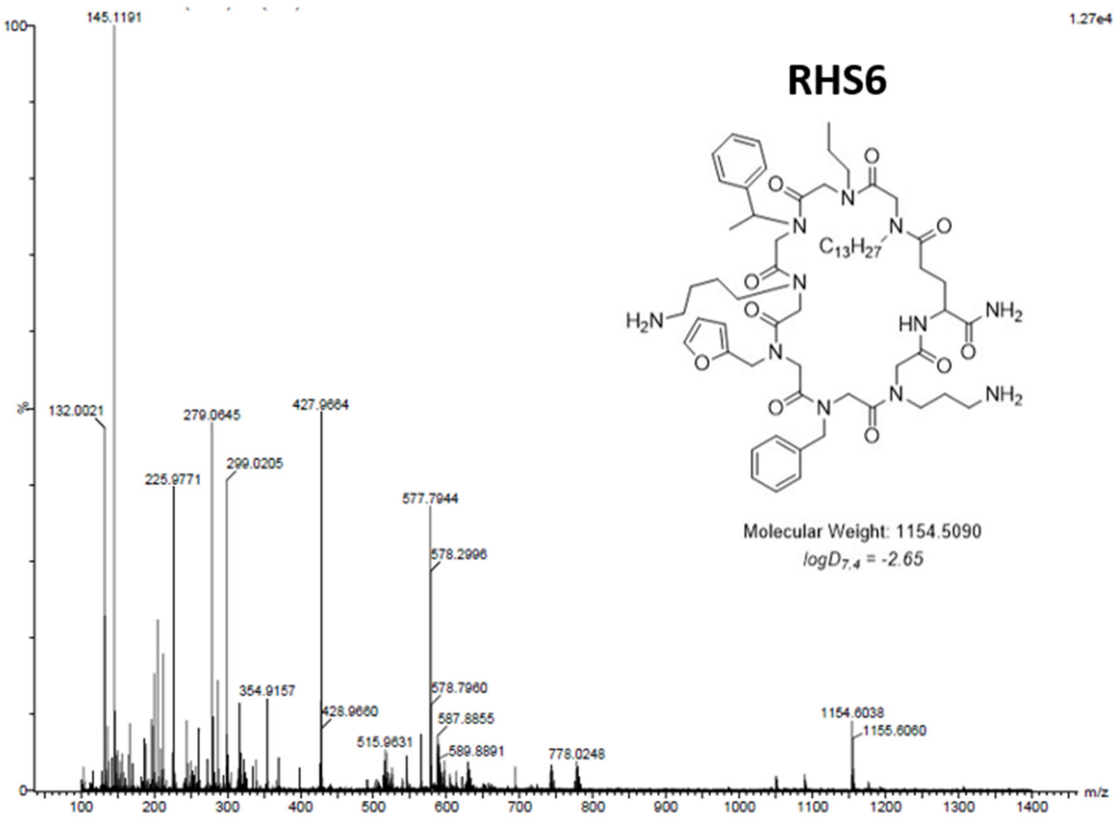


Figure 11. Mass spectra of RHS6. The spectra displays the expected m/1 peak of 1154 da and m/2 peak of 577 da.

In 2007, the first study confirming the successful head-to-tail cyclization of a linear peptoid into a macrocyclic product was published and sparked many other groups to investigate differences between linear and cyclic peptoids.[16] A later study compared the antimicrobial and hemolytic activities of 16 linear peptoids and their cyclic conformation, observing a general trend of enhanced antimicrobial activity with macrocyclization against gram-positive and gram-negative pathogens.[19] This same study identified four cyclic peptoids with a selectivity ratio >8 when compared to hRBCs though it is difficult to compare efficacy without testing RHS3 and RHS6 against the same pathogens. With respect to permeability, one study conducted against *Staphylococcus aureus* found that the macrocyclic conformation of a peptoid intercalated with anionic surface lipids to a greater extent than the linear conformation, suggesting an increase in permeability.[20] Therefore, the rationale behind the cyclization of RMG8-8 and RMG9-11 was largely driven by previous studies where the cyclization of linear peptoids yielded a cyclic conformer with increased antimicrobial activity and permeability relative to the linear conformation.

II. Minimum Inhibitory Concentration

Following synthesis and verification, the antifungal activity of RHS3 and RHS6 against *Cryptococcus neoformans* and *Candida albicans* was determined following standard broth microdilution techniques. The data indicate that the MIC_{90} for RHS3 against *Cryptococcus neoformans* was 3.13 µg/mL, a two-fold decrease in efficacy compared to the linear parent compound, RMG8-8, whose reported MIC_{90} was 1.56 µg/mL.[11] Additionally, data indicated that RHS6 was just as effective at inhibiting 90% of *Cryptococcus neoformans* growth as its linear parent compound, RMG9-11, both with a reported MIC_{90} of 3.13 µg/mL. Data indicated that the MIC_{90} for RHS6 against *Candida albicans* was 6.25 µg/mL, matching RMG9-11's MIC_{90} against the same fungal pathogen.[13] Additionally, data showed that RHS3 had a two-fold increase in efficacy against *Candida albicans* with a reported MIC_{90} of 12.5 µg/mL versus RMG8-8's MIC_{90} of 25 µg/mL. **Table 2** summarizes the MIC_{90} values of each peptoid against both fungal pathogens.

Table 2. MIC_{90} values against *Cryptococcus neoformans* and *Candida albicans*.

Organism	***MIC_{90} (µg/mL)***			
	RHS3	RMG8-8	RHS6	RMG9-11
C. neoformans	3.13	1.56	3.13	3.13
C. albicans	12.5	25	6.25	6.25

Generally, increased permeability across fungal cell membrane leads to the leakage of cellular components that ultimately increases rates of cell death.[21] In terms of RHS3 and RHS6, not all the data support this finding. For example, RHS3 was twice as effective at killing *Candida albicans* compared to its linear parent compound RMG8-8. However, when comparing RHS3's and RMG8-8's activity against *Cryptococcus neoformans*, the cyclized conformation yielded a two-fold decrease in efficacy. Similarly, when comparing RHS6 with its linear parent RMG9-11, the data indicates similar antifungal activity. From this, it could be posited that while the cyclization of peptoids may increase permeability, permeating the cell membrane may not be the mechanism of action of RHS3 and RHS6. A different interpretation of the data could be that permeability plays a role in increasing efficacy, but only when the MIC is relatively high as was the case for RHS8-8 against *Candida albicans*.

III. Mammalian Cytotoxicity

To gain an understanding of how cyclization affects cytotoxicity and selectivity, the mammalian cytotoxicity of RHS3 and RHS6 was tested against HepG2 liver cells and human red blood cells. Previous studies reported TD_{50} values of 189 ± 43 µg/mL for RMG8-8 and 114 ± 5 µg/mL for RMG9-11 against HepG2 liver cells.[11,13] Collected data indicate TD_{50} values of 159 ± 48 µg/mL and 86 ± 37 µg/mL for RHS3 and RHS6 respectively (**Table 3**). Based on the relatively large deviations within the data, it was difficult to make decisive conclusions on relative cytotoxicity, though toxicities do appear to be similar between the linear parent and cyclic derivative compounds.

Table 3. TD_{50} values against HepG2 liver cells.

Peptoid	***TD_{50} (µg/mL)***
RHS3 (cyclic RMG8-8)	159 ± 48 µg/mL
RMG8-8	189 ± 43 µg/mL
RHS6 (cyclic RMG9-11)	86 ± 37 µg/mL
RMG9-11	114 ± 5 µg/mL

To characterize the effect of cyclization on hemolytic activity, a hemolysis study was conducted using both the linear parent compounds and the cyclized derivatives. Since the hRBCs were single donor primary cells with a greater degree of natural variability in toxic susceptibility, it was important to determine the hemolytic activity all four compounds. Collected data indicate HC_{10} values of 19.9 ± 2.9 µg/mL for RHS3 versus 48.8 ± 1.3 µg/mL for RMG8-8. Additionally, data indicate HC_{10} values of 17.9 ± 7.5 µg/mL for RHS6 versus 14.3 ± 5.9 µg/mL for RMG9-11 (**Table 4**). Based on the data, RHS3 is roughly twice as hemolytic as its parent compound, RMG8-8, though RHS6 has a relatively similar HC_{10} value as its parent compound, RMG9-11.

Table 4. HC_{10} values against donor hRBCs.

Peptoid	***HC_{10} (µg/mL)***
RHS3 (cyclic RMG8-8)	19.9 ± 2.9 µg/mL
RMG8-8	48.8 ± 1.3 µg/mL
RHS6 (cyclic RMG9-11)	17.9 ± 7.5 µg/mL
RMG9-11	14.3 ± 5.9 µg/mL

IV. Data Summary

The selectivity ratio (SR) represents the probability of a peptoid to kill the fungal pathogen cell over the human cell line and is calculated as the TD_{50} or HC_{10} divided by the MIC_{90}. The cyclic derivative of RMG8-8, RHS3, had a two-fold decrease in efficacy against *Cryptococcus neoformans* (**Table 5**) which mathematically accounted for the approximately two-fold decrease in selectivity ratio despite having a relatively similar TD_{50}. RHS6 and RMG9-11 bear a similar SR due to the two sharing similar TD_{50} values and the same MIC_{90}. Generally, a SR of 100 or greater is required to initiate pre-clinical testing for a compound. Using this criterion, RMG8-8 was the only compound that could be potentially pushed into pre-clinical testing against *Cryptococcus neoformans,* though further optimization is desired to improve selectivity ratios for HepG2 and human red blood cells.

Table 5. Summary of data against *Cryptococcus neoformans.*

Peptoid	***MIC_{90} (µg/mL)***	***TD_{50} (µg/mL)***	***SR: HepG2 ($\frac{TD_{50}}{MIC}$)***	***HC_{10} (µg/mL)***	***SR: hRBC ($\frac{HC_{10}}{MIC}$)***
RHS3	3.13	159	51	20	6
RMG8-8	1.56	189	121	49	31
RHS6	3.13	86	27	18	6
RMG9-11	3.13	114	36	14	4

The cyclic derivative of RMG9-11, RHS6, had similar efficacy against *Candida albicans*, both with MIC_{90} values of 6.25 µg/mL as well as similar mammalian cytotoxicity against HepG2 cells (**Table 6**). Despite its two-fold increase in efficacy against *Candida albicans* relative to its parent compound, RHS3 was half as effective in terms of MIC_{90} compared to RHS6 and RMG9-11.

Table 6. Summary of data against *Candida albicans.*

Peptoid	***MIC_{90} (µg/mL)***	***TD_{50} (µg/mL)***	***SR: HepG2 $(\frac{TD_{50}}{MIC})$***	***HC_{10} (µg/mL)***	***SR: hRBC $(\frac{HC_{10}}{MIC})$***
RHS3	12.5	159	13	20	2
RMG8-8	25	189	8	49	2
RHS6	6.25	86	14	18	3
RMG9-11	6.25	114	18	14	2

Conclusions & Future Plans:

The intended goal of this project was to optimize the performance of two lead linear peptoids, RMG8-8 and RMG9-11, via cyclization with the intention of increasing fungicidal activity while preserving or improving upon mammalian and hemolytic cytotoxicity. With a selectivity ratio of 121, RMG8-8 remained the most promising peptoid in treating *Cryptococcus neoformans*, as well as the only peptoid with data supporting advancement into pre-clinical trials. Despite the desire to improve efficacy through cyclization, there was only one instance in which the cyclic derivative outperformed its parent compound: RHS3 had a two-fold increase in efficacy against *Candida albicans*, yielding an MIC_{90} value half that of RMG8-8. However, RHS6 and RMG9-11 both outperformed RHS3 in that same trial. Unfortunately, cyclization efforts in the creation of RHS3 and RHS6 did not yield significant improvement in the efficacy of the parent compounds RMG8-8 and RMG9-11. However, this project served as an exploration into previously uncharted territory within the Bicker Lab.

Despite the unlikelihood of RHS3 and RHS6 entering pre-clinical trials, it would be of interest to know if cyclization improves the ability of the linear parent compounds to cross the blood-brain barrier (BBB) as both *Cryptococcus neoformans* and *Candida albicans* can cross the BBB and infect brain tissue. Preliminary research conducted in the Bicker Lab using a parallel artificial membrane permeability assay (PAMPA) suggested that RMG9-11 has the ability to cross the BBB, though RMG8-8 does not. PAMPA will be conducted on both RHS3 and RHS6 to determine if permeability across the BBB changes as a result of cyclization.

References:

1. Bicanic, T. & Harrison, T. S. Cryptococcal Meningitis. *Br. Med. Bull.* **72**, 99 (2004).
2. Nobile, C. J. & Johnson, A. D. Candida Albicans Biofilms and Human Disease. *Annu. Rev. Microbiol.* **69**, 71 (2015).
3. Chen, S. C. A. & Sorrell, T. C. Antifungal Agents. *Med. J. Aust.* **187**, 404 (2007).
4. Lewis, R. E. Current Concepts in Antifungal Pharmacology. *Mayo Clin. Proc.* **86**, 805 (2011).
5. Hanson, K. E., Alexander, B. D., Perfect, J. & Mayers, D. L. *Antimicrobial Drug Resistance: Clinical and Epidemiological Aspects*. (2009).
6. Pierce, C. G. *et al.* The Candida Albicans Biofilm Matrix: Composition, Structure and Function. *J. fungi (Basel, Switzerland)* **3**, 14 (2017).
7. Mojsoska, B., Zuckermann, R. N. & Jenssen, H. Structure-Activity Relationship Study of Novel Peptoids That Mimic the Structure of Antimicrobial Peptides. *Antimicrob. Agents Chemother.* **59**, 4112 (2015).
8. Chongsiriwatana, N. P. *et al.* Short Alkylated Peptoid Mimics of Antimicrobial Lipopeptides. *Antimicrob. Agents Chemother.* **55**, 417 (2011).
9. Chongsiriwatana, N. P. *et al.* Peptoids That Mimic the Structure, Function, and Mechanism of Helical Antimicrobial Peptides. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 2794 (2008).
10. Shin, M.-K., Hyun, Y.-J., Lee, J. H. & Lim, H.-S. Comparison of Cell Permeability of Cyclic Peptoids and Linear Peptoids. *ACS Comb Sci* **20**, 237–242 (2018).

11. Green, R. M. & Bicker, K. L. Discovery and Characterization of a Rapidly Fungicidal and Minimally Toxic Peptoid against Cryptococcus neoformans. *ACS Med Chem Lett* **12**, 1470–1477 (2021).

12. Zuckermann, R. N., Kerr, J. M., Kent, S. B. H. & Moos, W. H. Efficient Method for the Preparation of Peptoids [Oligo (N-Substituted Glycines)] by Submonomer Solid-Phase Synthesis. *J. Am. Chem. Soc.* **114**, 10646 (1992).

13. Green, R. M. & Bicker, K. L. Development of an Anti-Biofilm Screening Technique Leads to the Discovery of a Peptoid with Efficacy against Candida albicans. *ACS Infect Dis* **8**, 310–320 (2022).

14. Pinto, P. M. *et al.* A. In Vitro Antifungal Susceptibility of Clinical Isolates of Candida Spp. Obtained from Patients with Different Predisposing Factors to Candidosis. *Microbiol. Res.* **163**, 579 (2008).

15. Taylor, R. D., MacCoss, M. & Lawson, A. D. G. Rings in drugs. *J. Med. Chem.* **57**, 5845 (2014).

16. Shin, S. B. Y., Yoo, B., Todaro, L. J. & Kirshenbaum, K. Cyclic Peptoids. *J Am Chem Soc* **129**, 3218–3225 (2007).

17. Rezai, T., Yu, B., Millhauser, G. L., Jacobson, M. P. & Lokey, R. S. Testing the Conformational Hypothesis of Passive Membrane Permeability Using Synthetic Cyclic Peptide Diastereomers. *J. Am. Chem. Soc.* **128**, 2510 (2006).

18. null, null. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Third Edition; CLSI Document M27-A3*. (2008).

19. Huang, M. L., Shin, S. B. Y., Kirshenbaum, K., Benson, M. A. & Torres, V. J. A comparison of linear and cyclic peptoid oligomers as potent antimicrobial agents. *ChemMedChem* **7**, 114-122–122 (2012).

20. Andreev, K. *et al.* Cyclization Improves Membrane Permeation by Antimicrobial Peptoids. *Langmuir* **32**, 12905–12913 (2016).

21. Hammoudi Halat, D., Younes, S., Mourad, N. & Rahal, M. Allylamines, Benzylamines, and Fungal Cell Permeability: A Review of Mechanistic Effects and Usefulness against Fungal Pathogens. *Membranes (Basel)* **12**, 1171 (2022).