Synthesis and Characterization of Antifungal Peptoids against *Cryptococcus* by Means of Structure Activity Relationship

By

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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 2018

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Dedication

For my parents and grandmother,
who believed in me before I could believe in myself.
For Jimmy Conwell, who dared me to jump.

Acknowledgments

Foremost, I would like to thank my primary research advisor, Dr. Kevin Bicker, who took a chance on a biology student. Dr. Bicker has been a role model to me throughout this entire process, and he is a professor I will always be grateful for. His mentorship, patience, and guidance has challenged me to strive for the highest level of academic and research success, and I am thankful for the opportunities he has provided for me while studying under him. Without his guidance this project would not have been possible.

I would like to thank my secondary research advisor, Dr. Erin McClelland, for her advice and expertise in testing our compounds with *Cryptococcus neoformans*.

I would also like to thank my teacher and lab partner, Scott Armstrong, who worked in collaboration with me on this project. Your kindness, diligence, and attention to detail in teaching me lab techniques was appreciated more than you know. Thank you to fellow lab mates Sabrina Spicer and Madison Green who assisted me countless times and answered tedious questions throughout my time within the research lab.

Further acknowledgements go to URECA for partial funding of this project and the MTSU Chemistry Department.

Abstract

The impending rise in antimicrobial resistance has necessitated alternative therapeutic options for resistant pathogens such as *Cryptococcus neoformans*. *C. neoformans* is the causative agent of cryptococcal meningitis, which can be deadly to immunocompromised individuals if it integrates into the central nervous system. Current research has been done with antimicrobial peptide mimics, termed peptoids, as a therapeutic agent for *C. neoformans* infections. Our primary goal is to optimize the therapeutic potential of the antifungal peptoid AEC5. A sarcosine scan was used to identify the most pharmacophorically important peptoid building blocks of AEC5, followed by sequential optimization of each building block through Structure Activity Relationship studies. We report an optimized antifungal peptoid from this study, β-5, has improved potency towards *C. neoformans* and decreased toxicity towards mammalian cells. Further studies are focusing on these antifungal peptoids' mechanism of action in *C. neoformans* cell death.

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Introduction

The rapid emergence and increase in antimicrobial resistance threatens antibiotic efficacy, and in doing so, endangers years of advancements in modern medicine (7). Due to the recent overuse and abuse of antibiotics and the lack of new antibiotic availability, antimicrobial resistance has become a worldwide crisis (3). Such a crisis calls for the production of new antimicrobial compounds to replace those with declining potency (7). One particular pathogen of interest is the fungus *Cryptococcus neoformans*, the causative agent of cryptococcal meningitis (3). This encapsulated yeast is found in the environment and is typically contracted through inhalation into the lungs and subsequent infection of pulmonary tissue (8, 12). The primary infection tends to produce pneumonia-like symptoms and can be controlled and eradicated by proper treatment in immunocompetent individuals, but if these individuals do not receive treatment, the infection can be fatal (8). Unfortunately, infections observed in immunocompromised individuals are at risk of spreading throughout the bloodstream to infect a variety of organs and tissues (7, 10, 12). Latent infections have shown a predilection of integrating the central nervous system, at which point *Cryptococcus* becomes much more dangerous (12). However, many other groups of individuals are susceptible to contracting *Cryptococcus* infections including organ transplant recipients, HIV-infected and nontransplant hosts. Other strains like *Cryptococcus gattii* can infect immunocompetent individuals, especially high-risk groups such as children, pregnant women, and those living in underserved areas with limited resources (10).

Some of the most recent estimates indicate approximately 220,000 patients with HIV/AIDS contract cryptococcal meningitis and over 181,000 of these cases proved fatal

(3). These infections predominate in Sub-Saharan Africa but pose a threat to other parts of the world (2). Severe infections are usually treated with amphotericin B formulation, with or without flucytosine, and fluconazole for maintenance therapy in HIV infected individuals (1, 6). However, these treatment methods have a high mammalian toxicity, further complicating these patients' healthcare with harsh side effects (10). Balancing patients' immunodeficiency care with cryptococcal meningoencephalitis can create a complicated clinical scenario, especially considering possible side effects of known antifungals (10). Developing pharmaceuticals with antifungal potency and reduced mammalian toxicity, thus reducing harsh side effects, is a priority in treating these patients. Furthermore, resistance to these treatments have been observed in certain species and strains ultimately leading to an extended therapy of the drug and even possible surgery if drug therapy fails, therefore necessitating new therapeutic options for such patients (1, 6). Patients with HIV/AIDS that contract cryptococcal meningitis and who survive after initial therapy may require prolonged treatment with antifungals to prevent disease relapse, which increases the risk of less susceptible cryptococcal strains, further fueling antimicrobial resistance (11).

Current research has identified antimicrobial peptides (AMPs) as promising therapeutic agents against a broad pathogen spectrum (5). AMPs are of particular interest because they are a naturally occurring front-line defense in varying organisms' immune responses, and they exhibit anti-infective agent potential and support wound healing (1, 5). Although many studies have proven AMPs are effective *in vitro*, they exhibit low resistance against proteolysis *in vivo* (5). Recent efforts in this field have focused on producing more naturally modified peptide structures, termed peptoids (5). Peptoids,

oligo-*N*-substituted glycines, are peptide mimics that differ structurally from peptides in that their side chain is attached to the nitrogen in the amide backbone instead of the alpha carbon (1, 5). This modification has increased peptoid half-life by improving their stability against proteases (5). Furthermore, peptoids lack hydrogen bonding, which prevents backbone aggregation, and they permeate cell membranes better than AMPs and may also evade immune recognition (5). Thus, peptoids exhibit properties indicative of excellent therapeutic potential against antimicrobials.

Background

Our lab is currently focused on the use of peptoid chemistry, which is based on established solid-phase synthesis methodology from the literature, and the development and screening of combinatorial peptoid libraries to determine alternative therapeutic options to antimicrobial peptides (9). AMPs are of interest because they display minimal mammalian toxicity and still specifically target fungal cells. Their mode of action includes disrupting the cell membrane by creating pores and causing cell leakage, resulting in death (2). Unfortunately, these antimicrobial peptides are degraded quickly by human proteases rendering too short a half-life for drug viability. Certain peptide mimics, termed peptoids, contain a slight structural difference where the side chain is attached to the amide backbone rather than to the alpha-carbon in peptides (**Figure 1**). This structural rearrangement increases peptoid stability *in vivo* extending compound half-life while maintaining potency.

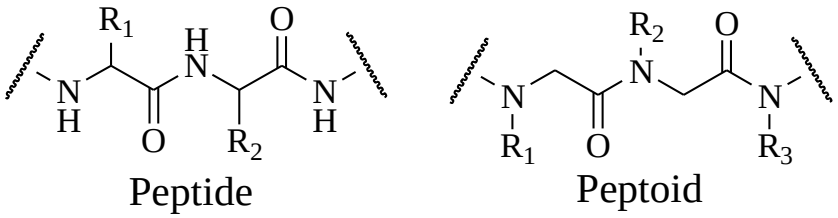


Figure 1: General peptide and peptoid structures. Peptoids contain a structural difference where the side chains are directly attached to the amide backbone rather than to the α-carbon as in peptides. This structural modification increases half-life while maintaining antimicrobial potency in peptoids.

Our lab utilizes a combinatorial library, which is a collection of chemical compounds of multiple different combinations of related chemical species, in our case peptoids. Our lab's combinatorial library high-throughput screening technique is termed the Peptoid Library Agar Diffusion (PLAD) assay, which utilizes a randomized split-and-

pool method to provide an efficient mode for discovery of viable antimicrobials (4). Prior work in the lab with this assay has produced AEC5 as a leading peptoid in this antifungal study due to its excellent potency against *C. neoformans* and minimal toxicity profile (4). AEC5's structure is sectioned into three major structural positions termed one, two, and three (**Figure 5A**). Position one is comprised of a hydrophobic alkyl tail; position two is a cationic amino side chain off the amide backbone; and position three is an aromatic heterocycle. The importance of each position can be explored using a Structure Activity Relationship (SAR) study, which is a rational design method that is used to study various modifications to positions of a compound. In this study, the SAR is separated into three rounds according to the three positions exhibited by AEC5. By use of the SAR, these sections can be sequentially studied by varying modifications to the three positions in an effort to increase potency and reduce toxicity.

Experimental Methods

Solid-Phase Peptoid Synthesis. Peptoids were synthesized on small Rink-Amide polystyrene beads which act as a solid-phase platform upon which the compound is built. For each synthesis, the resin (0.8 mmol/g) was deprotected twice with 20% piperidine in dimethylformamide (DMF) on the rocker for 20 minutes. Afterwards, the resin was acylated with 2 M bromoacetic acid in anhydrous DMF (1.5 mL) and 3.2 M diisopropylcarbodiimide (DIC) in anhydrous DMF (1.5 mL). This coupling was microwaved for 30 seconds at 10% power and softly rocked for 15 minutes before aspirating the solution and rinsing the resin three times with DMF. The amine necessary for the appropriate submonomer attachment was prepared in a 2 M solution of anhydrous DMF (3 mL) and microwaved at 10% power for 30 seconds, rocked 15 minutes, and washed three times with DMF. Acylations and aminations were verified colorimetrically via the ninhydrin test, where purple resin indicated a free amine and a clear, yellow resin indicated the amine was acylated. Thus, the deprotected resin was acylated, aminated with the amine heterocycle of interest, acylated, aminated with the cationic side chain of interest, acylated, and the tail of position one was attached by amination, or in the farnaesal tail's case, added by reductive amination.

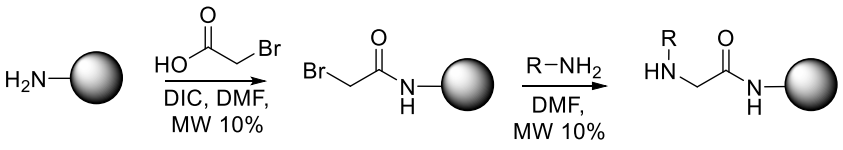


Figure 2. Peptoids were synthesized on small polystyrene rink amide beads through a series of acylations and aminations until the final structure was achieved. Acylation was achieved by reacting activated beads with bromoacetic acid in DIC and anhydrous DMF and microwaved on ten percent. Aminations were achieved by reacting with the amine of interest, the submomoner being attached, in anhydrous DMF and microwaved on ten percent.

Reductive Amination. The farnaesal tail installment was achieved by reductive amination for the synthesis of compounds (SA3, MM1, MM2, MM3, MM4). After the addition of the first two peptoid submonomers, Fmoc-Gly-OH was attached and deprotected as follows. Fmoc-Gly-OH and N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were combined in 5% N-methylmorpholine (NMM) in DMF and allowed to react for 10 minutes before addition to the resin. The resin was gently rocked with this solution for an hour and then aspirated and washed thrice with DMF. The Fmoc group was removed by treatment with 20% piperidine in DMF twice, 10 minutes each. The terpene alkyl tail was added by treatment with farnesal (1.58 mmol) in 9:1 DMF:methanol and rocked gently at 37ºC for 20 hours. The reaction was reduced with $NaBH_4$ (30 mg; 0.79 mmol) for 1 hour at 25 ˚C, warmed to 37ºC for over 3 hours, and washed with DMF.

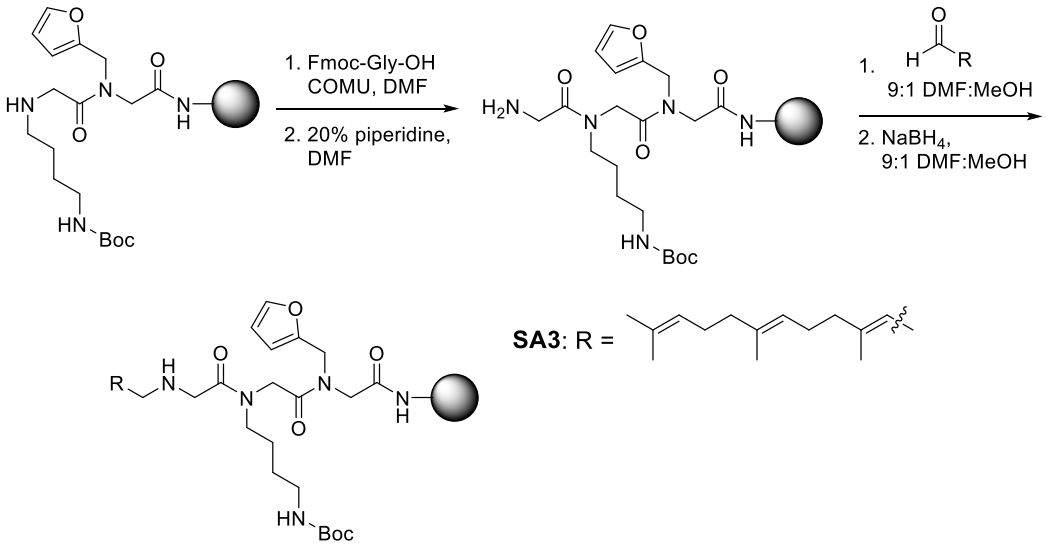


Figure 3: Compounds α-4 and α-5, with the farnesal and citral tails respectively, could not be synthesized via direct amination. After the addition of a cationic amine, F-moc protected glycine in a solution of COMU and DMF and 20% pipperidine in DMF were reacted with the beads to attach glycine. The tail of interest was added in a solution of 9:1 DMF:methanol and $NaBH_4$ in 9:1 DMF:methanol to produce the peptoid via reductive amination.

Synthesis of γ-5. Due to the reactivity of the pyridinyl side chain, γ-5 was synthesized following slightly modified submonomer conditions as previously described.[5] Briefly, Rink amide resin was prepared, coupled with bromoacetic acid, and coupled with 2-aminomethyl pyridine as described above. The subsequent acylation reactions for this compound were achieved by treating the resin with 0.4 M chloroacetic acid (1.7 mL) and 2 M DIC (0.4 mL) in anhydrous DMF and tumbling at 35 ˚C for 5 min. Amine couplings following chloroacylation were accomplished using 2 M amine solution in DMF (3 mL) at 35 ˚C for 90 min.

Synthesis of β-3. Peptoid synthesis was completed as described above using N-monomethoxytrityl-1,3-diaminopropane as the amine in position two to provide an orthogonal protecting group in this position. While still on the solid phase, the N-terminus of this peptoid was protected by treating with Boc anhydride (368 μL; 1.6 mmol; 10 eq) in 5% NMM in DMF (5 mL) for 1 h with gentle rocking. Resin was washed with DMF and CH_2Cl_2. The monomethoxytrityl protecting group was removed from the δ-amine in position two by treating 3x with 1% trifluoroacetic acid (TFA) in CH_2Cl_2 (5 mL) for 10 min, followed by washing with CH_2Cl_2 and DMF. Resin amines were freebased by treating with 5% NMM in DMF for 5 min prior to guanidinylation with pyrazole carboxamidine HCl (233 mg; 1.6 mmol; 10 eq) and catalyst dimethylaminopyridine (DMAP; 19.5 mg; 0.16 mmol; 1 eq) in 5% NMM in DMF (5 mL) for 20 h at 25 ˚C with gentle rocking. Resin was washed with DMF and CH_2Cl_2. The N-terminal Boc group was subsequently removed during compound cleavage from the resin.

Synthesis of β-4. Peptoid synthesis was completed as described above using N-monomethoxytrityl-1,4-diaminobutane as the amine in position two to provide an orthogonal protecting group in this position. While still on the solid phase, the N-terminus of this peptoid was protected by treating with Boc anhydride (368 μL; 1.6 mmol; 10 eq) in 5% NMM in DMF (5 mL) for 1 h with gentle rocking. Resin was washed with DMF and CH_2Cl_2. The monomethoxytrityl protecting group was removed from the ε-amine in position two by treating 3x with 1% trifluoroacetic acid (TFA) in CH_2Cl_2 (5 mL) for 10 min, followed by washing with CH_2Cl_2 and DMF. Resin amines were free based by treating with 5% NMM in DMF for 5 min prior to trimethylation with methyl iodide (99.6 μL; 1.6 mmol; 10 eq) and Cs_2CO_3 (130 mg; 0.4 mmol; 2.5 eq) in DMF (5 mL) for 20 h at 25 ˚C with gentle rocking. Resin was washed with DMF, water, DMF, and CH_2Cl_2. The N-terminal Boc group was subsequently removed during compound cleavage from the resin.

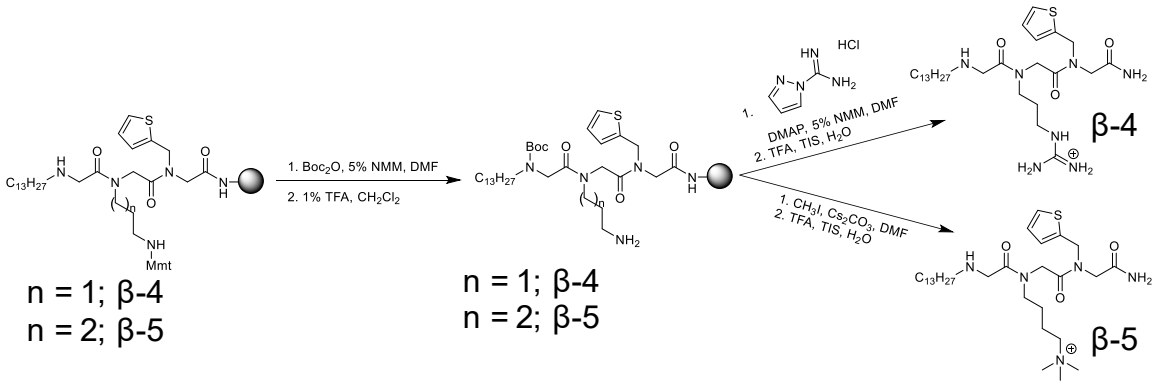


Figure 4. Synthesis of unique γ-2 derivatives with unique side chain modifications.

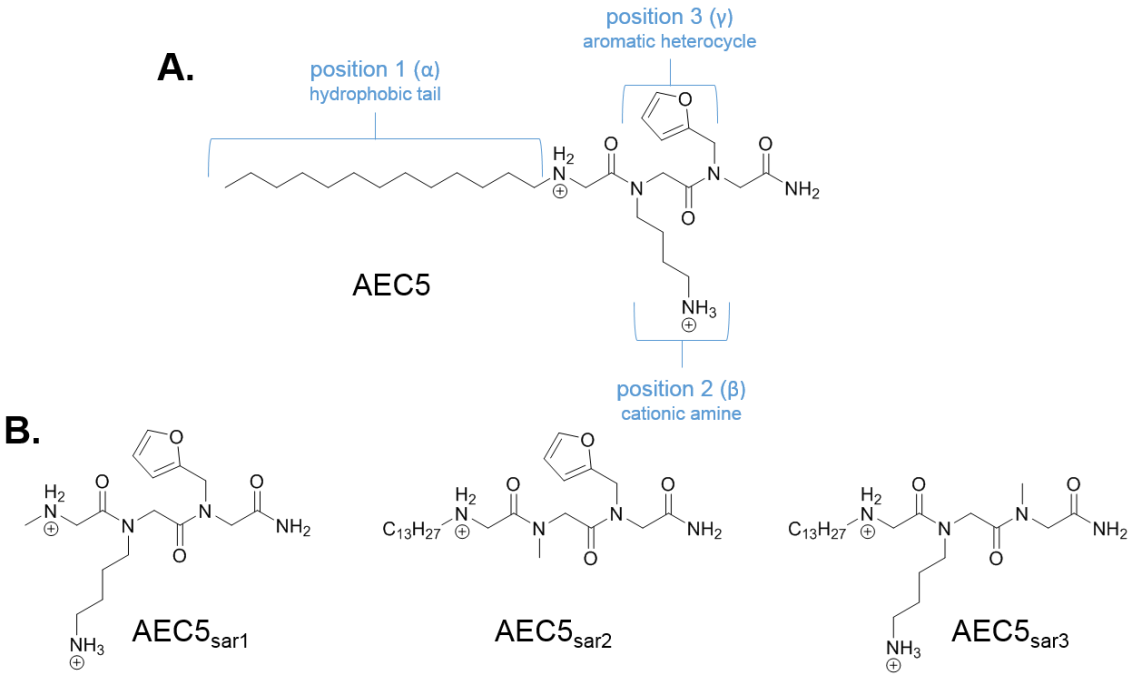
Peptoid Purification and Characterization. In preparation for purification of the derivatives, DMF was removed from the resin by washing it with CH_2Cl_2 three times and aspirated. Peptoid cleavage from the resin was achieved by rocking the resin in a mixture of 95% trifluoroacetic acid (TFA; 6.65 mL), 2.5% triisopropylsilane (TIS; 0.175 mL), and 2.5% H_2O (0.175 mL) for 1 hour. This solution was filtered to separate the compound from the resin, and the TFA was bubbled off. Cleaved peptoids were purified by HPLC, verified by MS, and lyophilized to produce the compound as a purified solid.

Minimum Inhibitory Concentration Assays. Antimicrobial peptoids were analyzed for potency via Minimum Inhibitory Concentration (MIC), which is a technique to test the lowest concentration of our particular peptoid needed to kill *C. neoformans* (13). To accomplish this, *C. neoformans* H99S (serotype A lab strain) was streaked from frozen stock for isolation and incubated for 96 hours. After which, a 5 mL saline solution was inoculated with *C. neoformans* colonies and vortexed for one minute to attain an optical density at 530 nm of 0.15 to 0.2 acquired by spectrophotometer. A 1:100 diluted solution of Roswell Park Memorial Institute (RPMI) media containing 3-morpholinopropane sulfonic acid (MOPS) and cells was made by adding 100 µL of inoculated saline to 9.9 mL of RPMI + 0.1 M MOPS and vortexed for one minute. 0.5 mL of 1:100 solution was added to 9.5 mL of RPMI + 0.1 M MOPS to make a 1:20 solution of cells and RPMI. This process was repeated until the desired amount of RPMI and cells was obtained.

Two-fold serial dilutions for the specific AEC5 derivative in water at 100x the concentration was prepared prior to plating. 198 µL of the 1:20 RPMI plus cells were vortexed and immediately added to a 96-well plate, followed by 2 µL of 100x compound or vehicle control in triplicate. Cells treated with 2 µL of amphotericin B (AmpB; 2 µg/mL)

were used as a positive control. The plate was incubated for 72 hours at 35 °C. For viability analysis, 20 μL of PrestoBlue was added to each well and incubated for another 8 hours at 35 °C. After which, the plate was analyzed with a M5 plate reader for cell presence. All triplicate assays were repeated twice on different days. The MIC was defined as the compound concentration resulting in greater than 90% inhibition of fungal growth consistently on multiple days.

Mammalian Cytotoxicity Testing. All peptoids underwent mammalian cytotoxicity testing on HepG2 hepatocarcinoma cells. Cells were grown in DMEM containing 10% Fetal Bovine Serum at 37 °C and 5% CO_2 atmosphere. For cytotoxicity testing, cells were seeded between 10,000 and 40,000 cells per well into 96-well plates in phenol red free DMEM and incubated for two hours to allow cell attachment. Cells were treated with various peptoid concentrations or vehicle in a 2-fold serial dilution, incubated for 72 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 20 μL; 5 mg/mL) was added to each well, and the plate was incubated for another 3.5 hours. Media was removed from the plate and 100 μL of DMSO was added and incubated at 37 °C for 10 minutes. The absorbance at 570 nm was read by spectrophotometer, percent inhibition found, and IC_{50} calculated using GraFit. All triplicate assays were repeated twice on different days.



C.

Compound	MW (g/mol)	$cLogD_{7.4}$	*C. neoformans* MIC (μg/mL)	HepG2 TD_{50} (μg/mL)	SR
AEC5	521.8	-1.18	6.3	56.2 ± 18.0	9
$AEC5_{sar1}$	353.4	-6.26	>400	>800	ND
$AEC5_{sar2}$	464.7	1.44	12.5	21.1 ± 1.5	2
$AEC5_{sar3}$	455.7	-1.96	50	54.3 ± 2.7	1

Figure 5. (**A.**) The structure of the antifungal peptoid AEC5 and each of its three submonomer positions. (**B.**) Sarcosine scan structures for AEC5 where each of the three submonomer positions were substituted with sarcosine. (**C.**) Data for AEC5 sarcosine scan containing calculated distribution coefficient ($cLogD_{7.4}$), *C. neoformans* antifungal potency (MIC), and HepG2 liver cell toxicity (TD_{50}). MW = molecular weight; $cLogD_{7.4}$ = calculated distribution coefficient at pH 7.4; MIC = minimum inhibitory concentration; TD_{50} = toxic dose 50%; SR = selectivity ratio (TD_{50}/MIC); ND = not determined.

AEC5 Sarcosine Scan

A sarcosine scan of AEC5 was completed, similar to an alanine scan of peptides, by synthesizing three AEC5 derivatives each with sarcosine in place of one of the submonomers (**Figure 5A**). Thus $AEC5_{sar1}$, $AEC5_{sar2}$, and $AEC5_{sar3}$ had positions one, two and three substituted for sarcosine respectively (**Figure 5B**). Substitution at any of the three

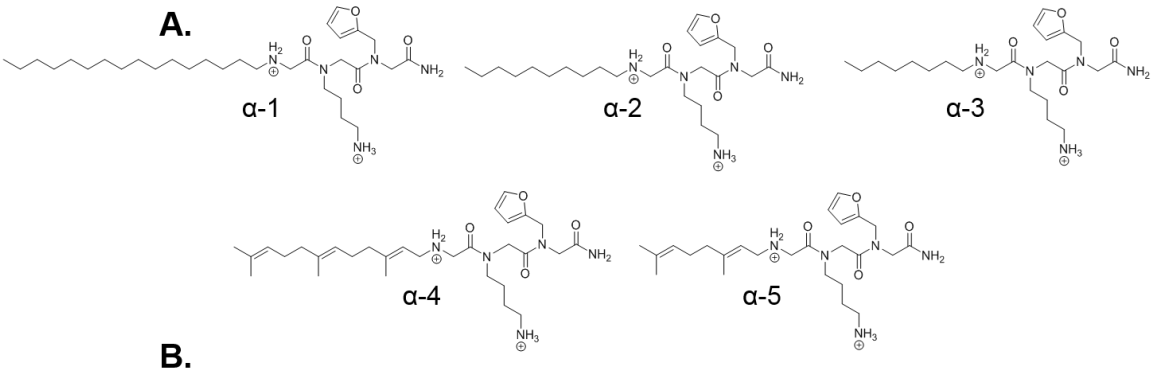
positions had deleterious effects on antifungal potency but mixed effects of mammalian cytotoxicity (**Figure 5C**).

Sarcosine substitution at position one for the hydrophobic alkyl tail resulted in the greatest deficit in potency. Observed fungal potency and mammalian toxicity fell above the highest concentrations of drug tested, 400 μg/mL and 800 μg/mL respectively, which indicates that position one contributes significantly in fungal potency (**Figure 5**). It also contributes a distinguishable mammalian toxicity, as expected from such a hydrophobic structure, though its antifungal properties are of greater interest.

Sarcosine substitution at position two for the cationic amine resulted in a slightly increased MIC of 12.5 μg/mL, but more significantly a two-fold increase in cytotoxicity from 56.2 μg/mL for AEC5 to 21.1 μg/mL for $AEC5_{sar2}$. Substitution at position two results in a significant increase in hydrophobicity as indicated by the change in calculated distribution coefficient $cLogD_{7.4,}$ which increased from -1.18 for AEC5 to 1.44. This increase in hydrophobicity is likely the cause of increased toxicity for $AEC5_{sar2}$ and stresses the importance of this submonomer in mitigating mammalian toxicity.

Interestingly, sarcosine substitution at position three for the heterocyclic amine resulted in a substantial 8-fold decrease in antifungal potency with little effect on mammalian toxicity compared to AEC5. These data indicate that the alkyl tail is the most important pharmacophoric moiety in AEC5 but that the heterocyclic amine contributes to fungal potency. Furthermore, it is important to note the cationic amine's presence reduces mammalian toxicity. We subsequently sought to optimize each of the three positions in three rounds of SAR.

A.



B.

Compound	MW (g/mol)	$cLogD_{7.4}$	*C. neoformans* MIC (μg/mL)	HepG2 TD_{50} (μg/mL)	SR
AEC5	521.8	-1.18	6.3	56.2 ± 18.0	9
α-1	563.8	0.16	3.13	40.4 ± 0.07	13
α-2	479.7	-2.51	400	ND	ND
α-3	451.6	-3.40	>800	ND	ND
α-4	543.8	-1.11	100	800	8
α-5	475.6	-2.77	400	ND	ND

Figure 6. (**A.**) Structures from round 1 SAR compounds with varied hydrophobic tails in position one. (**B.**) Data from round 1 SAR containing calculated distribution coefficient ($cLogD_{7.4}$), *C. neoformans* antifungal potency (MIC), and HepG2 liver cell toxicity (TD_{50}). MW = molecular weight; $cLogD_{7.4}$ = calculated distribution coefficient at pH 7.4; MIC = minimum inhibitory concentration; TD_{50} = toxic dose 50%; SR = selectivity ratio (TD_{50}/MIC); ND = not determined.

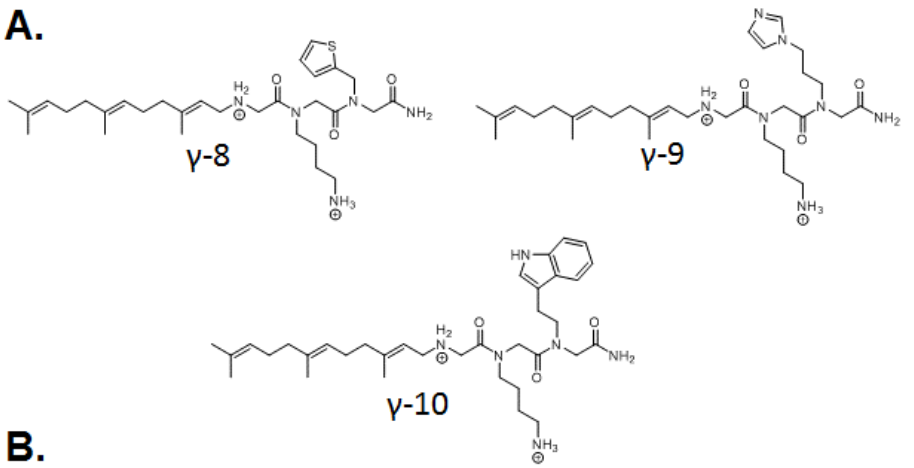
SAR Round 1

The first round of SAR sought to optimize position one, which we deemed to be the position with the most pharmacophoric potential from the sarcosine scan. Several studies have demonstrated the importance of alkyl tail length on potency and toxicity of lipophilic antimicrobial peptoids, thus compounds α-1, α-2, and α-3 were created to determine optimal hydrophobic tail length. AEC5 derivatives with sixteen, ten, and eight carbon alkyl tails in position 1, termed α-1, α-2, and α-3, respectively, were included in round one (**Figure 6A**). The known antifungal medication amphotericin B has a polyene structure we were interested in implementing in our peptoid derivatives. We hypothesized that the inclusion of polyene moieties in this position might improve antifungal potency.

Thus, the terpene aldehydes farnesal and citral were tested in position one affording the compounds α-4 and α-5, respectively (**Figure 6A**).

The *C. neoformans* MIC of these compounds was evaluated; however, significant decreases in potency were observed for compounds α-2 through α-5 (**Figure 6B**). This decrease in response to shortening the alkyl tail (compounds α-2 and α-3) was expected given that shorter tails tend to correspond to less hydrophobicity of the molecule overall. Although increasing the length of the alkyl tail from thirteen to sixteen carbons yielded a compound with a 2-fold improvement in *C. neoformans* potency, it subsequently resulted in significantly increased mammalian toxicity against HepG2 liver cells **(Figure 6B**). Neither polyene tail compounds resulted in improved potency; however, the farnesyl tail did show a large decrease in toxicity (>800 μg/mL) while possessing a similar $cLogD_{7.4}$, albeit with a 16-fold decrease in antifungal potency (**Figure 6B**).

A.



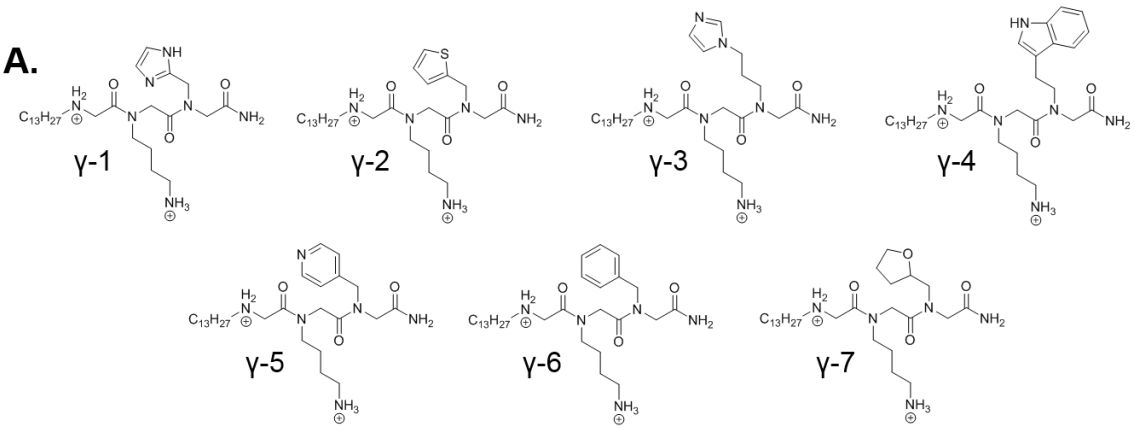
B.

Compound	MW (g/mol)	$cLogD_{7.4}$	*C. neoformans* MIC (μg/mL)	HepG2 TD_{50} (μg/mL)	SR
AEC5	521.8	-1.18	6.3	56.2 ± 18.0	8.9
γ-8	559.8	-0.25	400	ND	ND
γ-9	571.8	-1.99	100	ND	ND
γ-10	606.9	0.22	100	ND	ND

Figure 7. (**A.**) Round 2 SAR compounds containing the farnesal tail and varying the heterocyclic amine in position three. (**B.**) Data from round 2 SAR containing calculated distribution coefficient ($cLogD_{7.4}$), *C. neoformans* antifungal potency (MIC), and HepG2 liver cell toxicity (TD_{50}). MW = molecular weight; $cLogD_{7.4}$ = calculated distribution coefficient at pH 7.4; MIC = minimum inhibitory concentration; TD_{50} = toxic dose 50%; SR = selectivity ratio (TD_{50}/MIC); ND = not determined.

We carried over the farnesyl tail into round two in hopes that we could maintain the significant decrease in mammalian toxicity and recover antifungal potency through modification of the aromatic heterocycle in position three. This resulted in compounds γ-8, γ-9, and γ-10 (**Figure 7A**). However, none of these showed improved potency and the farnesyl tail was ultimately abandoned (**Figure 7B**). Therefore, it was concluded that the optimal alkyl tail in position one was the original tridecyl tail from AEC5.

A.



B.

Compound	MW (g/mol)	*cLogD*$_{7.4}$	*C. neoformans* MIC (μg/mL)	HepG2 TD$_{50}$ (μg/mL)	SR
AEC5	521.8	-1.18	6.3	56.2 ± 18.0	9
γ-1	521.8	-2.31	25	147.2 ± 15.8	6
γ-2	537.8	-0.32	3.13	79.3 ± 0.35	25
γ-3	549.8	-2.06	50	228.3 ± 22.5	5
γ-4	584.9	0.15	3.13	42.7 ± 5.6	14
γ-5	532.8	-1.46	25	104.1 ± 1.4	4
γ-6	531.8	-0.24	3.13	52.2 ± 8.8	17
γ-7	525.8	-1.54	12.5	170.4 ± 16.5	14

Figure 8. (**A.**) Round 2 SAR compounds containing the tridecyl tail from Round 1 and varying heterocyclic amines in position three. (**B.**) Data from round 2 SAR containing calculated distribution coefficient (*cLogD*$_{7.4}$), *C. neoformans* antifungal potency (MIC), and HepG2 liver cell toxicity (TD$_{50}$). MW = molecular weight; *cLogD*$_{7.4}$ = calculated distribution coefficient at pH 7.4; MIC = minimum inhibitory concentration; TD$_{50}$ = toxic dose 50%; SR = selectivity ratio (TD$_{50}$/MIC); ND = not determined.

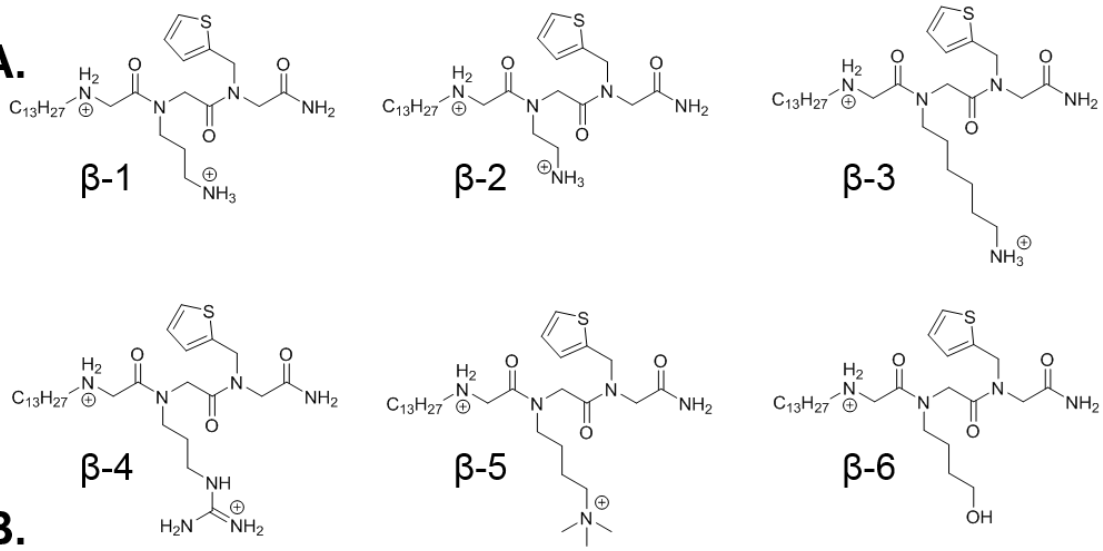
SAR Round 2

Following the alkyl tail in position one, the furan in position three appeared to have the second largest effect on antifungal potency from the sarcosine scan (**Figure 5**). AEC5 derivatives containing a variety of aromatic heterocycles in this position were synthesized for testing. These included imidazole derivatized compounds γ-1 and γ-3, compound γ-2 containing a thiophene side chain, γ-4 with an indole, and γ-5 displaying a pyridyl ring. Derivatives containing an aromatic phenyl side chain with no heteroatom (γ-6) and a

non-aromatic heterocyclic tetrahydrofuran (γ-7) were also synthesized to investigate the role of these individual properties (**Figure 8A**).

The *C. neoformans* potency and HepG2 toxicity of these compounds was determined (**Figure 8B**). Derivatives γ-1, γ-3, γ-5 all showed 4- to 5-fold decreases in antifungal potency as well as a 2- to 4-fold decrease in mammalian cell toxicity. It is interesting to note that this decrease in potency and toxicity accompanies a decrease in $cLogD_{7.4}$, supporting our earlier findings that overall lipophilicity of short antimicrobial peptoids is linked to mammalian toxicity (**Figure 8B**). The substitution with a non-aromatic heterocycle (γ-7) gave a modest 2-fold decrease in antifungal potency and a 3-fold decrease in mammalian cell toxicity. Interestingly, placement of an aromatic phenyl ring with no heteroatom (γ-6) resulted in a 2-fold improvement in antifungal potency and no significant change in mammalian cell toxicity. A 2-fold improvement in antifungal potency was observed with three of the round 2 derivatives; γ-2 with a thiophene side chain, γ-4 with an indole side chain, and γ-6, as mentioned, with a phenyl side chain. Two of these, γ-4 and γ-6, exhibited increased or unchanged mammalian cell toxicity compared to AEC5. However, γ-2 showed a decrease in HepG2 toxicity with a TD_{50} of 79.3 μg/mL compared to 56.2 μg/mL for AEC5. With improvement in both potency and toxicity, a selectivity ratio of 25 was calculated for γ-2, compared to 9 for AEC5. A higher selectivity ratio, defined as the TD_{50} divided by the MIC, is indicative of a compound that is more selective for pathogen over mammalian cells. Data from round two of SAR indicated that replacing the furan in position three with a thiophene greatly improved pathogen selectivity by increasing antifungal potency and decreasing mammalian toxicity. Therefore, the thiophene moiety was carried over into round three of SAR antifungal optimization.

A.



B.

Compound	MW (g/mol)	*cLogD*$_{7.4}$	*C. neoformans* MIC (μg/mL)	HepG2 TD$_{50}$ (μg/mL)	SR
γ-2	537.8	-0.32	3.13	79.3 ± 0.35	25
β-1	523.8	-0.73	3.13	47.3 ± 0.71	15
β-2	509.8	0.03	6.25	40.5 ± 0.71	6
β-3	565.4	0.35	3.13	56.2 ± 3.3	18
β-4	565.8	-1.16	12.5	40.8 ± 0.71	3
β-5	580.9	-1.28	3.13	91.2 ± 4.7	29
β-6	538.8	2.18	12.5	31 ± 12.7	3

Figure 9. (**A.**) Round 3 SAR compounds containing the tridecyl tail from Round 1, the thiophene moiety from Round 2, and varying cationic amine side chains in position two. (**B.**) Data from round 3 SAR containing calculated distribution coefficient (*cLogD*$_{7.4}$), *C. neoformans* antifungal potency (MIC), and HepG2 liver cell toxicity (TD$_{50}$). MW = molecular weight; *cLogD*$_{7.4}$ = calculated distribution coefficient at pH 7.4; MIC = minimum inhibitory concentration; TD$_{50}$ = toxic dose 50%; SR = selectivity ratio (TD$_{50}$/MIC); ND = not determined.

SAR Round 3

The final submonomer to optimize was the amino cation in position two. Derivatives of γ-2 were synthesized containing various chemical moieties in position two. These include compounds with ammonium groups similar to γ-2 attached via fewer methylene units (β-1 and β-2), or more methylene units (β-3), as well as an arginine mimic (β-4), a trimethylammonium lysine mimic (β-5), and a non-cationic hydroxyl group (β-6) (**Figure 9A**). β-6 was included in this study to evaluate the efficacy of a hydrogen bonding moiety without cationic nature in position two, as we anticipated that the cationic charge in position two might mitigate cytotoxicity from the sarcosine scan. The MIC of β-6 falls off 4-fold compared to γ-2 while the toxicity against HepG2 cells becomes more than 2-fold worse (**Figure 9B**). These data, combined with data from AEC_{sar2}, highlight the role of the cation in position two in improving *C. neoformans* potency, and perhaps more importantly, negating compound toxicity against mammalian cells. Bolt *et al.* recently showed that the chain length between peptoid backbone and amino cation affects mammalian cytotoxicity and antibacterial activity, although the nature of this relationship varied between organisms. Modifying the side-chain length of γ-2 had little effect on antifungal activity, with only β-2, possessing the two-carbon chain, showing a 2-fold decrease in potency. However, shortening the side-chain length was detrimental to mammalian cytotoxicity with β-1 (3 carbon linker) and β-2 having HepG2 cytotoxicity of 47.3 and 40.5 μg/mL, respectively. Similarly, lengthening the side-chain to six carbons (β-3) increased toxicity moderately to 56.2 μg/mL. This was an unexpected phenomenon considering the corresponding increased hydrophobicity and alkyl tail length observed in round one SAR.

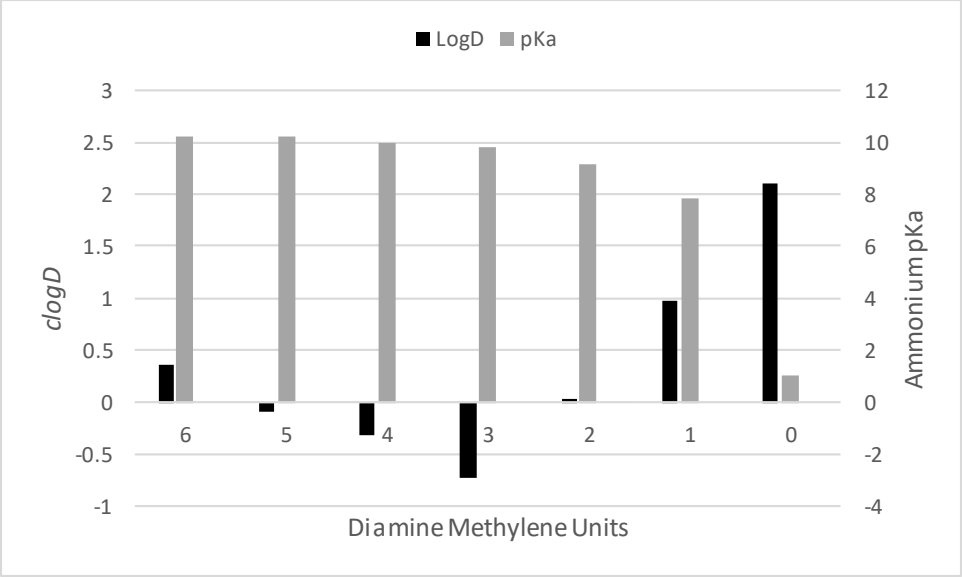


Figure 10. Calculated *logD* and pK_a values for γ-2 derivatives with varying diamine methylene units in the β position. All values calculated using MarvinSketch.

An interesting correlation between side-chain length and $cLogD_{7.4}$ was observed in the synthesized peptoids which was extrapolated out using distribution coefficients and pK_a values calculated in ChemAxon's MarvinSketch (**Figure 10**). β-3 had the highest distribution coefficient, as expected given the increased number of methylene units. As this side-chain is shortened to five, four (γ-2), or three methylenes (β-1), the distribution coefficient decreases, as expected. However, as this linker is shortened further to two methylenes (β-2), the distribution coefficient rises sharply, indicating that these compounds are becoming more hydrophobic, even though they possess fewer carbon/hydrogen groups. Given that the calculated distribution coefficient factors in compound ionization, an explanation for this trend can be obtained by observing the calculated pK_a values for the amino cation. These values remain relatively constant around a pK_a of 10 for compounds possessing side-chain linker lengths of 3-6 carbons. However, as the linker length becomes shorter than three, the calculated pK_a begins to drop, meaning that on average this side-

chain amino group becomes less ionized at neutral pH. Noting our observations regarding increased mammalian cytotoxicity with complete removal of the cation in position two, we hypothesize that this loss of ionization with shorter side-chain length makes peptoids slightly less potent against *C. neoformans* and significantly more cytotoxic to mammalian cells. This is further confirmed with the trimethylated γ-2 derivative β-5, which is permanently ionized. This compound exhibited no change in MIC but showed further improvement to mammalian cytotoxicity with a TD_{50} of 91.2 μg/mL.

The last derivative explored in this round of SAR possessed the cationic gaunidinium group of an arginine mimic (β-4). It was hypothesized that guanidinium containing peptoids have increased mammalian cytotoxicity, as well as improved antibacterial properties, but it was observed that substituting an arginine mimic in place of the lysine mimic in position 2 of γ-2 resulted in increased HepG2 toxicity as expected, but unexpectedly decreased antifungal potency 4-fold. Round 3 ultimately identified β-5 as the most promising antifungal peptoid from this study.

Conclusions

In conclusion, we report the improvement of our leading antifungal peptoid AEC5 through submonomer optimization by Structure Activity Relationship studies. This work identified beneficial modifications in positions two and three. Round two of SAR confirmed the most promising heterocyclic amine in position three, the thiophene aromatic heterocycle instead of a furan. This modification improved antifungal potency and selectivity through unknown mechanisms. Round three of SAR confirmed the trimethylated amino side chain as the best cationic amine in position two, supporting β-5 as our most promising antifungal peptoid. It is believed this cationic side chain's trimethylation locked its nitrogen in an ionized state, further reducing mammalian toxicity. This sequential optimization allowed for an improved selectivity ratio of 8 for AEC5 to 29 for β-5, as observed in mammalian liver cells. Current efforts are focused on elucidating these peptoids' mechanism of action, studying their resistance potential, and investigating their *in vivo* therapeutic properties.

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