Recent advances in the development of anti-infective peptoids

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Abstract:
In the search for new sources of antimicrobials many researchers have investigated antimicrobial peptides (AMPs) as templates for the design of innovative therapeutics. However, efforts to develop AMPs in this area has been severely hampered by their inherent susceptibility to enzymatic degradation. Given this only a handful of AMPs are currently in clinical trials. Peptide mimetics such as peptoids have emerged as highly promising alternatives to AMPs as they are inherently stable to enzymatic degradation and display potent antimicrobial properties. This feature article highlights the progress that has been made towards the development of novel anti-infective peptoids.

Biographies

Kevin L. Bicker obtained his Ph.D. in Organic Chemistry from the University of South Carolina working with Dr. John J. Lavigne and Dr. Paul R. Thompson developing peptide-based arrays to detect cancer-associated glycoproteins. He continued into postdoctoral studies with Dr. Paul R. Thompson at The Scripps Research Institute, Scripps Florida developing inhibitors and chemical probes for the protein arginine deaminase (PAD) family of enzymes. He is currently an Associate Professor of Bioorganic Chemistry at Middle Tennessee State University. His research group focuses on the high-throughput identification of antimicrobial compounds from peptoid libraries and the therapeutic development of these molecules.

Steven L Cobb obtained his Ph.D. in Bioorganic Fluorine Chemistry at the University of St. Andrews, working with Professor David O'Hagan on the biosynthesis of novel fluorinated natural products. He carried out postdoctoral studies on the development of new peptide-based antibiotics with Professor John C. Vederas FRS at the University of Alberta, Canada. He returned to the UK (Durham University) in 2008 as a Ramsay Memorial Fellow and he is currently an Associate Professor of Chemical Biology and Director of Biophysical Sciences Institute. His research interests focus on new peptide and peptoid based therapeutics for the treatment of antimicrobial infections.

1. Introduction

The innate immune system provides an effective first line of defence against infection and as such significant efforts have been directed towards exploiting the activities of antimicrobial peptides (AMPs), with a view to using them as templates for the design of innovative therapeutics.[1–8] AMPs are typically less than 40 amino acids in size, are cationic in nature and they play an important role in host defence, operating as both antimicrobial agents and modulators of the inflammatory response.[9,10] Their antimicrobial mode of action is typically based on the disruption of cellular membranes and as such the development of organism resistance is challenging. In the context of increasing antibiotic resistance, the antimicrobial and immunomodulatory actions of AMPs have some appeal for future treatment of infections.[1,10] However, AMPs like all peptides are susceptible to degradation by proteinases particularly at wound and inflammatory sites[4,11] thereby limiting their potential as novel therapeutics. Oligo *N*-substituted glycines (peptoids) are peptide isomers (**Fig. 1**) that combine many of the features of AMPs with the added advantage that they are resistant to proteinases.[12] Peptoids display a range of diverse biological activities[13] but a key area of expanding research interest is in their development as anti-infective agents.[14] Their antibacterial activity (in the low μm range), selectivity for bacterial cells and low hemolytic activity are comparable, and in many instances superior, to the values reported for leading AMP candidates.

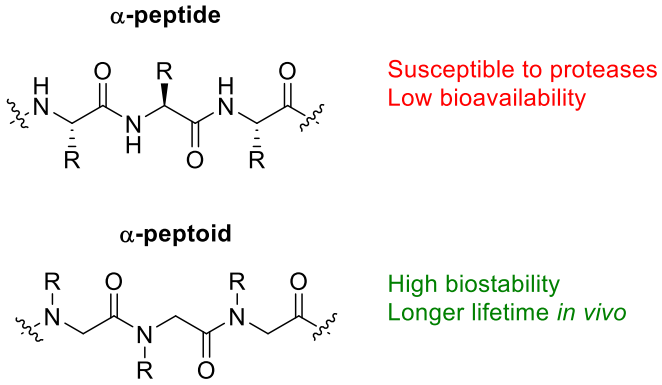


Fig. 1. Representative structure of the backbone of a peptide compared to a peptoid.

In this feature article we review the recent advances made in the development of anti-infective peptoids. While a discussion about peptoid activity against bacteria, fungi and parasitic infections is presented this review predominately focuses on molecules that fall within the structural class of alpha peptoids rather than beta-peptoids[15] or peptide-peptoid hybrids.[16,17]

2. Antibacterial Peptoids

Linear peptoids

One of the earliest examples of anti-infective peptoids was reported by Barron in 2003.[18] In this work a series of linear peptoid oligomers were designed to mimic the structural and physical properties of the well-studied cationic AMP, Magainin-2 Amide. An example of one of the oligomers prepared is (**1**) (**Fig. 2**) which is a linear peptoid comprised of 12 residues. (**1**) [H-(*N*Lys-*N*spe-*N*spe)$_4$-NH$_2$] contains a mixture of two monomers, *N*spe, a peptoid monomer analog of Phe, and *N*Lys, a peptoid monomer analog of Lys. Peptoid (**1**) was found to have low micromolar activity against both Gram-negative (*Escherichia coli*) and Gram-positive (*Bacillus subtilis*) bacterial strains and only 1.4% hemolysis was seen at the *E. coli* MIC (5.4 ±0.9 μM). The promising antibacterial properties observed for **1** were attributed to the fact that it had an overall

net positive charge, and a helical and facially amphipathic structure that resembled that of Magainin-2 Amide.

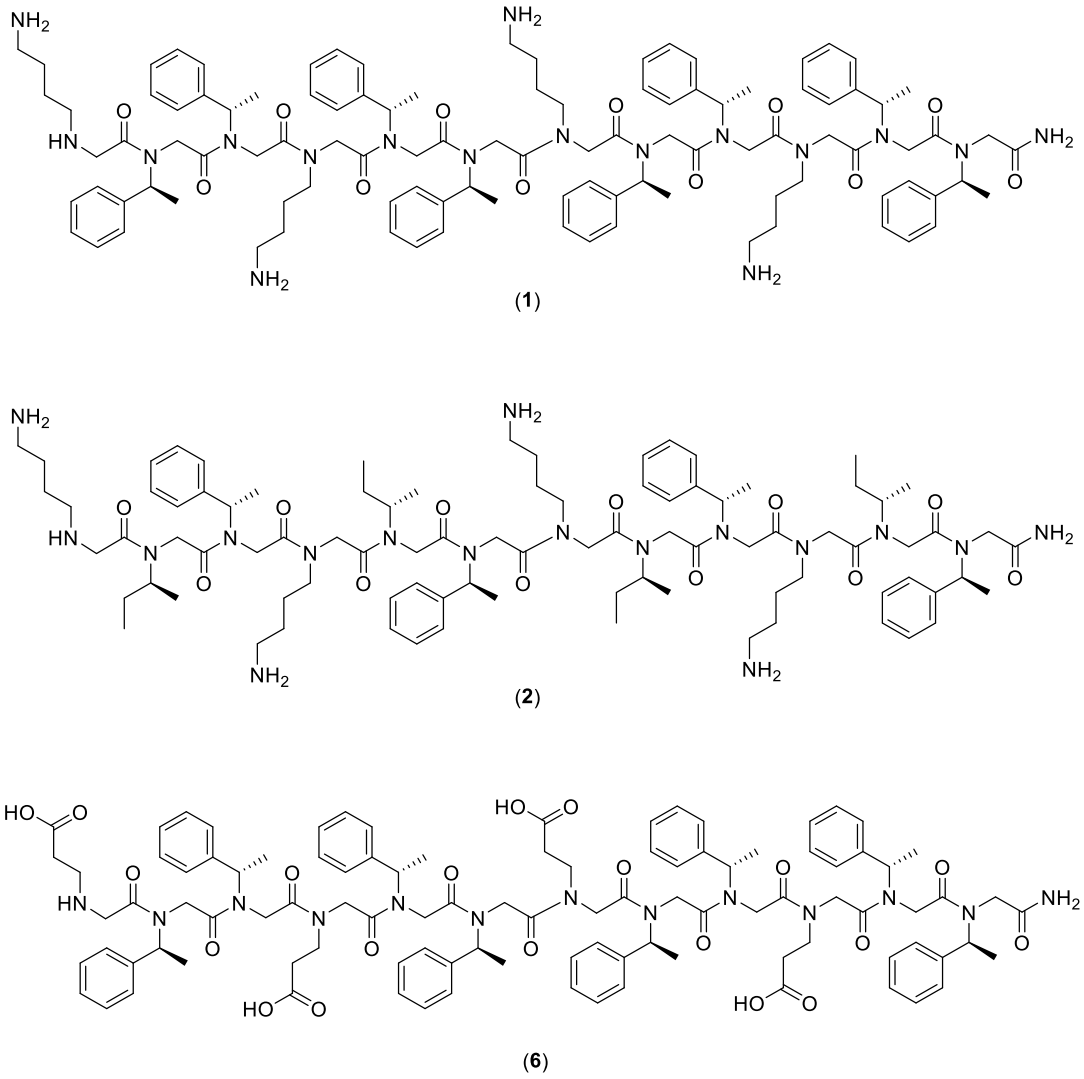


Fig. 2 Structures of ~~a~~ linear alpha peptoids developed by Barron and Zuckermann. ~~analog of~~ Magainin-2 amide [H-(*N*Lys-*N*spe-*N*spe)$_4$-NH$_2$] (**1**), H-(*N*Lys-*N*ssb-*N*spe)$_4$-NH$_2$ (**2**) and H-(*N*Glu-*N*spe-*N*spe)$_4$-NH$_2$ (**6**)

A more comprehensive study that built on this early work was reported by Barron and Zuckermann in 2008.[14] In this study a set of 15 linear peptoid analogs inspired by the structures of well-known cationic AMPs, such as melittin and also peptoid (**1**) were prepared and screened for biological activity against both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*). 10 of the 15 linear peptoids screened exhibited low-micromolar MICs against both *E. coli* and *B. subtilis* that were comparable to the AMPs also studied (melittin and pexiganan) (selected peptoids are presented in **Table 1**). The hemolytic properties of the peptoids were also determined along with an initial measure of selectivity (determined from the lytic activity of the peptoids against human erythrocytes) (**Table 1**).

Table 1 Early examples of linear peptoids with antibacterial properties.[14] MIC: minimum inhibitory concentration.

Peptoid sequence	*E. coli* (MIC, μM)	*B. subtilis* (MIC, μM)	Selectivity ratio (SR)*
H-(*N*Lys-*N*spe-*N*spe)$_4$-NH$_2$ (**1**)	3.5	0.88	6.0
H-(*N*Lys-*N*ssb-*N*spe)$_4$-NH$_2$ (**2**)	31	3.9	>3.9
H-(*N*Lys-*N*spe-*N*spe)$_2$-NH$_2$ (**3**)	27	27	>8.1
H-(*N*Lys-*N*spe-*N*spe)$_5$-NH$_2$ (**4**)	5.5	1.4	>0.55
H-(*N*Lys-*N*rpe-*N*rpe)$_4$-NH$_2$ (**5**)	3.5	0.88	4.6
H-(*N*Glu-*N*spe-*N*spe)$_4$-NH$_2$ (**6**)	>219	>219	*n/a*
H-(*N*Lys-*N*spe-*N*spe-*N*Glu-*N*spe-*N*spe)$_2$-NH$_2$ (**7**)	>110	6.9	>0.17
H-*N*Lys-*N*ssb-*N*spe-*N*ssb-*N*spe-*N*Lys-*N*spe*N*Lys-*N*ssb-*N*ssb-*N*spe-*N*Lys-NH$_2$ (**8**)	31	15	>3.9

* Selectivity ratio, SR (HD_{10})/(*E. coli* MIC)

Since this important study by Barron and Zuckermann was published several groups have made valuable contributions to the development of this field[13,16] and a selection of examples of linear α-peptoids with antibacterial properties are given in **Fig. 3**. As **Table 2** highlights linear α-peptoids have been found to be effective against a range of both Gram-positive and Gram-negative bacteria with MIC values ranging from 0.5 to >500 μg/mL (1 μM to > 50 μM). While the structures of the linear peptoids **9-19** vary considerably in terms of their monomer composition, it is worth noting that all of the peptoids reported to have antibacterial properties are overall cationic in nature. The charge within the peptoid sequence can be provided via the inclusion of a monomer(s) with an

amine functionality (e.g. *N*Lys, peptoids **9**, **10**, **15** and **16**), a guanidine functionality (e.g. *N*hArg, peptoid **11**), or a trimethylated ammonium (e.g. *N*aetm$^+$, peptoid **13** or *N*chtm$^+$ peptoid **14**). Examples where the positive charge within the peptoid is provided by more than one different type of cationic monomer (e.g. **Fig. 3**, peptoid **12**) are less common in the literature but recent advances in the synthetic approaches to access such peptoids is likely to change this.[19] At present it is not possible to draw any general conclusions as to~~o~~ which type of cationic monomer should be included to provide the most potent antibacterial properties and greatest SRs. This is due to the fact that the situation is highly complex given the number variables involved. For example, it has been found that that the position of the charged monomers within the peptoid sequence, the overall charge to hydrophobicity ratio and even the length of the side chain within the cationic monomer itself (e.g. *N*Lys versus *N*ah) can all affect the biological properties.[20]

While the sequence length can vary the majority of the antimicrobial peptoids reported in the literature are 8 to 12 residues long. However, work from Faure *et al.* (**Fig. 3**, peptoid **14**, 6 residues)[21] and Seo *et al.* (**Fig. 3**, peptoid **16**, 7 residues)[22] have demonstrated that shorter linear peptoids can be designed to maintain potent antibacterial properties and a good SR. We have also shown that lipidation of linear peptoids is a tool that can also be used to good effect to access shorter linear peptoid sequences with good biological activity profiles. [23,24]

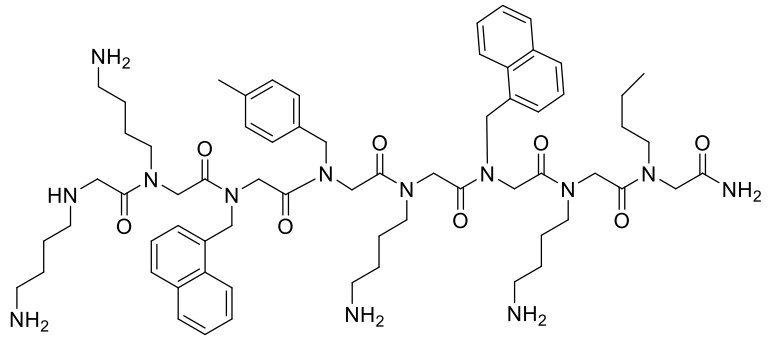
In terms of common structural features, it is also noteworthy that all of the linear peptoids reported are amphipathic, containing a mixture of both cationic and hydrophobic monomers. Just as for the cationic monomers a range of different hydrophobic monomers have been used in the design of linear antibacterial peptoids. The chiral aromatic monomer *N*spe which can be used to reinforce the helical properties of a linear peptoid can be found in many of the reported sequences (**Table 1**, peptoid **1**-**8** and **Fig. 3**, peptoids **11**-**17**). However, the inclusion of chiral residues is not essential

for activity and a range of peptoids that contain no chiral monomers (aromatic or aliphatic) have been found to have promising antibacterial properties (**Fig. 3**, peptoids **9** and **10**). The presence of substituents on the aromatic ring of a monomer has also been found to modulate peptoid activity. In particular, the inclusion of halogen atoms has been found to have either positive or negative effects in terms of both antibacterial activity and toxicity profiles. A wide range of halogenated monomers are commercially available and have been explored (**Fig. 3**. peptoids **15** and **17**). Both the Cobb group and those of Barron and Seo have reported that the inclusion of a fluorine atom on the aromatic ring of the monomer can enhance the biological activity of a linear α-peptoid compared to its non-fluorinated analog.[22] The results seen for fluorinated monomers in these linear α-peptoid systems were mirrored in a corresponding study on α-peptide-β-peptoids that was carried out by Franzyk and Hansen.[25] Interestingly, the inclusion of a chlorine atom on the aromatic ring of the monomer was found in both the studies carried out by Cobb and Barron and Seo to decrease the antibacterial activity observed. This is like to be due to the differences in hydrophobity that the inclusion of a F atom and Cl atom have on their respective monomers, and thus the linear peptoids into which they are incorporated.

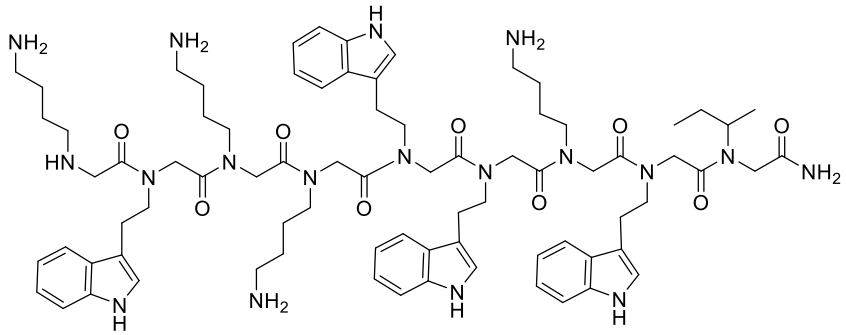
Table 2 Selected examples of linear α-peptoids with antibacterial properties.

Peptoid sequence	***E. coli* (MIC)**	***S. aureus* (MIC)**	**Reference**
H-*N*Lys-*N*Lys-*N*1Nal-*N*4MePhe-*N*Lys-*N*1Nal-*N*Lys-*N*Nle-NH_2 (**9**)	-	8 μg/mL	34
H-*N*Lys-*N*Trp-*N*Lys-*N*Lys-*N*Trp-*N*Trp-*N*Lys-*N*Trp-*N*Ile-NH_2 (**10**)	32 μg/mL	64 μg/mL	33,35
(*N*hArg-*N*spe-*N*spe)$_4$ (**11**)	6 μM	1 μM	20
H-(*N*Lys-*N*spe-*N*spe)$_2$(*N*hArg-*N*spe-*N*spe)$_2$-NH_2 (**12**)	17 μM	17 μM	19,20
H-(*N*aetm$^+$-*N*spe-*N*spe)$_4$ -NH_2 (**13**)	50 μM	6.3 μM	21
H-(*N*chtm$^+$-Nspe-*N*spe)$_2$ -NH_2 (**14**)	50 μM	3.1 μM	21
H-[(*N*Lys-*N*pfb*N*pfb)(*N*Lys*N*spe*N*spe)]$_2$-NH_2 (**15**)	6 μM	2 μM	20
H-(*N*Lys-*N*spe-*N*spe)$_3$-*N*Lys-NH_2 (**16**)	1.6 μM	0.8 μM	22
H-(*N*Lys-*N*spe(*p*Cl)-*N*spe(*p*Cl))$_4$-NH_2 (**17**)	>6.1 μM	6.1 μM	22
H-(*N*he-*N*spe-*N*Lys)$_2$-NH_2 (**18**)	*n.a**	*n.a**	26

******n.a* = not active against these specifc organisms but activity was observed against *Mycobacterium bovis* BCG, the laboratory strain *M. tuberculosis* H37Rv and the MDR clinical isolate *M. tuberculosis CSU87*. *Note* – Only one representive Gram-negative and one Gram-positive baterium were selected and the data given in this table does not cover the full range of antibacterial properties exhibited by peptoids **9**-**18**.



(**9**)



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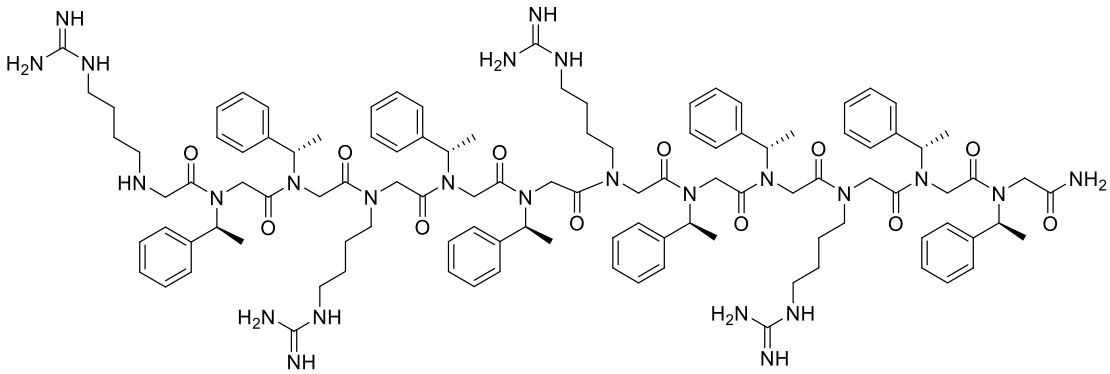

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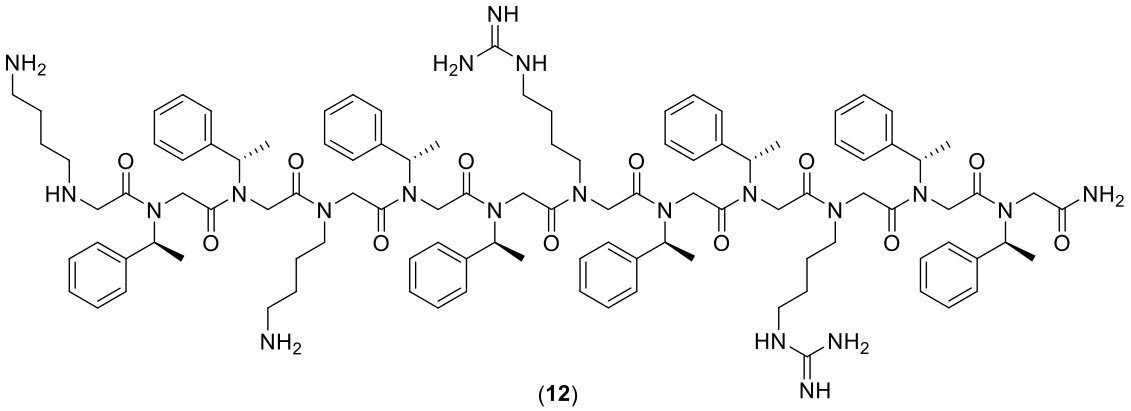

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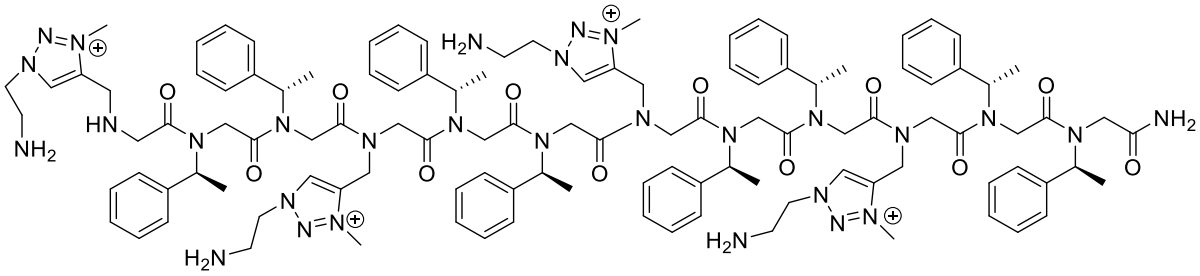

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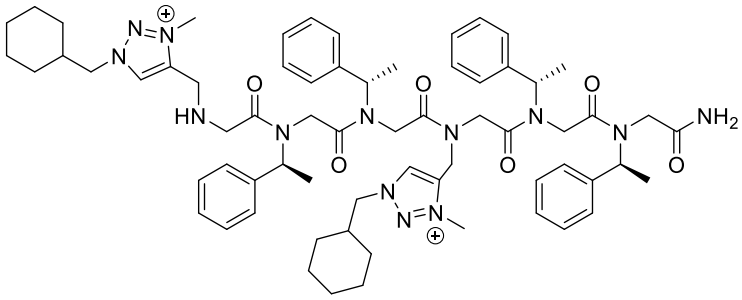

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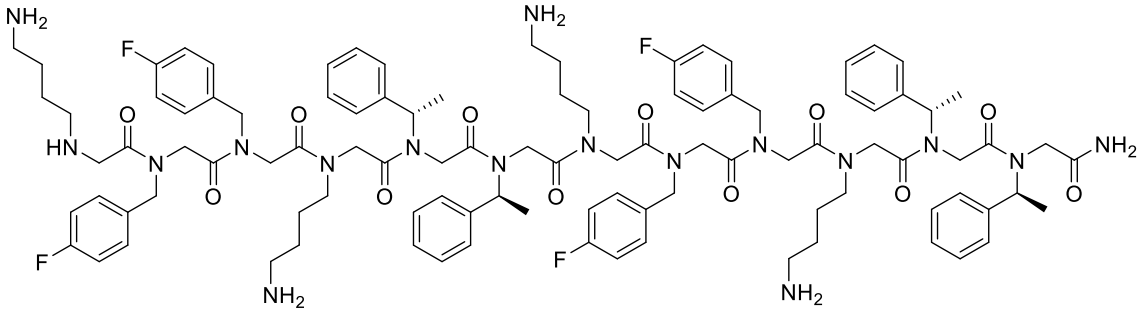

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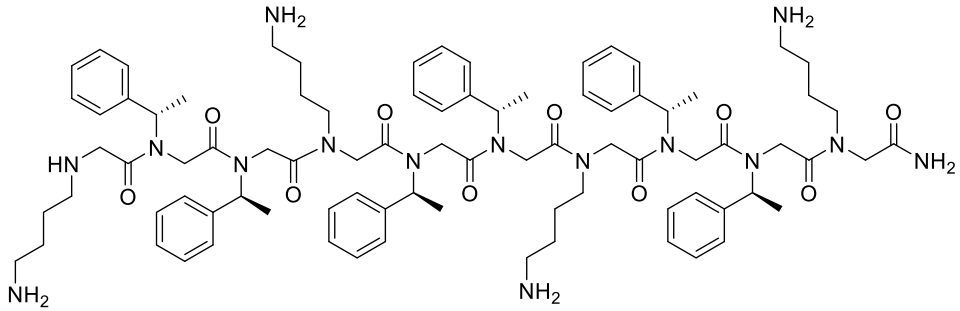
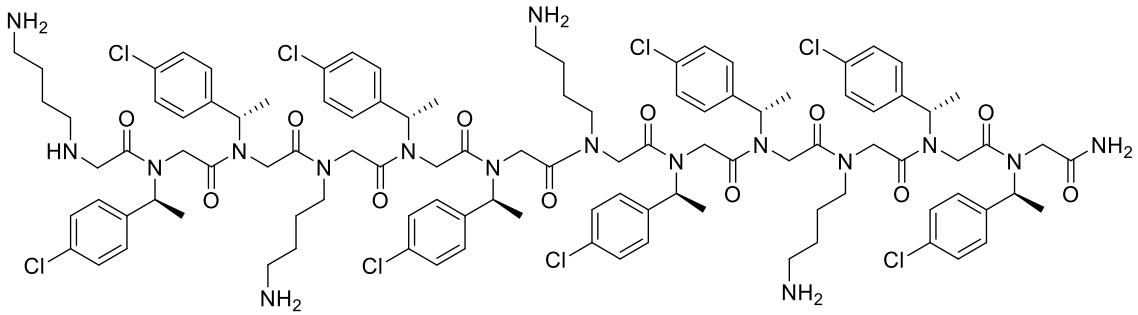
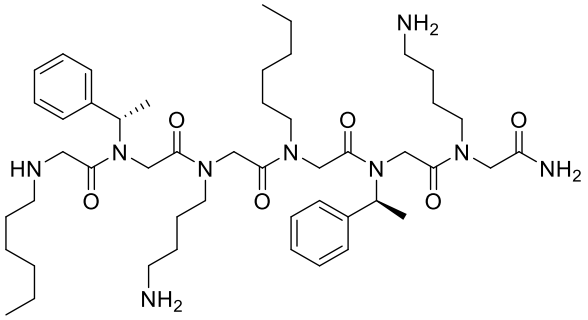


(**16**)



(**17**)



(**18**)

Fig. 3 Selected examples of linear α-peptoids with antibacterial properties.

Similar to the development of new AMP based antibiotics a challenge in this area relates to the design of peptoid sequences where the selectivity ratios (SRs) are suitable for further development. The application of machine learning approaches have helped to enable the rationale design of AMPs with improved properties and SR's but the application of such methods in the area of antimicrobial peptoid design has not been widely applied.[27–29] One of the only examples of work

in this area was reported by Barron and Jenssen in 2016.[30] In this work the authors were able to take 27 diverse peptoid sequences and use them to develop a new QSAR model, which could be used to correlate antimicrobial peptoid structure with antimicrobial activity. This study makes a valuable contribution to the field as it demonstrates the clear potential that machine learning approaches could offer in terms of the rationale design of anti-infective peptoids. A second more recent study in a related area was published by Lee *et al*. who used a training set derived from a series of AMPs to predict the bacterial membrane disruptive properties of several antibiotics. Within the compounds that were investigated in this study were a series of cyclic peptoids developed by the Kirshenbaum group.[31]

Being able to identify peptoid properties ~~that peptoids have which~~ that could ~~an~~ be used as descriptors of biological activity, offers a route by which to rationally design new sequences for screening. The Cobb group have sought to correlate physical properties such as log D (distribution coefficient) with the biological data obtained for linear peptoids.[32] It is likely that this area will see further activity in the future as new approaches to aid in the rationale and computational design of biological activity and selective linear peptoids are sought.

A further area that is likely to influence and aid in the future design of more active and selective antibacterial linear peptoids is mode of action studies. Several groups have demonstrated that linear peptoids can cause bacterial cell membrane disruption ultimately leading to cell death.[21,33–35] Just like AMPs, the exact mechanisms by which this cellular membrane disruption occurs is likely to vary given factors such as peptoid length, charge and cationic/ hydrophobic ratios. It has recently been demonstrated that like some linear AMPs (e.g. Indolicidin)[36] linear peptoids can also operate via an intracellular mode of action.[37] While this later discovery is an exciting one and it

potentially offers a route by which to design more selective anti-infective peptoids the identification of more peptoids with intracellular modes of action will be needed.

Lipopeptoids

Lipopeptides such as polymyxin B, daptomycin, and caspofungin are valuable antimicrobial agents used in the clinic. The earliest exploration of lipidating peptoids was done by the group of Annelise Barron. This work indicated that relatively long (12-mer) antibacterial peptoids could be shortened and lipidated with long alkyl tails to retain antibacterial activity.[38] Decreasing the overall size of peptoids simplifies synthesis and improves pharmacological properties. Not surprisingly, both antibacterial efficacy and hemolytic activity increased as the alkyl tail was lengthened. The most promising compound from this initial study, $C13_{4mer}$ (**19**)(**Fig.4**) was later shown to be effective against *M. tuberculosis* and *P. aeruginosa* biofilms with MIC values ranging from 6-25 μM.[39,40] Additionally, the Schweizer group explored a series of guanidylated lipopeptoids that showed improved efficacy over lipopeptides against Gram-negative bacteria.[41]

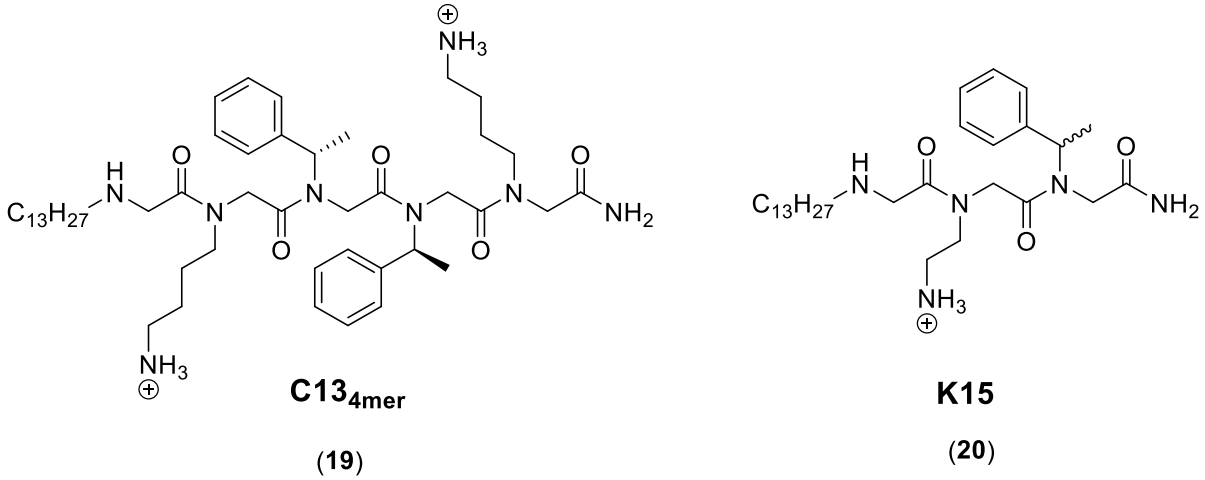
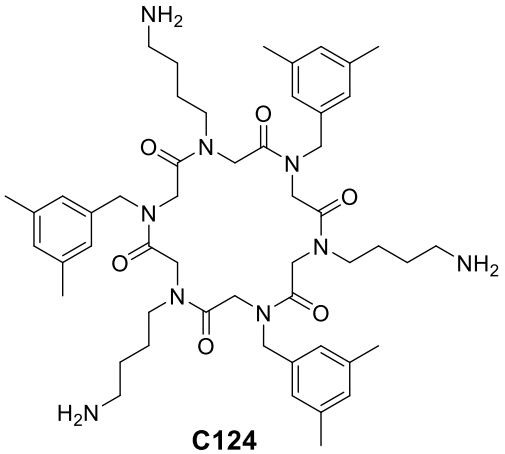


Fig.4 Examples of lipopeptoids with antibacterial activity. $C13_{4mer}$ (**19**), an early lipopeptoid with activity against *M. tuberculosis* and *P. aeruginosa* biofilms. K15 (**20**), a lipopeptoid identified by the PLAD assay with activity against the ESKAPE bacteria.

Inspired by these seminal explorations, the Bicker lab utilized a high-throughput Peptoid Library Agar Diffusion (PLAD) assay to identify antibacterial lipopeptoids from combinatorial libraries. Screening of a relatively small library resulted in a lipotripeptoid, K15 (**20**) (**Fig. 4**), with modest antibacterial efficacy against the ESKAPE pathogens.[42] Further study interrogated the relationship between alkyl tail length, antibacterial efficacy, and mammalian cytotoxicity using K15 (**20**). This work indicated that increased alkyl tail length was necessary for antibacterial activity but also increased cytotoxicity and hemolytic activity, confirming earlier studies.[23] However, alkyl tail length was not solely responsible for cytotoxicity and hemolytic activity and we realized that these toxic effects could be mitigated by increasing the hydrophilicity of lipopeptoid monomers. This work also indicated that lipidation of short peptoid libraries improved hit rate and identification of antibacterial compounds during PLAD screening. Our most recent study in this field explored the conversion of antimicrobial lipopeptides into lipopeptoids.[24] Peptoid mimics had similar or slightly diminished antimicrobial activity, an improved toxicity profile, and excellent proteolytic stability compared to their peptide counterparts.

Cyclic Peptoids

Cyclic peptoids are of interest as antimicrobials because they are conformationally ordered, more rigid, and have better cell permeability than linear peptoids.[43] An early study demonstrated that cyclization of peptoids improved antibacterial and antifungal activity compared to linear counterparts regardless of monomer composition.[44] The further development of antibacterial cyclic peptoids was spearheaded by Kent Kirshenbaum's group over the past several years. A seminal study from Kirshenbaum *et al*. showed that macrocyclization of amphiphilic peptoids improved antibacterial activity by as much as eight fold.[45] Furthermore, these compounds were effective against both Gram-positive and Gram-negative bacteria with MIC values ranging from 0.5 to >500 μg/mL depending on macrocycle size and composition and importantly had relatively little hemolytic activity. Follow-up studies by Kirshenbaum *et al*. indicated that some macrocyclic peptoids were efficacious and selective for clinical strains of methicillin resistant *Staphylococcus aureus* (MRSA), exerting antibacterial activity through membrane pore formation.[46] One compound in particular, C124 (**21**)(**Fig. 5**), had an MIC against MRSA of 3.9 μg/mL and 32 to 45-fold selectivity for MRSA over mammalian cells. Further detailed studies using electron microscopy and X-ray scattering analysis of Langmuir monolayers indicated that peptoid macrocyclization improved membrane permeation[47] and that tuning hydrophobicity could control cyclic peptoid selectivity by regulating their intercalation into anionic lipid membranes.[48]



C124
(**21**)

Fig. 5. Structure of cyclic peptoid, C124 (**21**), with selective antibacterial activity against methicillin resistant *Staphylococcus aureus*.

3. Antifungal Peptoids

Nearly all research into antifungal peptoids has focused on identifying compounds with efficacy against *Candida albicans* and *Cryptococcus neoformans*, two yeast like pathogens responsible for most fungal infections in humans. *C. albicans*, the leading fungal pathogen in hospital originated infections, primarily infects mucosal surfaces and can lead to invasive candidiasis, a dangerous blood borne infection.[49] *C. neoformans* principally infects immunocompromised individuals, originating as a pulmonary infection that leads to deadly cryptococcal meningitis if left untreated.[50] An early report evaluated the antimicrobial activity of lysine rich peptoid-peptide hybrids effective against both *C. albicans* and *C. neoformans* with MICs ranging from ≤1.6 to 12.5 μg/mL.[51] The Franzyk group also evaluated a series of hybrids, specifically β-peptoid-peptide hybrids, against *C. albicans* with modest efficacy and low hemolytic activity, though these compounds exhibited better antibacterial that antifungal efficacy.[15] Around the same time, Barron *et al*. demonstrated the antifungal activity of relatively short lipopeptoids incorporating pentyl, decyl, and tridecyl

tails.[38] Anti-*Candida* activity was generally improved with the addition of longer alkyl tails. Intrigued by these results, the groups of Riccardis and Izzo explored the synthesis and antifungal activity of cyclic peptoids.[44] Amphipathic and N-lysine rich cyclic peptoids were more effective against *C. albicans* and *C. neoformans* than bacterial pathogens, with minimal hemolytic activity. Additionally, cyclic compounds were more effective than their linear counterparts regardless of the microbe tested. While the focus of most antifungal peptoid studies has been human pathogens, the antifungal efficacy of peptoids against plant pathogens has also been explored, namely against *Fusarium virguliforme* and *Fusarium lateritium*.[52]

Relatively little is known regarding the mechanism of action for antifungal peptoids. It is hypothesized that most peptoids exert antimicrobial activity through membrane disruption, analogous to AMPs, though intracellular targets are possible. Being eukaryotes, fungi and mammalian cells share similar membrane compositions, making antifungal selectivity challenging for compounds exerting their effects via the membrane. One early study on anti-*Candida* peptoids indicated that cells exposed to these compounds decreased phenotypic switching to the pathogenic hyphal phenotype and displayed marked cellular and organellular stress, suggesting a possible intracellular target.[53]

The most recent efforts in antifungal peptoids have come from our own labs, focusing on *C. albicans* biofilms (Cobb) and *C. neoformans* (Bicker). Most clinical infections of bacteria and fungi exist in biofilm form, often resulting in resistance to antimicrobials that are effective against planktonic microbes. Additionally, biofilms are rarely monospecial but often exist polymicrobially and can include both bacterial and fungal pathogens, resulting in synergistic sensing, growth, and resistance. Cobb and Lundy sought to explore the utility of peptoids as anti-biofilm agents while developing a novel modified quantitative PCR method for evaluating cross-kingdom biofilm

viability.[54] Unlike traditional methods of biofilm quantification, the modified qPCR method allows researchers to detect viable but non-culturable microbes as well as delineate the viability of different species in a polymicrobial biofilm. From this study, peptoid **17** (**22**) (**Fig. 6**) was the most effective anti-biofilm peptoid against monospecial and cross-kingdom polymicrobial biofilms of *C. albicans*, *S. aureus*, and *E. coli* at concentrations as low as 10 μM. Additionally, this peptoid had no observable cytotoxicity below 100 μM and data suggest peptoid **17** (**22**) exerts its antifungal effects through membrane disruption, which would decrease the likelihood of antifungal resistance development. This was the first study demonstrating peptoid efficacy against fungal biofilms, an important step towards developing peptoids as antifungal therapeutic agents.

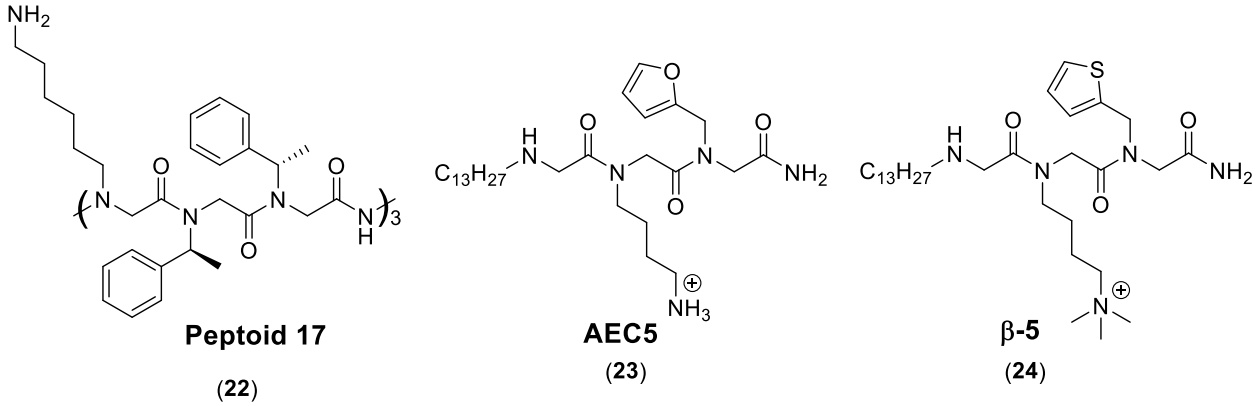


Fig. 6 Structures of the most recent antifungal peptoids, including Peptoid 17 (**22**) effective against cross-kingdom polymicrobial biofilms, as well as AEC5 (**23**) and β-5 (**24**) effective against *C. neoformans*.

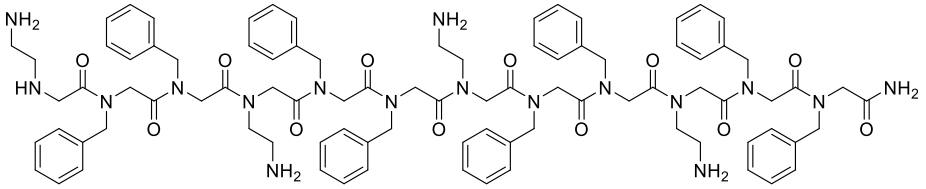
The Bicker lab adapted a high-throughput phenotypic screen, known as the Peptoid Library Agar Diffusion (PLAD) assay, toward identifying antifungal peptoids against *C. neoformans* from combinatorial libraries.[55] One lipotripeptoid, termed AEC5 (**23**)(**Fig. 6**), had an MIC of 6.25 μg/mL against *C. neoformans* H99S, as well as efficacy against clinical strains of *C. neoformans* and *C. gattii*, a more virulent cryptococcal strain capable of infecting immunocompetent

individuals. Additionally, AEC5 (**23**) demonstrated minimal toxicity against several mammalian cell lines, giving a reasonable lead compound for further development. In an effort to contribute valuable preclinical information on antifungal peptoids we characterized some pharmacokinetic and therapeutic parameters of AEC5 (**23**). Namely, we determined that this peptoid kills *C. neoformans* rapidly (~50% reduction in 30 minutes; complete killing in 3 hours), has an excellent *in vivo* half-life of 20+ hours, and was not toxic during a 28-day sub-chronic study with daily doses of AEC5 as high as 50 mg/kg in mice.[56] Concomitantly, we undertook an iterative structure-activity relationship study to improve the selectivity of AEC5 by individually optimizing the peptoid monomers. Slight modifications to AEC5, namely substituting a thiophene into the third position and trimethylating the N-lysine derivative in the second position resulted in peptoid β-5 (**24**) (**Fig. 6**) with improved efficacy against *C. neoformans* and decreased toxicity against human hepatic cells.[57] Taken together, the most recent data on antifungal peptoids indicate that they could supplement the small collection of clinically used antifungal agents wrought with resistance and toxicity issues.

4. Antiparasitic peptoids

Research efforts from our own group (Cobb)[6,7] and others[58] have identified AMPs derived from amphibians as potential anti-parasitic compounds. A wide variety of other AMPs derived from mammalian sources, invertebrates, or synthetic AMPs have been shown to have activity against protozoan species.[59] In contrast and despite the encouraging results reported on the antibacterial properties of peptoids very few groups have sought to investigate the effects of peptoids against protozoan parasites. An early study from Olsen *et al.* tested α-peptide β-peptoid chimeras against *Plasmodium falciparum*, the causative agent of malaria. For some sequences within this study, sub-hemolytic doses showed promising anti-plasmodial activities.[60] Short linear peptoids have

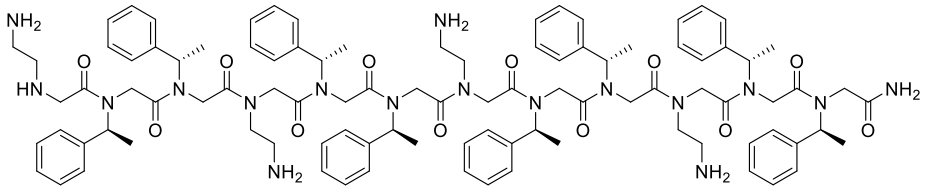
also been found to be inhibitors of trypanothione reductase, an enzyme vital to both *Trypanosoma* and *Leishmania* species.[61] Recently, work in our group (Cobb) has focused upon the anti-parasitic action of linear peptoids against *Leishmania mexicana*, the causative agent of cutaneous leishmanaisis (CL).[62,63] In these structure activity investigations, some key factors for anti-Leishmanial activity against the amastigote (clinically relevant form) of the parasite have been identified (**Fig. 7**, Peptoids **15**, **25**-**27**). In a given series of linear peptoids anti-parasitic activity increased as the chain length was increased from 6 monomers to 12 monomers.[62] In addition the inclusion of a fluorine atom on the aromatic monomers used within a sequence (e.g. **Fig, 7**, Peptoid **15**) led to an increase in the anti-Leishmanial when compared to the analogous non-fluorinated systems. Interestingly, it was also found that peptoids such as **27** (**Fig. 7**), where the overall charge had been reduced to +2 were found to have more potent anti-Leishmanial activity compared to their corresponding +4 analogs. This result is the opposite of what is typically seen in studies carried out to design potent antibacterial peptoids where an increase in cationic properties and overall net charge generally results in increased biological properties. In addition, it was also found that in the libraries of linear peptoids screened most were less active against the axenic amastigote forms of the *L. mexicana* parasite. This result mirrors what is commonly seen with linear AMPs tested against *leishmania spp*. and the differences in anti-parasitic activity between the different parasite life stages provides evidence that the linear α-peptoids studied are exerting their mode of action via membrane disruption. However, more work is needed to confirm if this is actually the case. As part of our work in this area we have also been able to design a peptoid (**27**)(**Fig. 7**) with an ED_{50} of 1.6 μM against *Leishmania* infected mammalian macrophages.[63]



(**25**)

(*Nae*N*phe*N*phe*)$_4$

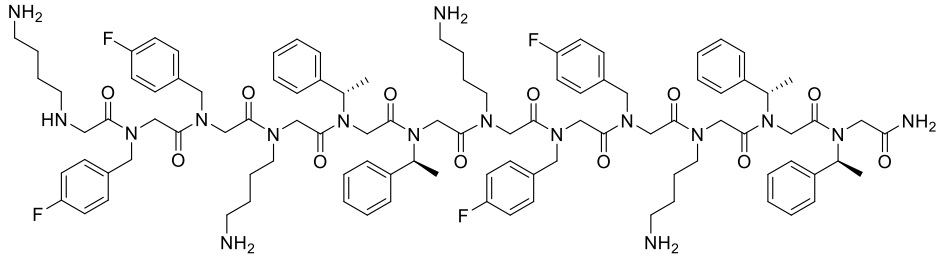
L. mexicana promastigotes ED_{50} = 21 μM
L. mexicana axenic amastigotes ED_{50} = >100 μM



(**26**)

(*Nae*N*spe*N*spe*)$_4$

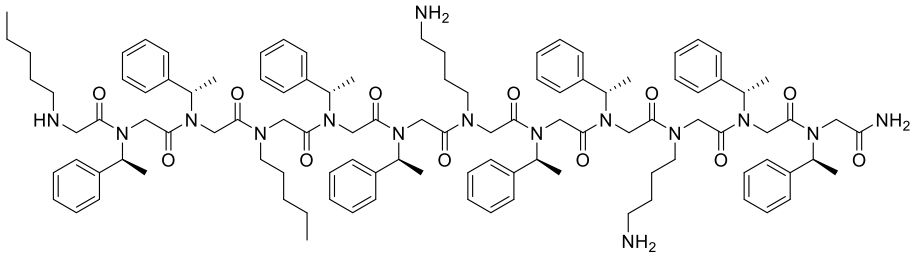
L. mexicana promastigotes ED_{50} = 7μM
L. mexicana axenic amastigotes ED_{50} = 17 μM



(**15**)

[(*N*Lys*N*pfb*N*pfb)(*N*Lys*N*spe*N*spe)]$_2$

L. mexicana promastigotes ED_{50} = 6μM
L. mexicana axenic amastigotes ED_{50} = 21μM



(**27**)

(*N*amy*N*spe*N*spe)$_2$(*N*Lys*N*spe*N*spe)$_2$

L. mexicana promastigotes ED_{50} = 11μM
L. mexicana axenic amastigotes ED_{50} = 16μM

Fig. 7 Representative structures of linear peptoids **15**, **25-27**, with activity against *L. mexicana*.

5. ~~Conclusions and~~Future outlook

Peptoids with antimicrobial activity against a host of pathogens from different kingdoms have been developed over the past many years. While peptoids offer many advantages over AMPs or classical antibiotics, we highlight below some of the challenges that remain in the transition of antimicrobial peptoids to the clinical space. An important measure of a compound's therapeutic value is the selectivity ratio (SR) or therapeutic index (TI). SR is calculated as the toxicity value divided by the antimicrobial efficacy (*e.g.*, SR = TD_{50}/MIC) and serves as a measure of the selectivity of a compound. SR values greater than 10 are characteristic of lead molecules but much higher SR values must be achieved for clinically relevant antimicrobial compounds. As shown in **Table 1**, early antibacterial linear peptoids developed by Barron had SR values ranging from 8 to <1.[14] Initial antifungal lead peptoids developed by the Bicker lab had SR values around 10 and were improved to >30 through iterative SAR studies.[57] Initial antiparasitic peptoids developed by the Cobb group had SR values ranging from 2.4 to 6.[62] Exploring expanded chemical space yielded peptoids with improved antiparasitic activity, but increased toxicity as well, limiting SR values to 1 or less.[63] In general, peptoid toxicity towards mammalian cells increases with increasing antimicrobial activity, regardless of pathogen. However, there has been a focus in recent years to understand this correlation and develop antimicrobial peptoids with higher SR values. One particular phenomenon observed by our labs and others is the unique correlation between toxicity and side-chain length for cationic residues.[17,20,57] Cationic monomers with longer side chains (*i.e.*, five or six methylenes) have increased toxicity compared to the monomer with four methylenes (*N*Lys) due to increased C-H group hydrophobicity. Interestingly, as side chains are shortened to three or two methylenes, hydrophobicity and toxicity increase. The exact reason for this remains unknown, though it could be due to loss of entropic freedom or depression of pKa as the cationic

group approaches the amide backbone. Through these explorations, antimicrobial peptoids with excellent SR values have been identified, namely "Peptoid 7" (**Fig. 3**, Peptoid **16** in this article) identified by Seo and Barron with an SR of approximately 50[22] and peptide/peptoids hybrids identified by Franzyk with SR values >100.[17] Additionally, one of the cyclic peptoids identified by Kirshenbaum had modest toxicity, possessing SR values >32.[46] The SR value of antimicrobial peptoids must increase for them to gain clinical traction and factors that contribute to peptoid toxicity remain an area for continued exploration.

Literature indicates that the major driver of mammalian cytotoxicity in antimicrobial peptoids is overall compound hydrophobicity.[17,20] In lipopeptoids the majority of this hydrophobic character comes from the aliphatic tail. Efforts from the Bicker lab to reduce toxicity of lipopeptoids by shortening the aliphatic tail concomitantly lead to a decrease in antimicrobial activity.[23,57] Similar studies early on by the Barron lab had the same results.[38] Yet one thing we realized in our studies was that the toxicity of the aliphatic tail could be mitigated by using more hydrophilic monomers while still retaining antimicrobial activity. The chemical diversity of antimicrobial peptoids reported in the literature is somewhat limited. Most compounds are composed of the monomers *S*-phenylethylamine (*N*spe), benzylamine (*N*phe), and length derivatives of the cationic 1,4-diaminobutane (*N*Lys). Though these have proven useful, the path forward to clinical antimicrobial peptoids will undoubtedly require us to explore greater chemical diversity. High-throughput screening using the Peptoid Library Agar Diffusion (PLAD) assay has allowed the Bicker lab to rapidly interrogate the antimicrobial utility of nontraditional monomers.[42,55] Following the discovery of an antifungal peptoid containing a furan monomer and subsequent optimization to a thiophene, our lab continues to explore the utility of aromatic heterocycles. The Cobb, Barron/Seo and Franzyk groups have all explored halogenated aromatic

monomers with particular success using fluorinated derivatives (e.g. Peptoid **15** in **Table 2** and **Fig. 3**).[20,25] The primary method for synthesizing peptoids allows for the incorporation of a tremendous number of monomers and the continued exploration of this chemical space and the development of new monomers[64–66] will be key in developing more effective and selective antimicrobial peptoids.

Peptoids are generally thought to exert antimicrobial activity through non-specific membrane disruption and permeabilization, much like their natural peptide counterparts.[14,67] Membrane disruption may occur through depolarization or insertion into the membrane, causing a change in membrane fluidity that leads to leakage of cytoplasmic components. Andreev *et al*. recently demonstrated that insertion of cyclic peptoids into bacterial membranes resulted in pore formation.[48] A common technique for investigating membrane disruption is Live/Dead staining. In this assay, a membrane permeable dye is used to visualize live cells while a dye only capable of traversing compromised membranes before intercalating into DNA and fluorescing is used to indicate dead cells. Though direct membrane permeabilization would result in increased "dead" staining, so would a mechanism of action with intracellular targets that triggers rapid cell death. Therefore, Live/Dead staining is best followed with other assays to confirm membrane disruption, such as liposomal assays and visualization of damaged membranes by electron microscopy. A recent study by Mojsoska *et al*. nicely demonstrated this series of techniques to conclude that peptoids with different structural properties exhibit differing degrees of membrane disruption.[33] Additionally, this study showed that peptoids may work by both membrane disruption and binding of intracellular targets that disrupt protein or DNA synthesis. Recent studies by the Hansen group showed that peptide/peptoid hybrids exerted antimicrobial activity through membrane disruption at higher compound concentrations while inhibiting cell wall biosynthesis at sub-MIC

concentrations.[34,68] Studies into antifungal or antiparasitic peptoids suggest a membrane disruption mechanism of action, yet remain somewhat inconclusive and require further study.[56] Though the traditional mechanistic model for antimicrobial peptoids remains membrane disruption, these studies from the past few years highlight the need for detailed study into the mechanism of action for newly discovered antimicrobial peptoids, which may use more complex mechanisms of pathogen killing.

One last frontier that we identify as being poised to contribute to this field is the use of machine learning and predictive modeling. Two studies have reported *in silico* characterization and modeling of antimicrobial peptoids. The first by Czyzewski *et al*. used a set of Peptoid 1 (**1**) (**Fig. 2**) derivatives to build a training set capable of predicting antimicrobial activity based on compound structure.[30] Using this training set they were able to predict an MIC against *E. coli* very similar to the experimental MIC for Peptoid 1 (**1**). A second study by Lee *et al*. used an antimicrobial peptide training set to predict the bacterial membrane disruptive properties of several antibiotics, including cyclic peptoids developed by the Kirshenbaum group.[31] These studies demonstrate the utility of computer models in relating peptoid structure to antimicrobial activity and could be beneficial in the exploration of new peptoid monomers and efforts to improve SR values. However, accurate computer modeling relies on sizeable data training sets. As groups from around the world continue to explore the antibacterial, antifungal, and antiparasitic activity of peptoids, collaborative efforts to share and assemble data will be critical in using machine learning to propose and predict the physicochemical properties of antimicrobial peptoids that could progress towards the clinic.

6. Conclusions

Nearly 30 years since their initial development, peptoids remain an exciting source of promising antimicrobial agents. Within these feature article we have highlighted the progress made by our groups and others over the past several years into peptoids with antibacterial, antifungal, and antiparasitic properties. Notable progress has been made recently in exploring new monomers, relating peptoid structure to function, and broadening the scope of targeted pathogens. The research of our individual groups has focused on antiparasitic (Cobb) and antifungal (Bicker) peptoids, while both continuing to explore antibacterial peptoids as well. We are thankful for the opportunity to share progress from our own groups among others doing impactful research in this field. However, we hope that inclusion of a future outlook perspective will inspire readers to participate in this field, working to address the challenges remaining in the push to bring an antimicrobial peptoid to clinic.

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