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Antimicrobial β-peptoids by a block synthesis approach

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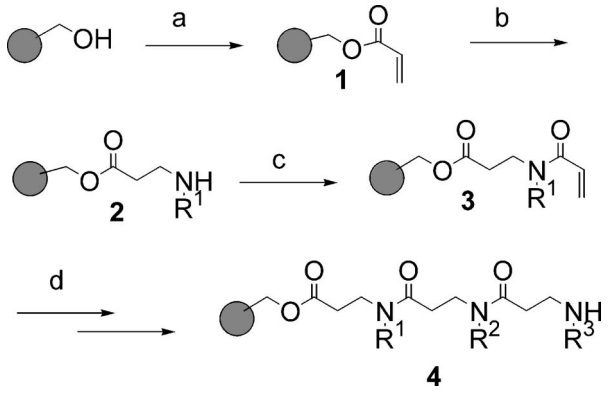
Abstract—Antimicrobial peptides and their mimetics offer a potential new disinfective tool. β-Peptoids (oligo-*N*-substituted β-alanines) were synthesized and investigated for antimicrobial activity. A block approach whereby di- and tri-β-peptoids were first prepared and then ligated via amide bond formation to synthesize larger β-peptoids was developed. The β-peptoids were found to possess moderate activity in an *Escheridchia coli* assay.

Antimicrobial peptides are found in most living organisms and are part of the innate immune system in humans. There has been longstanding interest in these peptides due to their interesting structures, and the fact that they possess a different mode of action from most commercial antibiotics.

One common motif among antimicrobial peptides is a cationic amphiphilic structure in which the peptides contain a high number of cationic residues which are segregated from a lipophilic domain. These peptides are thought to act by interaction with, and disruption of, the bacterial cell membrane, leading to pore formation.[1] Peptides can obtain an amphipathic structure in a variety of ways including formation of amphiphilic helices, where one face is cationic and the other lipophilic, formation of hydrophobic and hydrophilic patches or formation of β-sheets which segregate the hydrophobic and cationic domains. Many natural, α-helical, and β-sheet antimicrobial peptides are known including cecropin,[2] magainin,[3] and defensins.[4] Both helical and β-sheet cationic amphipathic peptides have been designed from first principles and shown to be antimicrobial.[5] Although many helical antimicrobial structures are known, it is clear that a pre-formed helix is not necessary for activity. Indeed, it has been shown that many natural peptides adopt amphiphilic structure only in the presence of a membrane,[6] and compounds which cannot adopt helical structures can still be good antimicrobials.[7]

Several classes of antimicrobial peptidomimetics have been studied. These include β-peptides,[8] α-peptoids,[9] and several types of polymers.[10] Another class of potential peptidomimetics, which has been much less studied, are the β-peptoids (poly-*N*-substituted β-alanine). We became interested in studying these molecules as potential antimicrobials, since they may have an interesting combination of properties; they are isosteric to β-peptides yet potentially easier to prepare since no chiral β-amino acids need be made, they, like α-peptoids, are expected to be protease resistant, and they may be more tunable than synthetic polymers.

Scheme 1 shows the synthesis of β-peptoids, first described by Hamper.[11] Reaction of a solid support with



Scheme 1. Reagents and conditions: (a) acryloyl chloride (2.0 equiv), DIEA (3.0 equiv), THF 3 h; (b) RNH_2 (20 equiv), 2-propanol, 50 °C, 48 h; (c) acryloyl chloride (2.0 equiv), DIEA (3.0 equiv), THF 3 h; (d) RNH_2 (20 equiv), 2-propanol, 50 °C, 48 h; acryloyl chloride (2.0 equiv), DIEA (3.0 equiv), THF 3 h; RNH_2 (20 equiv), 2-propanol, 50 °C, 48 h.

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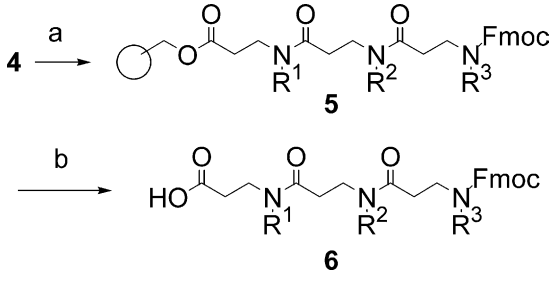
acrylic acid or acryloyl chloride to generate acrylate resin **1**, followed by a Michael-type addition of a primary amine, gave secondary amines **2**. Iterative reaction with acryl chloride and primary amines then led to the β-peptoids **4**. We found this chemistry to work well for several cycles, but had difficulty generating longer β-peptoids, as the yields fell off after approximately the pentamer stage. Antimicrobial testing of these short oligomers showed no measurable activity (data not shown).

One hypothesis concerning the mechanism of antimicrobial peptides supposes that oligomers need to be able to span the membrane and we would therefore need β-peptoids greater than 5 residues in length to obtain good activity.[12] Since we were unable to prepare longer oligomers by the published route, we decided to explore the use of a building block approach whereby we could synthesize blocks of di- or tri-β-peptoids and then ligate them together on solid phase via amide bond formation to achieve the desired length β-peptoids. Our first approach utilized Hamper's synthesis to prepare di- and tri-β-peptoid blocks. The amine terminus of the β-peptoid blocks was then protected with Fmoc to give **5**, and the building blocks **6** released from the resin, as shown in Scheme 2, by treatment with TFA.

While the solid phase synthesis of the building blocks was convenient for smaller quantities, to enable synthesis of longer oligomers by a block approach we required a method to quickly produce larger quantities of the building blocks. We explored several solution phase synthetic routes including preparation and amide coupling of *N*-substituted β-alanines. We found the method described in Scheme 3 to be most efficient. Reaction of a primary amine with *t*-butyl acrylate in a Michael-type addition gave the *N*-substituted β-alanine **7**. Reaction of the crude mixture of **7** with excess acryloyl chloride gave acrylamide **8** which could be purified by flash chromatography. Repeating this cycle of Michael additions with primary amines and acryoylation, dimers and trimers **9** were constructed. The intermediates were purified at each acrylamide stage by flash chromatography. When the desired block was complete, its amine terminus was protected with an Fmoc group to give **10** and the *t*-butyl group cleaved by formic acid treatment to provide the final building blocks, **6**. In this way, multi-gram quantities of the β-peptoid building blocks could be accessed.

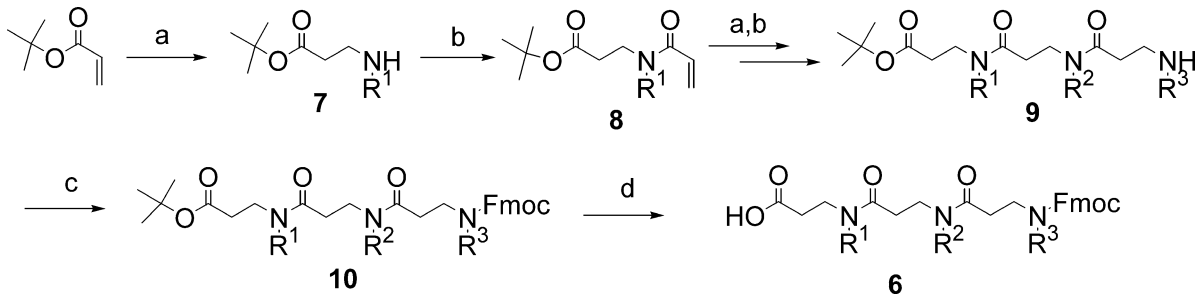


Scheme 2. Protection of and cleavage from the resin of β-peptoid blocks. Reagents and conditions: (a) Fmoc-Cl (2.0 equiv), DIEA (3.0 equiv), NMP, 3 h; (b) 50% TFA/DCM, 1 h.

To make an initial scouting set of β-peptoids, a group of side chains was chosen in which hydrophobic and cationic groups were represented. Figure 1 shows the composition of the side chains for the scouting library. The isobutyl and benzyl groups were chosen for hydrophobic side chains, while the 2-aminoethyl, 4-aminobutyl, and *N*,*N*-dimethyl-3-aminopropyl groups were chosen for cationic groups. The dimethylaminopropyl side chains could be incorporated directly into the β-peptoids, whereas the aminobutyl and aminoethyl groups required protection of the terminal amine to allow the blocks to be synthesized.

Ten different building blocks were synthesized by the solution and solid phase routes described above. The composition of the building blocks is listed in Table 1. The blocks were designed to probe the effects of the ratio of cationic to hydrophobic groups, the importance of the side-chain composition, and the effect of the relative position of the cationic and hydrophobic groups. Our initial hypothesis being that if the group has sufficient conformational freedom, it could adopt an amphiphilic structure in the presence of a bacterial membrane. The blocks were designed to cover a hydrophobic content range from 33 to 66% but because of the oligomer synthesis limitations encountered, we are limited to 50–66%, which is still within the optimal range found for idealized helical peptides which has been found to be 50–60% Figure 2.[13]

The final β-peptoids were constructed using a solid phase synthesis analogous to peptide synthesis (Scheme 4).



Scheme 3. Solution synthesis of β-peptoid blocks. Reagents and conditions: (a) RNH_2 5.0 equiv, 50 C MeOH 48 h; (b) acryloyl chloride, 1.2 equiv, DIEA 2.0 equiv, DCM 3 h; (c) FMOC-Cl 1.3 equiv, DIEA 2.0 equiv, DCM 12 h; (d) HCOOH, 12 h.

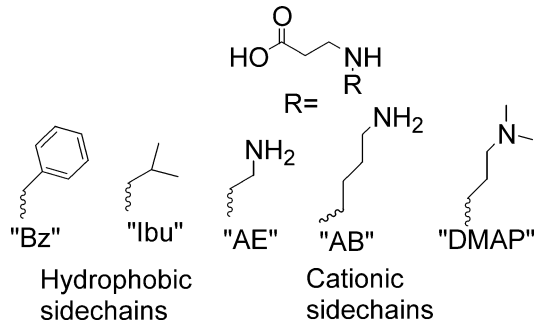


Figure 1. Side-chain composition for the β-peptoids.

Table 1. Composition of the di- and tri-β-peptoid building blocks used to construct the final β-peptoids

Compound	R^1	R^2	R^3
6a	Ibu	DMAP	Bz
6b	DMAP	Ibu	Bz
6c	DMAP	Ibu	
6d	Ibu	DMAP	
6e	DMAP	Ibu	DMAP
6f	AE	Ibu	Ibu
6g	AE	Ibu	
6h	AE	AE	Ibu
6i	AB	Ibu	Ibu
6j	AB	Ibu	

When R^3 is blank, this denotes a di-peptoid. The designations Ibu, etc., denote the side-chain content and are described in Figure 1.

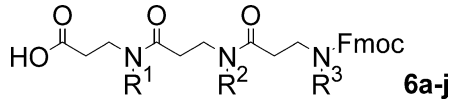


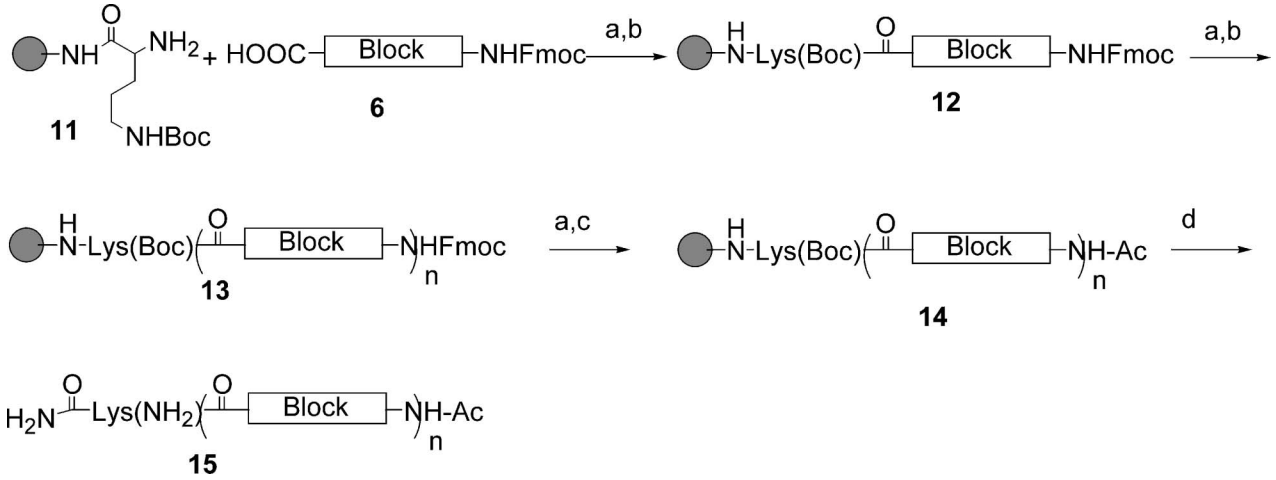
Figure 2.

Repetitive cycles of coupling to resin bound amine followed by deprotection of the Fmoc group served to build the desired β-peptoids. The resin was first reacted with Fmoc(Boc)lysine to ensure high initial loadings of the resin and to conserve the β-peptoid blocks. While coupling of the secondary amines with carboxylic acids was more difficult than a standard peptide bond formation, adequate results were obtained.[14] When the desired β-peptoid was complete, the final Fmoc group was removed and the β-peptoid capped with acetic anhydride to give the N-terminal acetamide. After cleavage from the resin by treatment with TFA (which also removed the Boc from the lysine linker), the Cbz-side-chain protection, if present, was removed by hydrogenation to give the final β-peptoids. Purification was achieved by prep HPLC, and all compounds were identified by mass spectral analysis. The purified β-peptoids were assayed for antimicrobial activity.[15]

The development of the block synthesis approach enabled synthesis of longer β-peptoids for this study. Although the conditions developed for coupling the blocks to form the tertiary amides which were generally useful, when the side chain of the N-terminal group to be coupled contained the dimethylaminopropyl group (**6d**, **6e**), the reaction failed to yield any recoverable oligomers. A strategy in which blocks terminating in an uncharged group were instead constructed and used to prepare the oligomers. We were unable to prepare blocks with two dimethylaminopropyl containing β-peptoid residues, presumably for the same reason. When blocks containing two protected primary amine bearing side chains were used, we were able to prepare the blocks, but the synthesis failed for oligomers longer than hexamers for reasons we don't understand.

We chose to synthesize β-peptoids from 9 to 18 mers to demonstrate proof of principle for the synthesis method and to get initial results of the efficacy of this class of compounds as antimicrobials. Table 2 shows the MIC values obtained for the β-peptoids.

Maximal activity of 128 μg/mL was seen for the β-peptoids which is about an order of magnitude lower than that of natural antimicrobials (For comparison, Magainin and the idealized Leu-Lys helical peptides have MIC



Scheme 4. Method for condensation of the β-peptoid blocks into oligo β-peptoids. Reagents and conditions: (a) HATU (1.6 equiv), DIEA (1.3 equiv), NMP, 20 h; (b) 20% piperidine/DMF, 1 h; (c) Ac_2O/TEA/DMF 1:2:1, 1 h; (d) 50% TFA/DCM, 1 h.

Table 2. Composition of the β-peptoid test compounds and their mic values against *E. coli*

Compound	Peptoid block (**6**)	Number of repeat (*n*)	MIC (μg/mL)
15a	**6a**	3	128
15b	**6a**	5	128
15c	**6a**	6	128
15d	**6b**	3	512
15e	**6b**	4	128
15f	**6b**	5	128
15g	**6c**	5	>512
15h	**6c**	7	>512
15i	**6c**	8	>512
15j	**6f**	3	>512
15k	**6f**	4	256
15l	**6f**	5	256
15m	**6g**	5	256
15n	**6g**	7	128
15o	**6g**	8	256
15p	**6h**	2	>512
15q	**6i**	1	>512
15r	**6i**	2	>512
15s	**6i**	3	>512
15t	**6j**	2	>512
15u	**6j**	3	512

of 16 μg/mL in this assay). This initial library demonstrates that β-peptoids can be prepared with antimicrobial activity. More compounds are needed to draw conclusions as to the minimal length necessary for activity, the ideal hydrophobic/cationic content, and preferred side-chain composition.

A method for preparing oligo-β-peptoids was developed by using a block approach. The method is far more efficient than synthesis by sequential sub-monomer approach which failed in our hands after about the pentamer stage. The method was successfully used to prepare oligomers up to 18 mers which were tested in an antimicrobial assay. This route opens the way for a more detailed look at this class of peptide-mimetic oligomers. More work is needed to fully elucidate the importance of length, hydrophobic content, and side-chain composition on the SAR. In addition, elucidation of secondary structure for these materials compared to that of peptides, β-peptides and α-peptoids is now possible.

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14. Rink-Fmoc-Lys(BOC)-OH resin (0.042 mmol) was treated with 3.0 mL of 25% piperidine/THF for 1.0 h. The resin was then drained, washed, and dried under N_2 pressure. Added **6a–j** (0.126 mmol) as a 0.5 mg/μL stock in NMP and DIEA (0.168 mmol) to the resin. Prepared HATU (0.210 mmol) in 1.0 mL NMP, added to the resin, and agitated at 25 °C for 20 h under N_2.
15. The minimal inhibitory concentration (MIC) was determined in sterile microtiter plates in a final volume of 200 μL using trypticase soy broth (TSB) as the growth medium. Serial 2-fold dilutions of the β-peptoid stock solutions were made in the plate wells such that concentrations ranged from 1024 to 2 μg/mL in a volume of 100 μL. Each well was then inoculated with 100 μL of a dilute solution of bacteria (*E. coli* ATCC 25922) in TSB yielding a final concentration of 1×10^{-4} bacteria/mL. The final β-peptoid concentrations ranged from 512 to 1 μg/mL. The assay plates were incubated at 37 °C for 24 h in a bioscreen C microtiter plate reader. The optical density of the medium at 600 nm was recorded every 20 min to monitor cell growth. The lowest concentration of β-peptoid preventing bacterial growth at 24 h was defined as the MIC.