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Combinatorial Discovery Process Yields Antimicrobial Peptoids

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Abstract—*N*-alkylated glycine trimers are generically referred to as peptoids. The identification of antimicrobial peptoids from a statistically unbiased diverse combinatorial chemistry library led to the design of the optimization peptoid library that we describe in this manuscript. This optimization library was designed using structural information from the most active peptoids in the unbiased library. Screening of the optimization library for antimicrobial activity identified a single pool of peptoids with activity against both *Staphylococcus aureus* and *Escherichia coli*. The active peptoids from this pool were active against drug sensitive and drug resistant organisms and represent novel antibacterial compounds. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Clearly, there is a worldwide need for new antimicrobial agents.[1] In the last 10 years there has been a dramatic escalation in drug resistance among microorganisms.[2] This resistance issue, already problematic in the hospital setting, threatens to spread to the community.[3] The urgency of this problem has resulted in many companies adding antimicrobial screens to their drug discovery repertoires.

We have screened a number of combinatorial libraries in a microbial growth inhibition assay to evaluate pools for antimicrobial activity. The peptoid pools in our library are statistically unbiased and diverse[4] with 324 compounds per pool, yet in the antimicrobial assay we discovered growth inhibitory activity in many of the pools. These unbiased libraries were made with redundant sets of substituents (substituents: the free amines that are brought together to make peptoids via the submonomer synthesis technology). We suspected that the high frequency of activity from these libraries was indicative of a small set of substituents that were active regardless of their position in the trimer peptoid.

On this basis, a small non-redundant library (the substituent pool of free amines was unique for each position) was synthesized. The free amines for each position were chosen based on the structures of the most active peptoids of the unbiased peptoid libraries. This type of combinatorial-chemistry optimization library allows two things to be accomplished: (1) a variety of different pools are synthesized, and (2) within each pool a variety of related analogues are available. This paper describes the screening of the resulting optimization library which led to the identification of many anti-*Staphylococcus* peptoid pools and a small set of peptoids with an extended host range. Further development of these peptoids could lead to a new class of antibacterial drugs.

Results and Discussion

Deconvolutions from the unbiased peptoid libraries referred to above showed a high frequency of the amine dehydroabietylamine (DHAA) as a component in many, (at positions R1 or R2 or R3) though not all, antimicrobial peptoids. To rapidly synthesize a variety of analogues with DHAA and with other amines frequently found in active peptoids, we made a biased library of 65 pools of 13 compounds each. This library was made as a 'one-mix' library as shown in Figure 1. The free amine selection was based on the frequency and position of the free amines observed in antimicrobial peptoids from previous unbiased library

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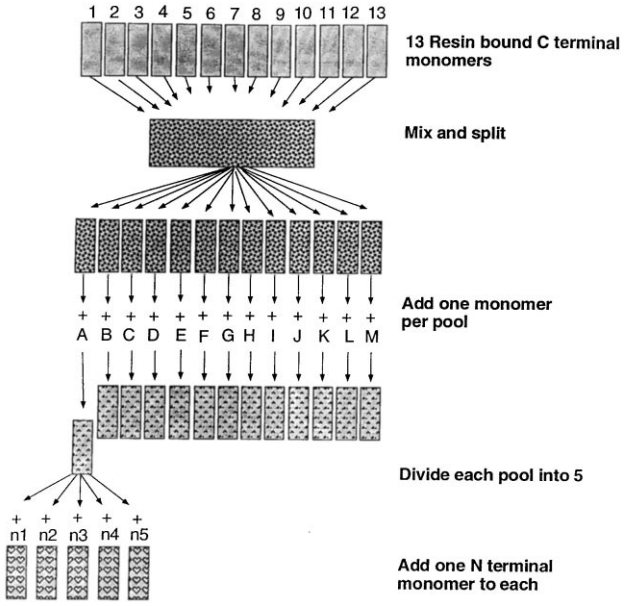


Figure 1. A one-mix library. A total of 13 amines were attached through the C-terminus to Rink type resin. The amine resin was then mixed and the population containing 13 different resins was split into 13 tubes. To each mixed resin containing tube a defined second position amine was added. Each of the 13 tubes were then split into five tubes so that five different defined N-terminal amines could be added to each pool. This protocol results in 65 pools of 13 compounds, in which the N-terminal and middle positions are defined.

screens. An attempt was made to maintain similar chemotypes without exact duplications for the middle and C-terminal amine sets. The optimization library free amine substituents are shown in Figure 2.

Peptoid pool synthesis, deconvolution, and purification

The synthesis of an equimolar peptoid mixture for a one-mix library uses the same synthesis protocol as described for individual peptoids[5] but requires two additional steps that involve the transfer of resin particles: (a) splitting and distributing resin slurries into a variable number of reaction vessels equally, and (b) transferring and recombining resin slurries from individual reaction vessels into the mixing chamber, as shown in Figure 1. Equimolar resin mixtures were achieved by using the resin-splitting method on an automated robotic synthesizer.[6] Pools were deconvoluted as previously described.[7] HPLC purification conditions for individually synthesized peptoids were as follows: C4 reverse-phase column (22×250 mm) 5–85% B linear gradient in which A: 0.1% TFA in water, and B: 0.1% TFA in acetonitrile, flow rate 10 mL/min, gradient length 80 min. Collected fractions were lyophilized, rinsed with acetic acid and relyophilized. The final products were resuspended in DMSO. Final peptoid stocks were 20 mM.

Growth inhibition assay

Pools were screened at 1 μM in the growth inhibition assay. This assay measures growth, as evaluated by

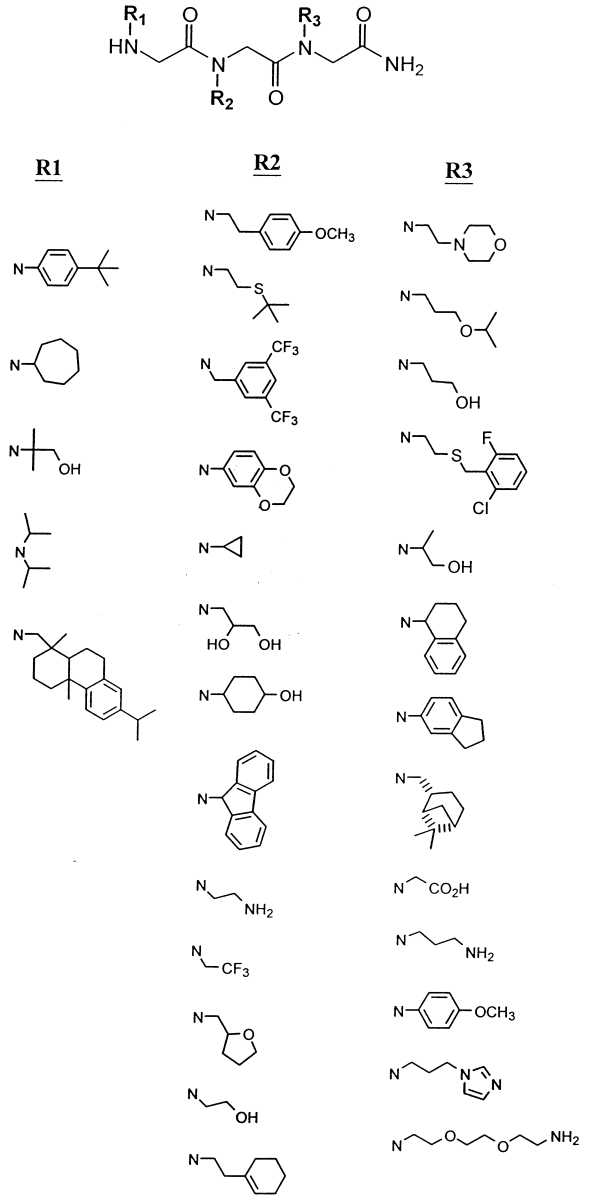


Figure 2. Amines used in the synthesis of the peptoid library. The top of the figure shows the peptoid scaffold. The 5 N-terminal amines used in the library are defined in column one. Dehydroabietylamine (DHAA) is the last amine in the R1 column. Column 2 illustrates position 2 amines. Position 3 defines the pool of C-terminal amines used on the starting resin for the synthesis of each pool. Note that the CHIR29017 pool that is mentioned in Discussion has the R2 substituent 2-(4-methoxyphenyl) ethyl amine that is shown as the top amine in the R2 list. Rather than define the Chiron ID for each of these 65 pools, the pool of interest and their deconvolution products are described by number in Figure 4.

OD_{600} absorption read by a molecular devices plate reader. The molecular devices plate reader was programmed to read the 96-well plate every 30 min and to generate a kinetic trace over a 24-h period. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were grown at 37° in the presence and absence of 1 μM peptoid pools. Assays were performed in Meuller Hinton cation adjusted (MHCA) broth at 37° in the presence of 1% DMSO to keep insoluble pools in solution. Comparative studies showed minimal interference

from the DMSO in the assay. The initial bacterial inoculum per well was between 10^4 and 10^5 colony forming units (cfu).[8] Plate tops were treated with a defogging agent (Fogpruf, Mine Safety Appliances Company) to prevent condensation interference of optical density readings. Minimum inhibitory concentrations (MIC) for individual peptoids were obtained by the microdilution method as described in the NCCLS guidelines.[8]

Figure 3 shows a bar graph representing the profile from *S. aureus* and *E. coli* growth inhibition assays of the optimization library pools. The first observation to be made is the regular pattern of inhibition of *S. aureus* growth in every fifth pool. This activity corresponds to the pools that have the N-terminal DHAA. This confirmed our earlier suspicion that this amine was superior to the others as a growth inhibitor. The growth inhibition results from the *E. coli* assay were more surprising since previous peptoids had never demonstrated appreciable activity against gram negative organisms such as *E. coli* (ATCC 25922) or *Psuedomonas aeruginosa* (ATCC 27853).

Intrigued by this expansion in the antibacterial spectrum of peptoid activity, we deconvoluted the pool that exhibited this activity. Figure 4B shows the structures of the deconvolution products for the extended spectrum pool. The antimicrobial activity against 19 drug sensitive or drug resistant strains is shown in Table 1. Only 11 of 13 peptoid single products are shown, as two syntheses (CHIR29493 and CHIR29497) failed in both pool and deconvolution synthesis attempts. Notably, not all peptoids with an N-terminal DHAA were active, (six of 11 show poor or no activity in the range tested) and the four most active also showed varying levels of gram negative activity (*E. coli, P. aeruginosa*). Additionally, those which were active were most active against the gram positive strains tested (*Staphylococcus, Streptococcus, Enterococcus*), whether the strains were resistant to other antibiotics or not.

As shown in Table 1, DHAA itself had significant gram positive antibacterial activity though its spectrum is limited to gram positive organisms (it is not active against *E. coli* even at 100 μm) and it is also less active on the gram positive *Enterococcus* than the peptoids. The optimization library indicated that at least for the N-terminal position, DHAA was superior to the other four N-terminal candidates.

The diaminoethane in position 2 of pool CHIR29057 appears to be responsible for extension of the peptoid spectrum to include some gram negative organisms. This is evident in that none of the other position 2 amines caused this expansion in host range. Since there were amines offered for the C-terminal position which included basic amines, we deconvoluted another pool in which the N-terminal position was also DHAA but position 2 was: 2-(4-methoxyphenyl) ethyl amine (CHIR29017; see Fig. 2 legend). We did this to determine whether, in the absence of a basic charge on the central amine, a C-terminal basic amine would have any

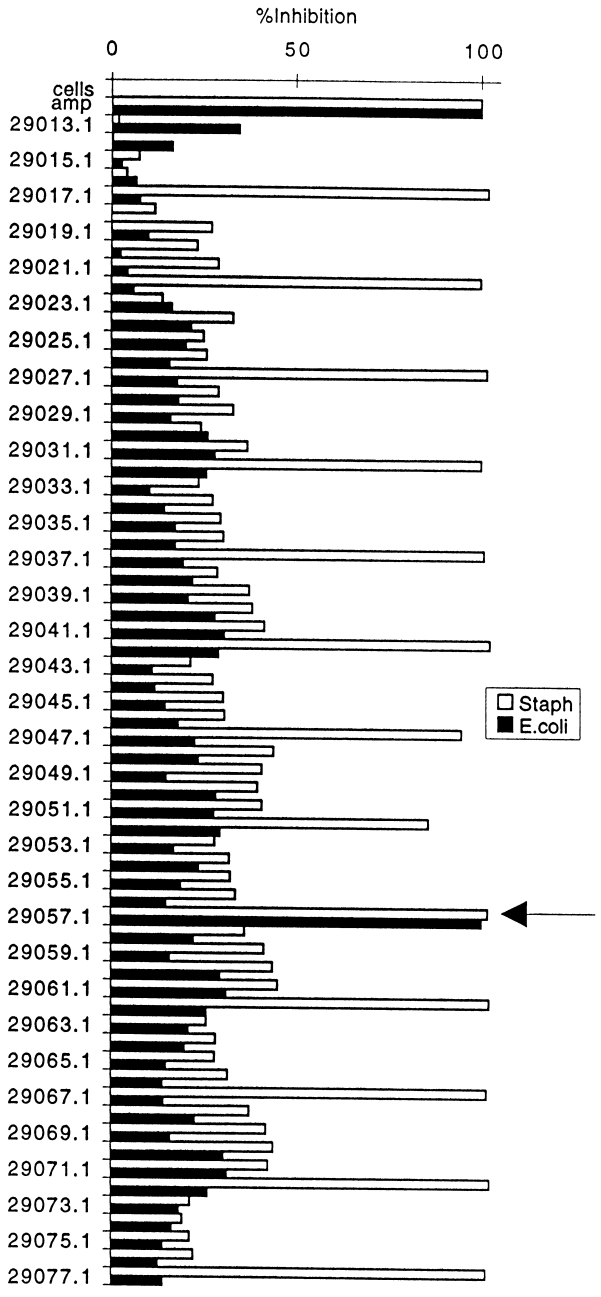
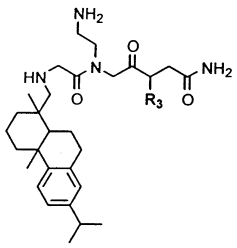


Figure 3. Screening results from the peptoid library. The bar graph represents percent inhibitory activity measured at 1 μM concentration of each pool in a growth inhibition assay. The open boxes represent the screening results from a *Staphylococcus aureus* growth inhibition assay and the dark boxes represent the screening results from an *Escherichia coli* growth inhibition assay. The data are represented as % inhibition as normalized against controls without drug or with 100% inhibitory levels of ampicillin. The data are represented above in total, though only every other Chiron ID number is shown below the corresponding pair of bars.

affect on host range. In fact, the only broad spectrum activity from this pool was observed for the three peptoids that had basic amines: 1-(3-amino propyl) imidazole, 2,2 (ethylenedioxy) bis (ethylamine) and diaminopropane (shown in position 3 amines list for CHIR29057 in both Figs 2 and 4B). These three compounds, derived from the pool CHIR29017, were two- to fourfold less potent against the gram negative strains

A.)



B.)

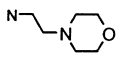
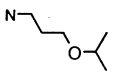
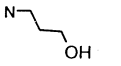
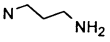
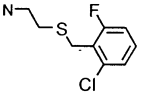
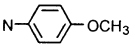
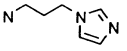
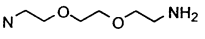
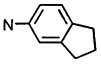
Chiron ID for Peptoid	R_3 Monomer	Chiron ID for Peptoid	R_3 Monomer
29493		29500	
29494		29501	
29495		29502	
29496		29503	
29497		29504	
29498		29505	
29499			

Figure 4. CHIR29057 deconvolution. The pool, CHIR29057, which was active against both *Escherichia coli* and *Staphylococcus aureus*, was deconvoluted to single compounds. Eleven of 13 syntheses were successful and the products HPLC purified. The N-terminal position and the middle amine for each compound are shown in part A of the figure. Part B of the figure shows the C-terminal position amines (depicted as R3 in part A of the figure) and the Chiron identification number that corresponds to peptoids in which the R group is defined by the amines in the illustration.

tested than the most active compounds from the CHIR29057 pool, and the other 10 sister compounds from the CH29017 deconvolution lacked broad spectrum activity (data not shown). Since only three of 13 from the second deconvolution had weak extended spectrum activity, this activity was not detected at the levels used in the antimicrobial assay of pools shown in Figure 3. Nonetheless the observation that the CHIR29017 compounds containing basic residues had some *E. coli* and *P. aeruginosa* inhibitory activity supports our hypothesis that a basic amine in the middle or C-terminal position expands the host range of these peptoids. It is notable however, that when both the middle position and the C-terminus were occupied by basic amines (such as the CHIR29057 deconvolution products with the basic C-terminal amines: CHIR29504: 1-(3-amino propyl) imidazole, CHIR29505: 2,2 (ethylenedioxy) bis (ethylamine) and CHIR29502: diaminopropane; see Fig. 4B), the compounds were inactive. We suspect that there may be tolerance for variation around the specific amines at each position, however a certain combination of hydrophobicity and basic residues must be retained for the peptoid to maintain its greatest antimicrobial activity. In fact, we have made antimicrobial peptoids of free amines that do not have independent antimicrobial activity and again we have seen that some ratio of charge to hydrophobicity seems to be important. Similar observations have been made for the antifungal family of alkylamines.[9] Molecules such as the alkylamines and the peptoids may use these structural features to access membrane localized targets.

We have demonstrated that although DHAA alone has antibacterial activity, this activity can be enhanced by attaching DHAA to a peptoid scaffold containing a primary amine and a hydrophobic amine. The enhancement of activity is seen as an expansion of host range rather than an increase in potency against all organisms. Both of these parameters, potency and host range, are important in the discovery and improvement

Table 1. Minimum inhibitory concentration (MIC) values for CHIR29057 deconvolution products[a]

Strain	MIC (μM) data													
	29494	29495	29496	29498	29499	29500	29501	29502	29503	29504	29505	DHAA	GM	Van
Staphylococcus aureus ATCC 29213	>40	>40	20	20	20	20	>40	>40	40	>40	>40	20	≤1	≤1
Staphylococcus aureus SA188 GM/CIP/IM	>40	>40	10–20	10–20	20	20	>40	>40	40	>40	>40	20	128	≤1
Staphylococcus aureus SA244MRSA βlac+	>40	>40	10	10	20	10	>40	>40	40	>40	>40	20	8	≤1
Staphylococcus epidermidis CNS25OX/CIP	40	>40	5–10	10	10	10	>40	20	20	40	40	20	8	2
Staphylococcus epidermidis CNS112 OX/CIP/GM	40	5	10	10	10	>40	>40	40	20	>40	>40	20	32	4
Staphylococcus haemolyticus CNS 66 OX/CIP	40	≪40	10	10	10	10	>40	20	20	>40	>40	40	64	2
Streptococcus pneumonia ATCC 49619	40	>40	10	10	10	10	>40	>40	20	>40	>40	20	2	≤1
Streptococcus pneumonia BT2 P M SXT	40	>40	10	10	10	10	>40	>40	20	>40	>40	40	8	≤1
Enterococcus faecalis ATCC 29212	40	>40	10	10	10	10	>40	40	20	>40	>40	40	32	2
Enterococcus faecalis ENT 141 Van B/CIP/GM	>40	>40	5–10	10	10	10	>40	>40	20	>40	>40	>40	>128	128
Enterococcus faecium ATCC 35667	40	>40	5	10	10	10	>40	>40	20	>40	>40	>40	4	≤1
Enterococcus faecium ENTC 129 Van A	40	>40	20	5	10	10	>40	>40	20	>40	>40	40	8	>128
Escherichia coli ATCC 25922	>40	>40	10–20	20	20	20–40	>40	>40	40	>40	>40	>40	≤1	>128
Escherichia coli Misc 239TEM 10	>40	>40	10–20	10–20	20	20	>40	>40	40	>40	>40	>40	≤1	>128
Klebsiella pneumonia Thai K21 CIP/TEM/SHV	>40	>40	20–40	20	20	>40	>40	>40	>40	>40	>40	>40	16	>128
Enterobacter cloacae ENTB 122 CIP/IMI	>40	>40	>40	20	40	>40	>40	>40	40	>40	>40	>40	≤1	>128
Serratia marcescens SER69 CAZ/GM	>40	>40	20	20–40	40	>40	>40	>40	>40	>40	>40	>40	32	>128
Pseudomonas aeruginosa ATCC 27853	>40	>40	20	20	40	40	>40	>40	>40	>40	>40	>40	2	>128
Pseudomonas aeruginosa 197 IM/CIP/GM	>40	>40	20	20–40	>40	40	>40	>40	>40	>40	>40	>40	>128	>128

[a] Values are represented in μM. Molecular weight values for compounds from left to right are: 599, 557, 701, 692, 615, 635, 557, 556, 605, 607, 630, 285. Strains above the solid line are gram positive, below the line are negative. Drug resistant profiles are abbreviated next to each name. Separate assays show that DHAA does not inhibit gram negative strains on this panel even at 100 μM (data not shown). Drug and drug resistance abbreviations: GM, gentamicin; CIP, ciprofloxacin; IM, imipenem; MRSA, methicillin resistant *Staphylococcus aureus*; βlac, β-lactamase positive; TEM (*bla*TEM-1) and SHV (*bla*SHV-4); OX, oxicillin; SXT, sulfamethoxazole trimethoprim; Van A, high level resistance to vancomycin and teicoplanin; Van B, moderate level resistance to vancomycin; CAZ, ceftazidime.

of antimicrobial leads. In a separate manuscript we describe a more extensive characterization of CHIR29498 and analogues.[10]

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