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Pyrrolidine bis-cyclic guanidines with antimicrobial activity against drug-resistant Gram-positive pathogens identified from a mixture-based combinatorial library

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Abstract—The rapid rise in antibiotic-resistant Gram-positive bacterial infections prompted us to explore the development of novel strategies for synthesis of large chemical libraries amenable to high-throughput screening for antimicrobial activities. Here we report the solid-phase synthesis of a 738,192 member pyrrolidine bis-cyclic guanidine chemical library with 26 different amino acids at three positions of diversity and 42 carboxylic acids at the fourth position. This synthetic combinatorial library was developed for positional scanning and screened for bacteriostatic and bactericidal activities against the important human pathogen methicillin-resistant *Staphylococcus aureus* (MRSA). The eight compound mixtures exhibiting bactericidal activity (10 μg/mL) against MRSA were used to direct the synthesis of 36 individual compounds that were then screened for activity against MRSA, vancomycin-resistant *Enterococcus faecalis* (VRE), and two Gram-negative bacterial species. At least 20 individual compounds were bactericidal for MRSA at $\leqslant$2.5 μg/mL, with a subset of these compounds showing bactericidal activities ($\leqslant$10 μg/mL) against the other species tested. This approach demonstrates the capability to synthesize and screen a complex library to yield promising antimicrobials that address a critical need for novel infectious disease therapeutics.

The rising incidence of infections caused by antibiotic-resistant bacteria has become a major concern for clinicians and the public health system. In the past several years, these types of infections have increased in severity, and their treatment has become far more complex and costly.[1] Once restricted to nosocomial infections within the healthcare setting, antibiotic-resistant bacterial strains are now emerging in high prevalence among community-acquired infections.[2] Worrisome trends are particularly evident in the preeminent Gram-positive bacterial pathogen *Staphylococcus aureus*, which is increasingly unresponsive to first-line antibiotic therapies including β-lactams (e.g., methicillin and cephalexin). *S. aureus* is associated with a spectrum of disease ranging from skin boils to life-threatening toxic shock syndrome, and is the single leading cause of bacteremia, hospital-acquired infections, skin and soft tissue infections, and bone and joint infections. Prevalence of methicillin resistance among strains of *S. aureus* (MRSA) now ranges from 33% to 55% in U.S. hospitals.[3] Another troublesome trend is the emergence of vancomycin-resistant isolates of *Enterococcus* species (VRE), nosocomial pathogenic species with frequent multi-drug resistance to other agents such as ampicillin and aminoglycosides. Over 28% of *Enterococcus* spp. responsible for intensive care unit infections in the U.S. are now vancomycin-resistant.[4] Clearly, the expeditious discovery and development of novel classes of antibiotics is critically important in order to effectively combat these and other complex antibiotic-resistant pathogens.

A half-century of synthesizing analogs based on a handful of classical antibacterial scaffolds has resulted in the development and marketing of more than 100 antibiotics, however, few new synthetic templates have emerged to address the burgeoning problems of resistance. The recent introduction of readily accessible synthetic combinatorial libraries (SCLs) of compounds has generated a fundamental shift in the process of drug discovery.

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus*; PS-SCL, positional scanning synthetic combinatorial library; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; ATCC, American Type Culture Collection; THA, Todd–Hewitt agar; THB, Todd–Hewitt broth.

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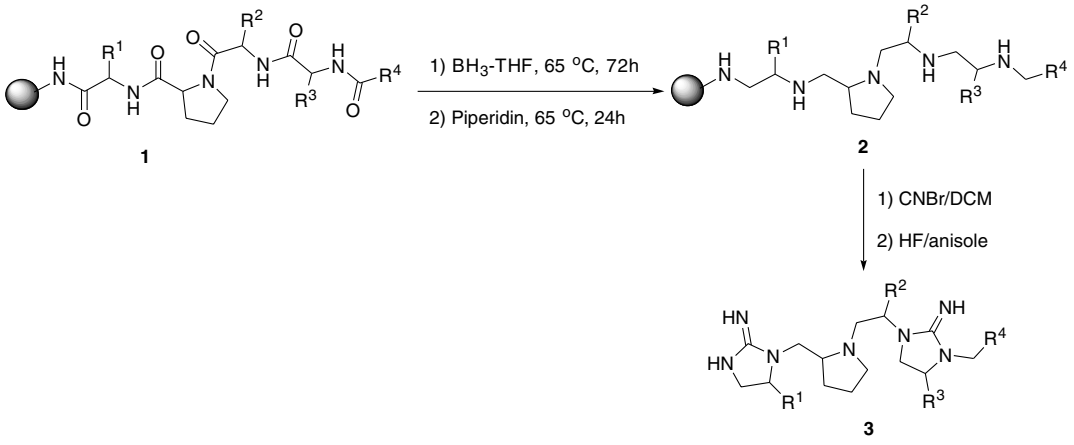
This revolutionary concept enables hundreds to thousands-fold more compounds to be synthesized and screened compared to traditional approaches,[5] and offers tremendous enhancement toward the identification of new therapeutic candidates. The preparation of structurally complex and diverse compounds results in a broader population of chemical space and facilitates effective probing of biological space.[5] Early work from our laboratory has shown the utility of mixture-based chemical libraries of small molecules for the de novo identification of highly active antimicrobial compounds, novel antitumor agents, and potent analgesics.[6–9] These libraries represent chemical collections of low molecular weight heterocyclic and acyclic compounds. The diversity of these chemical structures, as well as the large number of compounds making up each class of structures, greatly increases the probability of identifying compounds having useful chemical characteristics.

In the current work, we describe the solid-phase construction of a pyrrolidine bis-cyclic guanidine mixture-based library designed to analyze chemical diversity at four defined positions. All 738,192 members of this library were rapidly screened in a 96-well plate format. This approach facilitated the identification of 36 novel individual compounds which exhibited marked bactericidal activity against known human pathogens and which may represent a novel class of antimicrobial therapeutics.

Library synthesis. An efficient method for the solid-phase synthesis of pyrrolidine bis-cyclic guanidine library from resin-bound proline-containing acylated tetrapeptides was achieved (Scheme 1). Starting from resin-bound amino acids (diversity R_1), Boc-proline was coupled using standard SPPS coupling reagents,[10,11] followed by Boc deprotection and subsequent coupling of two Boc-amino acids (diversities R_2 and R_3). The N-terminal Boc was cleaved and the generated primary amine was N-acylated with different commercially available carboxylic acids (diversity R_4). The generated resin-bound N-acylated tetrapeptide was exhaustively reduced using borane–THF.[12,13] Our approach involved the use of proline as a spacer, which, following the exhaustive reduction of the amide groups, yielded a resin-bound pentaamine containing two pairs of secondary amines separated by a pyrrolidine ring. The resulting pairs of secondary amines were treated with cyanogen bromide to generate the corresponding resin-bound pyrrolidine bis-cyclic guanidines. Twenty-six different amino acids were selected for R_1, R_2, and R_3, and 42 carboxylic acids for R_4 to prepare a library of pyrrolidine bis-cyclic guanidine containing 738, 192 individual compounds in positional scanning format.[14] The intermediate resin-bound polyamine was also used as an intermediate for the generation of different pyrrolidine bis-heterocyclic compounds.

Positional scanning synthetic combinatorial libraries (PS-SCLs) are composed of one sublibrary for each variable position. In the case of single position defined PS-SCLs, each compound present in a given mixture has a common individual building block at a given position, while the remaining positions are composed of mixtures of all of the building blocks used to prepare the library; a common single building block thus defines each relevant mixture. The sublibraries for each position represent the same collection of individual compounds, and they differ only by the location of the defined position. The screening data permit the identification of key functionalities at each diversity position. It is important to note, however, that the activity found for a mixture is due to the presence of specific active compound(s) within the mixture, and not the individual functionalities as separate independent entities. The combination of all positional functional groups identified as key elements leads to the identification of individual active compound(s). As outlined in Figure 1, a PS-SCL, having four sites of diversity, consists of four separate sublibraries, each having a single defined position (R) and three mixtures position (X). In our case, the use of PS-SCL enables the most active groups at each position of the pyrrolidine bis-cyclic guanidine library to be determined directly from the initial screening data. The library was screened for bactericidal activity against MRSA. Screening the four sets of mixtures, totaling 120 mixtures (26 + 26 + 26 + 42) in the same assay, yielded information about the most important groups of each position in the pyrrolidine bis-cyclic guanidine and, following the parallel individual synthesis of all



Scheme 1.

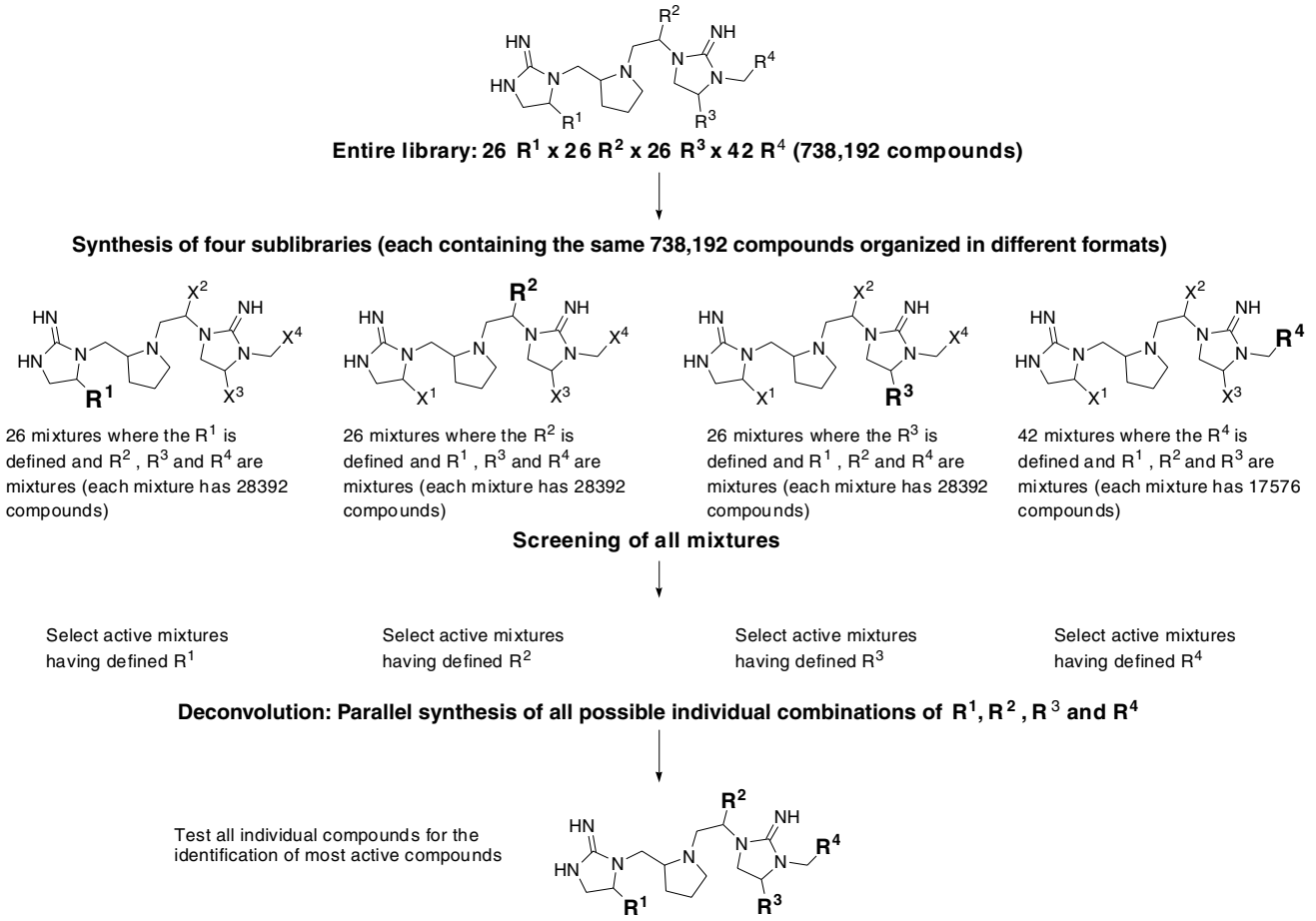


Figure 1. Synthesis of the entire pyrrolidine bis-cyclic guanidine library and details of the four sublibraries (the synthesis of the 738,192 member pyrrolidine bis-cyclic guanidine chemical library was done in four weeks).

possible combinations, led to the identification of the most active compounds (Fig. 1).

Guanidine compounds are known for their antimicrobial activity.[15] We screened the 120 mixtures of the pyrrolidine bis-cyclic guanidine library in a 96-well plate format for bactericidal activity against MRSA, scanning positions R^1, R^2, R^3, and R^4. The cutoff for significant bactericidal activity was arbitrarily set at <10 μg/mL to limit the number of hits to only the most active mixtures and also such that the compound activity was within one log of the activity of the positive control (vancomycin, with MBC of 0.613–1.25 μg/mL against the test strain of MRSA). We found that compound mixtures with a defined R^1 showed redundancy in activity, suggesting that the R^1 position contributes less to specific activity. Therefore, when individual compounds were synthesized, R^1 was fixed at either a phenylalanine or alanine side chain. From compound mixtures with fixed R^2 or R^3 groups, three groups each were identified as contributing significantly to activity (dark bars in Fig. 2), while two groups were identified from mixtures containing a fixed R^4 group (dark bars in Fig. 2).

Consequently, this initial screen against MRSA identified eight compound mixtures with an MBC < 10 μg/mL, suggesting that the identity of R^2, R^3, and R^4 contributes significantly to the anti-MRSA activity of the molecule. Based on the screening results, 36 individual compounds ($2 \times 3 \times 3 \times 2$) were synthesized and screened for bacteriostatic and bactericidal activity (Figs. 3 and 4).

Although our primary interest was in the identification of compound leads targeting MRSA, we also assessed antimicrobial activity of the individual compounds against VRE, *Escherichia coli*, and *Pseudomonas aeruginosa*. Twenty of the 36 compounds were highly active (MBC $\leqslant$ 2.5 μg/mL) against MRSA; the MBC of eight of these compounds was $\leqslant$2.5 μg/mL for VRE as well (Table 1). Three of these compounds appeared to be slightly more active against VRE than MRSA (Table 1). Moderate activity (MBC $\leqslant$ 10 μg/mL) of 10 compounds was also noted against *E. coli*; while 10 of these were active against MRSA, three were not active at $\leqslant$10 μg/mL against VRE. Figure 5 illustrates the exclusivity or overlapping MBCs of all 36 individual compounds against MRSA, VRE, and *E. coli*. Both the MIC and MBC of all 36 of these individual compounds were ges10μg/mL against *P. aeruginosa*; several of these compounds were inactive against *P. aeruginosa* up to 100 μg/mL.

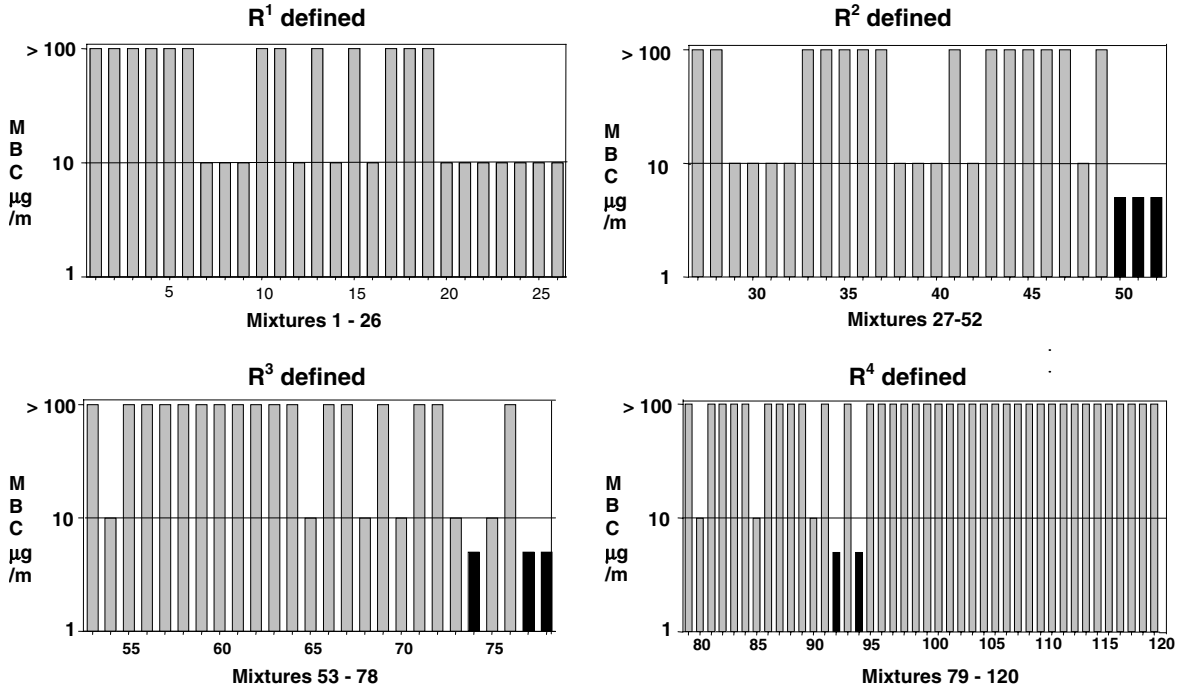


Figure 2. Results of screening the 738,312 member compound library for bactericidal activity against MRSA. Minimal bactericidal concentration (MBC) is expressed in μg/mL for each of the 120 compound mixtures. Mixtures that exhibited MBC < 10 μg/mL (dark bars) were used for determination of highly active individual compounds.

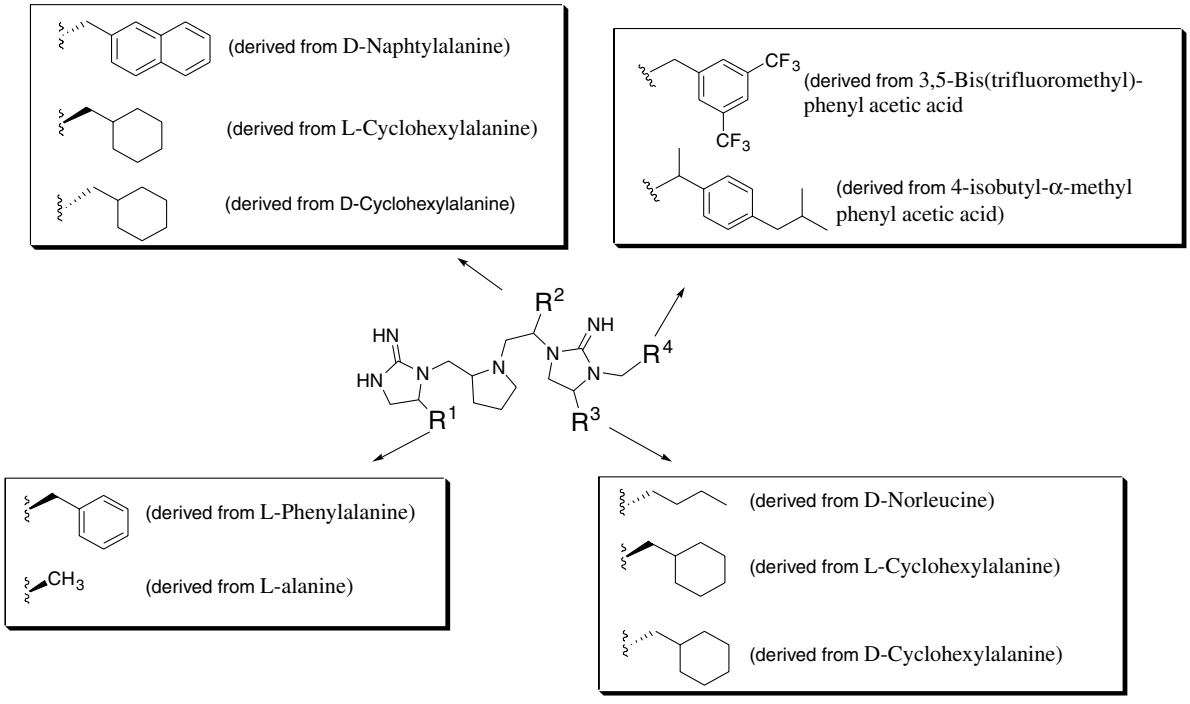


Figure 3. Identification of active mixtures at defined positions R^1–R^4.

An examination of each R group in the individual compound screen reveals preferences for some groups over others for bactericidal activity against MRSA. With R_1 defined, 12 of the 18 containing the L-phenylalanine derivative were highly active against MRSA. With R_2 defined there appeared to be no preferential activity attributable to any of the three groups examined in this screen. When R_3 was defined, eight of 12 compounds containing either the L- or D-cyclohexylalanine derivative showed more potent bactericidal activity against MRSA. When the R_4 group was defined, 12 of the 18 compounds containing the bis(trifluoromethyl)-phenyl acetic acid derivative exhibited bactericidal activity against MRSA.

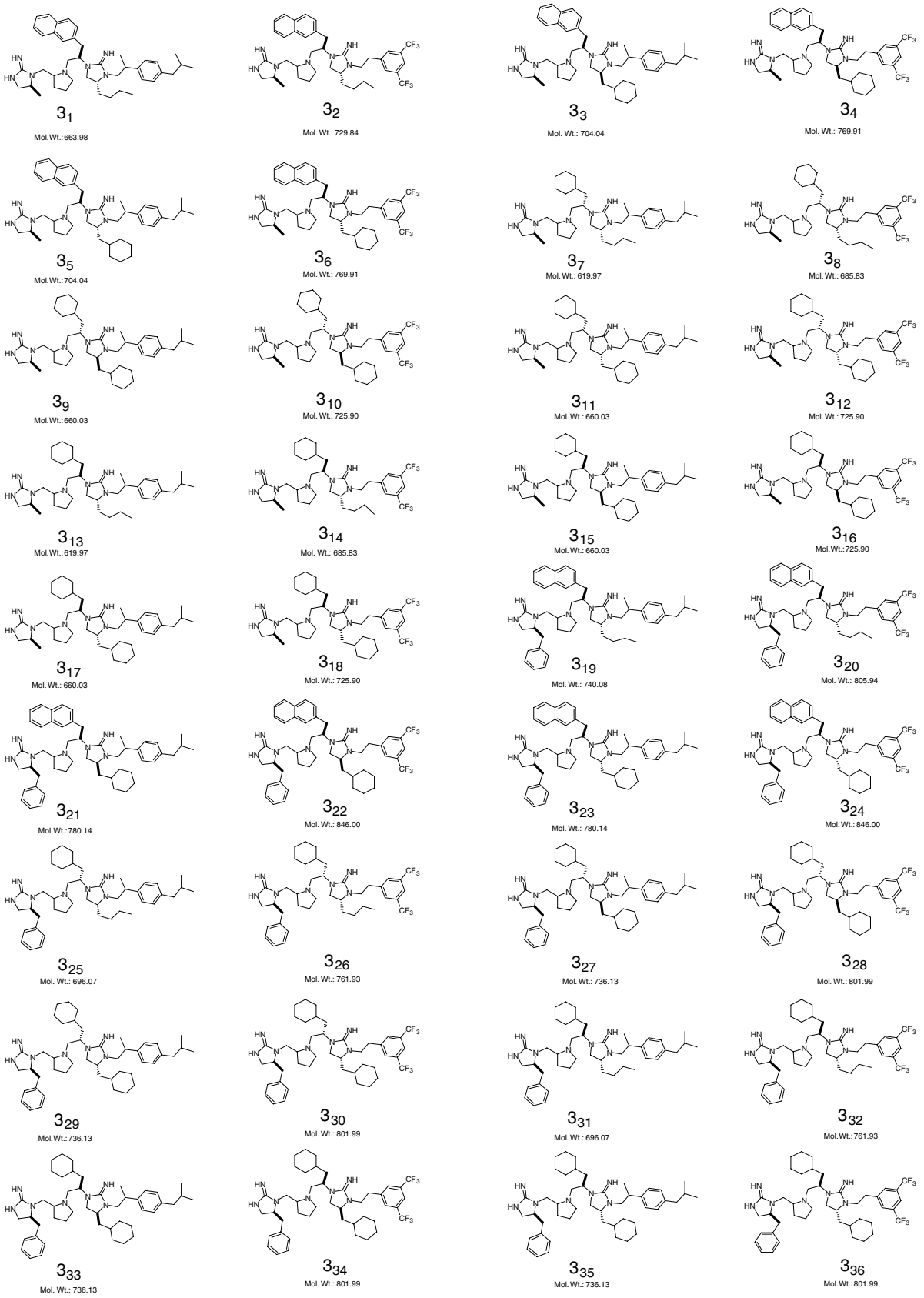


Figure 4. Structures of the most individual compounds identified in the initial library screen with highest bactericidal activity against MRSA.

We present the synthesis and the antibacterial screening results of a pyrrolidine bis-cyclic guanidine-based library consisting of 738,192 unique compounds. A PS-SCL approach utilizing four defined sites of diversity was employed in four separate sublibraries with a single defined position (R) and three mixtures position

Table 1. LMIC and MBC of individual compounds $\mathbf{3}_1$–$\mathbf{3}_{36}$ against MRSA, VRE, and *E. coli*. Values were calculated as described for the in vitro biological assay

Compound	MRSA		VRE		*E. coli*	
	MIC	MBC	MIC	MBC	MIC	MBC
$\mathbf{3}_1$	◑	◑	●	◑	◔	◔
$\mathbf{3}_2$	●	◑	●	○	○	○
$\mathbf{3}_3$	◑	◑	○	◑	○	○
$\mathbf{3}_4$	●	●	●	●	◔	◔
$\mathbf{3}_5$	●	●	●	●	○	○
$\mathbf{3}_6$	●	●	●	●	○	○
$\mathbf{3}_7$	◑	◑	●	○	○	○
$\mathbf{3}_8$	◑	◑	○	○	○	○
$\mathbf{3}_9$	●	◑	●	○	◔	◔
$\mathbf{3}_{10}$	●	●	●	○	○	○
$\mathbf{3}_{11}$	●	●	●	○	○	○
$\mathbf{3}_{12}$	●	●	●	○	○	○
$\mathbf{3}_{13}$	●	◑	●	○	○	○
$\mathbf{3}_{14}$	●	●	○	○	○	○
$\mathbf{3}_{15}$	●	●	●	○	◔	◔
$\mathbf{3}_{16}$	●	◑	●	◑	◔	◔
$\mathbf{3}_{17}$	●	◑	●	●	◔	◔
$\mathbf{3}_{18}$	●	◑	●	○	◔	◔
$\mathbf{3}_{19}$	●	◑	●	○	○	○
$\mathbf{3}_{20}$	●	●	●	○	○	○
$\mathbf{3}_{21}$	●	●	●	○	○	○
$\mathbf{3}_{22}$	●	●	●	●	○	○
$\mathbf{3}_{23}$	●	◑	●	○	○	○
$\mathbf{3}_{24}$	●	◑	●	○	○	○
$\mathbf{3}_{25}$	●	◑	●	●	○	○
$\mathbf{3}_{26}$	●	●	●	●	○	○
$\mathbf{3}_{27}$	●	●	●	●	○	○
$\mathbf{3}_{28}$	●	●	●	○	○	○
$\mathbf{3}_{29}$	●	●	●	●	○	○
$\mathbf{3}_{30}$	●	●	●	●	◔	◔
$\mathbf{3}_{31}$	●	●	●	●	○	○
$\mathbf{3}_{32}$	●	◑	●	●	◔	◔
$\mathbf{3}_{33}$	●	◑	●	○	○	○
$\mathbf{3}_{34}$	●	●	◑	○	○	○
$\mathbf{3}_{35}$	●	●	◑	○	◔	◔
$\mathbf{3}_{36}$	●	●	◑	◑	○	○

Key

● < 2.5 μg/mL

◑ 2.5 – 5 μg/mL

◔ 5 – 10 μg/mL

○ > 10 μg/mL

(X). Our results indicate that of the four positions examined, varying the R_1 position had a minimal effect on bactericidal activity against MRSA, whereas our scanning revealed multiple groups with high activity on the remaining three positions examined (MBC < 10 μg/mL). Using this information, synthesis of 36 individual compounds from this library was completed. Screening these compounds on two Gram-positive pathogens (MRSA and VRE) and two Gram-negative pathogens (*E. coli* and *P. aeruginosa*) revealed an array of compounds with specificity for MRSA, exhibiting MBC of $\leqslant$2.5 to $\leqslant$10 μg/mL, while other subsets of compounds were active against VRE or *E. coli*. Interestingly, neither the mixtures nor individual compounds exhibited any bacteriostatic or bactericidal activity against *P. aeruginosa*, a Gram-negative opportunistic pathogen. It is possible the mucoid alginate polysaccharide produced by this species[16] provides an effective defense against our compounds.

In summary, we have identified multiple compounds from a large complex library using a demonstrated synthetic and screening approach. The individual compounds identified from this library exhibit potent bactericidal activity primarily against MRSA, with subsets of these compounds also showing bactericidal activ-

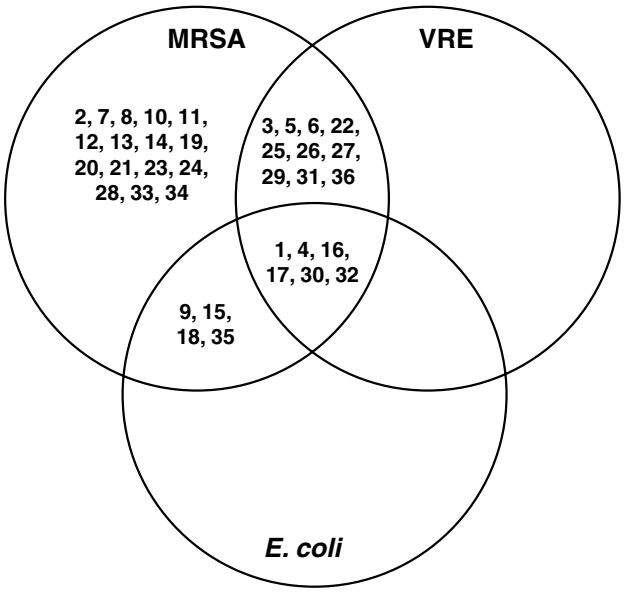


Figure 5. Venn diagram illustrating MBC selectivity (for MBC < 10 μg/mL) against MRSA, VRE, and *E. coli* of individual compounds $\mathbf{3}_1$–$\mathbf{3}_{36}$ identified in the TPI-1346 library.

ity against other known human pathogens. Of note, narrow-spectrum antibiotics targeting MRSA could possess an attractive therapeutic profile by minimizing alteration of normal bowel flora and the attendant side effects. This described approach using PS-SCL provides a rapid and powerful method to identify antibiotic candidates to potentially fill a critical niche in drug discovery and in the identification of novel therapeutics. Future studies of the bactericidal compounds identified from this library will focus on determining mechanism of action against MRSA and other susceptible pathogens.

In vitro biological assay. Todd–Hewitt broth (THB) and solid medium (THA) were freshly prepared per standard protocol. Microorganisms used in the screening include MRSA (ATCC # 33591), VRE (ATCC # 51299), *E. coli* (ATCC # 25922), and *P. aeruginosa* (ATCC # 27853). Compound/mixture dilutions were prepared in THB in sterile flat-bottomed 96-well polystyrene plates (Costar # 7593). Screening controls for every assay included vancomycin (Abbott Laboratories, Chicago, IL) at the same concentration as the compounds/mixtures, vehicle alone, and no bacteria. Screening was done by inoculating 200 μL THB containing the diluted compound mixtures with an overnight culture of the desired bacterial strain using a Boekel replicator. The inoculated plates were incubated at 37 °C overnight. The minimal inhibitory concentration (MIC, in μg/mL), the lowest concentration of the antimicrobial agent that prevents visible growth after overnight incubation, was determined turbidimetrically at A_{595}. The bacteria were then transferred via a Boekel replicator to Todd–Hewitt agar plates in the absence of antibiotic. The agar plates were incubated at 37 °C overnight, and the presence or absence of bacterial growth was assessed the following day. The minimal bactericidal concentration (MBC, in μg/mL), the lowest concentration of antimicrobial agent that completely kills bacteria (i.e., prevents bacterial growth after removal of the antimicrobial agent), was determined by visual examination of the agar plates. Each set of screening assays was repeated three times.

Acknowledgments

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