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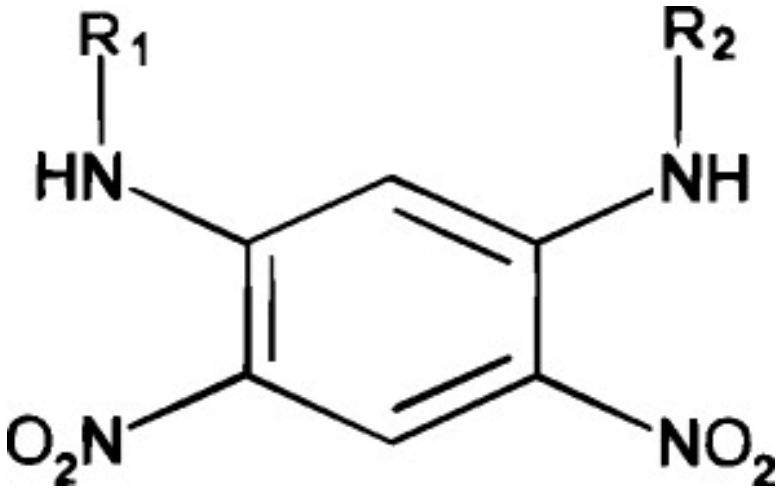

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Solution-Phase Synthesis of a 1,5-Dialkylamino-2,4-dinitrobenzene Library and the Identification of Novel Antibacterial Compounds from This Library

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In this report we demonstrate that a 1,5-dialkylamino-2,4-dinitrobenzene small molecule library can be generated by a highly efficient solution-phase synthesis method. From this 2485-member library, a series of novel compounds with antibacterial activity were isolated. The significance of this report is that the synthetic scheme is extremely simple, with minimal number of liquid handling steps, and the solvents and reagents left in the final library preparation are fully compatible with cell-based assays.

Combinatorial chemistry is a powerful tool for drug discovery and optimization of lead compounds.[1−5] While the "on-bead" binding or functional screening methods for the "one-bead one-compound" combinatorial library are highly efficient,[6,7] there are biological assays that need to be done in a solution-phase format. Most solution-phase chemical libraries are synthesized on solid support, after which the chemical linkers are cleaved and the library of compounds is released for solution-phase biological assays. The synthesis of such libraries requires thorough washing of the solid support during each coupling step to remove the reactants and side products and the addition of cleavage agents such as trifluoroacetic acid for releasing the products from the solid support. Unless a volatile cleavage agent, such as HF or NH_3 vapor, is used or a photolabile linker is involved, the cleavage agents must be removed prior to biological assays. It is certainly advantageous if one can generate a chemical library using a solution-phase synthesis approach by adding the appropriate building blocks sequentially and at the end of the synthesis no further manipulation of the resins or compound extraction are needed.[8−11] In this paper, we describe the design and solution-phase synthesis of a 1,5-dialkylamino-2,4-dinitrobenzene solution-phase library (Figure 1) and the identification of a series of novel compounds with antibacterial activities from this small molecule library. 1,5-Difluoro-2,4-dinitrobenzene, the scaffolding for this solution-phase library, has been used as cross-linker for protein conjugation and cross-linking since 1984.[12] The chemical reaction is based on the reactivity of two fluoro groups at the *ortho* position of two aromatic nitro groups. Through nucleophilic substitutions of the two fluoro groups sequentially with two amines, a bifunctional 1,5-dialkylamino-2,4-dinitrobenzene small molecule library can be generated in solution phase. This generic random small molecule library is suitable for lead identification for a variety of different targets.

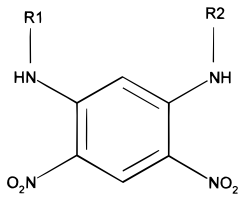

Figure 1. Chemical structure of the 1,5-dialkylamino-2,4-dinitrobenzene small molecule library.

Results

Library Design and Synthesis. The 1,5-dialkylamino-2,4-dinitrobenzene small molecule library was designed with six important criteria in mind: (1) efficient coupling of two "R" groups onto a rigid aromatic scaffold, (2) minimal amount of side products are expected to be produced, (3) coupling reactions occur in solution phase and not on solid phase so that resin washing and linker cleavage steps are not needed, and only minimal number of liquid handling steps are required, (4) the solvents and reagents used and left in the final library preparation are compatible with cell-based assays, (5) the compounds in the library are spatially addressable, and (6) the synthetic process and assay steps can be easily scaled up and fully automated. We believe the 1,5-dialkylamino-2,4-dinitrobenzene library described in this report fulfills essentially all the above six criteria. Seventy commercially available primary amines (Figure 2) were chosen as building blocks for this library. Since 1,5-difluoro-2,4-dinitrobenzene is planar and symmetrical, the actual number of final products is less than the expected 70 × 70 or 4900 compounds. Instead the actual total number of compounds is 2485, with 2415 heterobifunctional and 70 homobifunctional compounds. The synthetic scheme for this small molecule library is shown in Figure 3. In reaction I,

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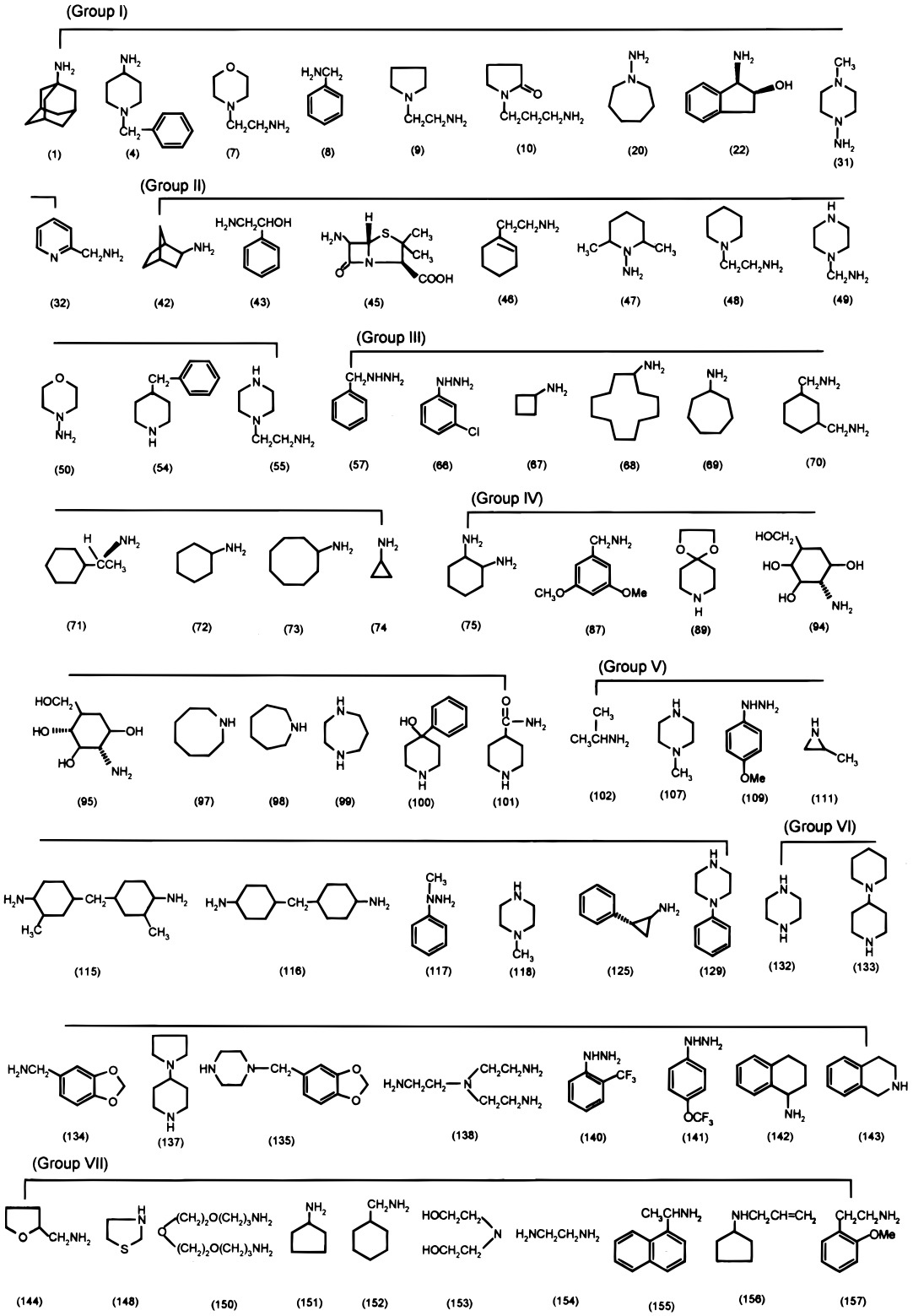


Figure 2. Amines that were used in library synthesis. The 70 building blocks were assigned to seven groups with 10 amines for each group.

70 amines were each added to 1,5-difluoro-2,4-dinitrobenzene in 70 separate wells to form 70 different 1-alkylamino-5-fluoro-2,4-dinitrobenzenes. After that, contents from wells #1−10 were pooled and redistributed into 70 different wells of a new plate. This step was repeated until all the products from reaction I were pooled (10 wells each) and redistributed. At this time, there were seven plates of pooled 1-alkylamino-5-fluoro-2,4-dinitrobenzenes. In reaction II, the same 70 amines were each added to the corresponding well of each of the seven plates from reaction I. At the end of the synthesis, there were a total of 490 wells, and each well contained 10 compounds at a concentration estimated to be 0.01 M for each compound (Figure 3B). As indicated above, because of the planarity and symmetry nature of the cross-

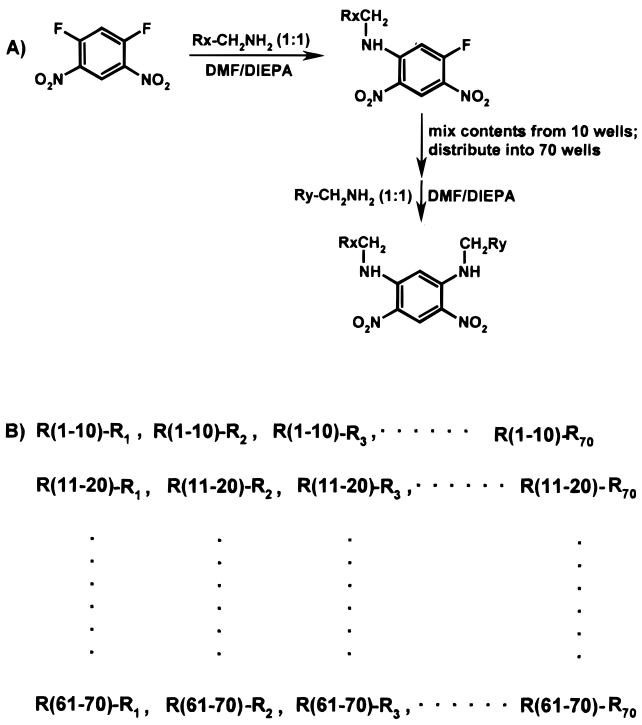


Figure 3. (A) Synthetic scheme for the 1,5-dialkylamino-2,4-dinitrobenzene small molecular library, and (B) the matrix indicates the content in each well of the final 490-well library.

linker, some of the final compounds are represented twice in two different wells.

Efficiency of Coupling. The nucleophilic substitution of the first fluoro group of 1,5-difluoro-2,4-dinitrobenzene with amine was highly efficient, with quantitative yield after mixing the two reactants at room temperature for 3 h. The HPLC profile of two of the representative products, 1-fluoro-5-*N*-benzylamino-2,4-dinitrobenzene and 1-fluoro-5-*N*-(4-methylpiperazine)amino-2,4-dinitrobenzene, without purification, are shown in Figure 4. Only one single major peak was seen for each of the reactions, and mass spectrometric analysis confirmed the identity of the intended products. Nucleophilic substitution of the second fluoro group, however, was slower and required overnight incubation for the reaction to complete. Figures 5 and 6 each demonstrates a single major peak with intended product confirmed by mass spectrometry. In another experiment, we pooled the 10 intermediate compounds after the first nucleophilic substitution with group I amines. To initiate the second nucleophilic substitution, we added 1,4′-dioxa-8-azaspiro(4,5)-decane to this mixture. The HPLC profile of the final products is shown in Figure 7, showing 10 distinct peaks and a few minor peaks. This mixture was then analyzed by LC-MS, and the result of the MS for each of peaks is shown in Table 1. All the 10 intended products were further characterized and confirmed by ^{1}H NMR after resynthesis in a large scale and purification by a semipreparative HPLC on the C_{18} column. The results of two representative NMR experiments are shown in Figure 8.

Identification of Antibacterial Agents from the 1,5-Dialkylamino-2,4-dinitrobenzene Library. The resulting 2485-member small molecule library was tested for antibacterial activities against *Staphylococcus aureus* (ATCC

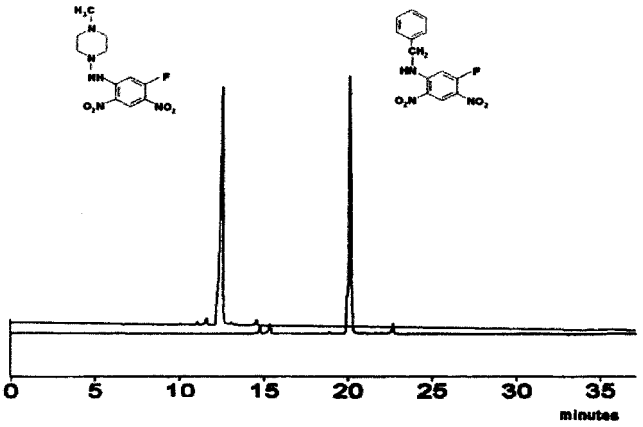


Figure 4. HPLC profile of products from two separate reactions. A single major peak for each of the reactions indicates that the reactions were quantitative. The intended products of the two reactions are 1-fluoro-5-*N*-benzylamino-2,4-dinitrobenzene and 1-fluoro-5-*N*-(4-methylpiperazine)amino-2,4-dinitrobenzene, respectively.

25923) or *Enterococcus fecalis* (ATCC 51299) at a final concentration of 10 μM for each compound. The bacteria were incubated with the test compounds at 35 °C for 18−24 h. Inhibition of bacterial growth was discovered in five wells that all contained 4,4′-methylenebis(2-methylcyclohexylamine) (amine #115). Four of these wells used amine #115 in reaction II (Figure 3) and the following pools of amines in reaction I: group I (amines #1, 7, 8, 9, 10, 20, 22, 31, and 32), group III (amines #57, 66, 67, 68, 69, 70, 71, 72, 73, and 74), group IV (amines #75, 87, 89, 94, 95, 97, 98, 99, 100, and 101), and group VI (amines #131, 133, 134, 135, 137, 138, 140, 141, 142, and 143). The remaining one positive well used amine #49 in reaction II and group V amines (amines #102, 107, 109, 111, 115, 116, 117, 118, 125, and 129) in reaction I. All the 50 possible individual compounds were resynthesized by solution phase as individual compounds. Ten of these compounds were tested to be positive against both bacteria. All these 10 compounds contained amine #115. Their chemical structures are shown in Figure 9.

Since all these 10 compounds have a free amino group at the 4,4′-methylenebis(2-methylcyclohexylamine) portion of the molecule, they can be readily synthesized on solid support (2-chloride trityl resin) with a synthetic scheme shown in Figure 10. Solid-phase synthetic approach was chosen as an alternative method for the synthesis of these final products. This will avoid the formation of cross-linking products by the bifunctional 4,4′-methylenebis(2-methylcyclohexyl)-amine. After the compounds were released from resin by 5% TFA/DCM, their purity was over 95% with a correct molecular weight as determined by electrospray mass spectrometry (ESI). These compounds were further tested for their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Table 2)[13,14] by using amines #8, 9, 32, 49, 70, 73, 75, 99, 132, 137, and 115 as controls (all of them were inactive under tested conditions). Some of these compounds not only killed the low-level vancomycin-resistant *E. faecalis* (ATCC 51299), but also killed the standard *S. aureus* (ATCC 25923) strain at a concentration between 5 and 20 μg/mL.

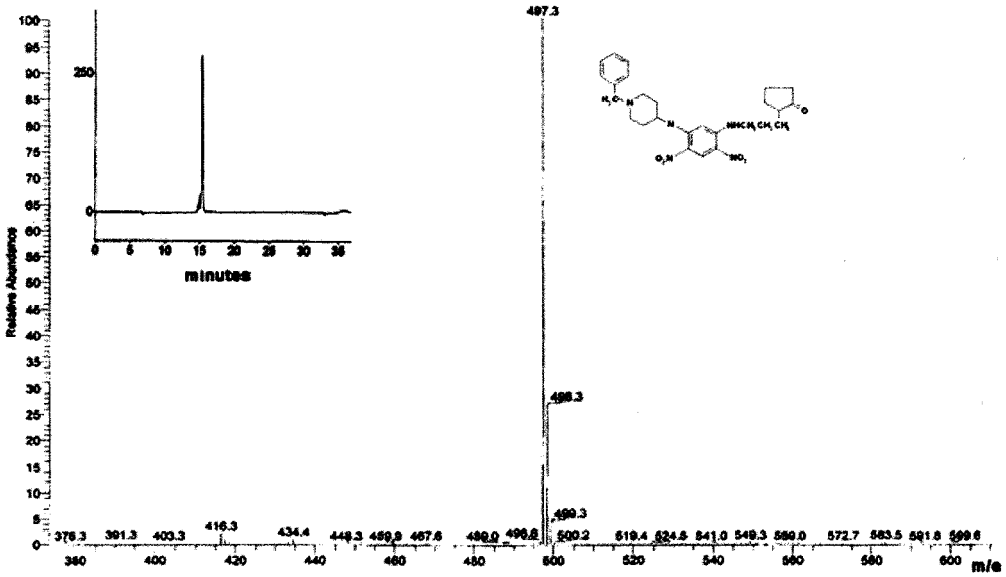


Figure 5. The 1-*N*-(1-benzylpiperidine-4-amino)-5-*N*′-[3-(2-pyrrolidinone)]propylamino-2,4-dinitrobenzene was synthesized by a solution-phase method that gives us high purity and correct molecular weight by HPLC (254 nm wavelength) and ESI spectrum. The reaction mixture was analyzed without prior purification.

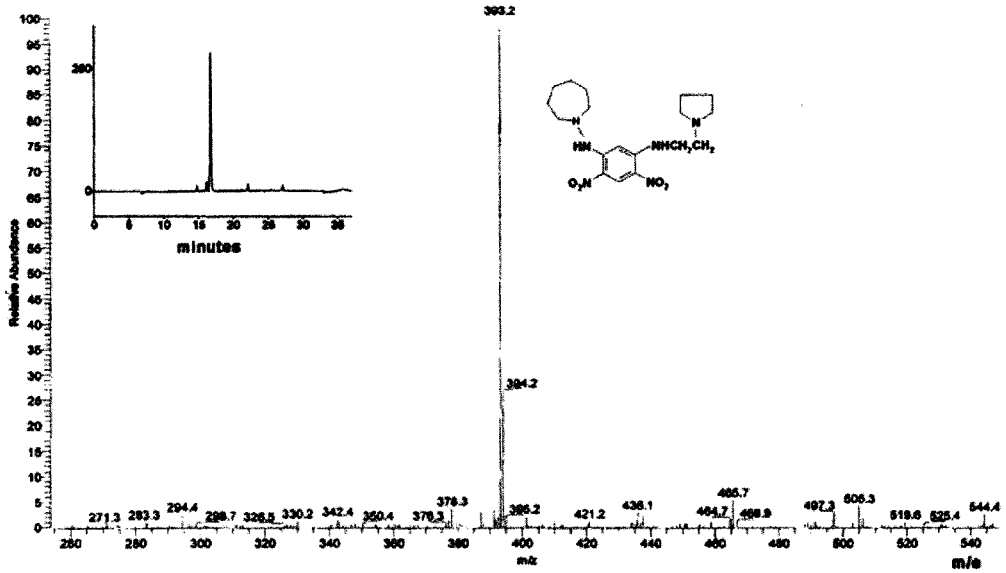


Figure 6. HPLC and ESI spectrum show that 1-*N*-(homopiperidine-1-amino)-5-*N*′-(2-pyrrolidine)ethylamino-2,4-dinitrobenzene synthesized by a solution-phase method was relatively pure and has the correct molecule weight. The reaction mixture was analyzed without prior purification.

Discussion

Gram positive bacteria have become increasingly resistant to antimicrobial agents.[15,16] In fact, multi-drug-resistant bacterial pathogens have now become a major problem in clinical medicine.[17] For example, *S. aureus* is a common human pathogen that has become increasingly difficult to treat because of resistance to antimicrobial agents.[15−17] Vancomycin now remains the main antimicrobial treatment for infections caused by *S. aureus* strains that are resistant to pencillinase-resistant antibiotics. The emergence of vancomycin-resistant *Enterococcus* species raises the threat of possible transfer of resistance factors to *S. aureus*.[18,19] Vancomycin-resistant *Stapylococcus* clinical isolates have already been discovered in Japan.[20] There is a definite need for the discovery of new antimicrobial agents.

In this report we demonstrate that a small molecule library can be generated by a highly efficient solution-phase synthesis method and that we can also isolate a series of novel compounds with antibacterial activity from this 2485-member library. The antibacterial activities of these compounds are only modest with MIC no less than 11 μg/mL for *S. aureus*. As a comparison, MIC of vancomycin for *S. aureus* is about 1 μg/mL. Since the compounds reported here are just lead compounds, there are rooms for further optimization. The significance of this report is that the synthetic scheme is extremely simple, with minimal number of liquid handling steps, and the solvents and reagents used and left in the final library preparation are fully compatible with cell-based assays. In contrast to solid-phase synthesis, no resin washing steps or linker cleavage steps are needed here. The sequential addition of the reagents is straightforward and can easily be automated, and the synthesis of the

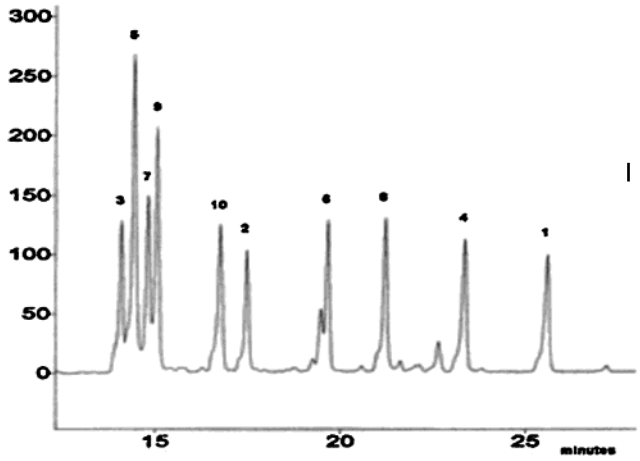
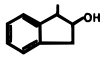
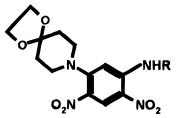


Figure 7. HPLC profile of a pool containing 10 compounds with correct molecular weights determined by LC-MS (the numbers over each peak correspond to the compound numbers in Table 1).

Table 1. Physical Data of a Typical Pool Containing 10 Compounds[#]

Comp No.*	R	MWt (Cal.)	MWt (Found)
1		458.5	458.5
2		497.6	497.5
3		437.5	437.5
4		414.4	414.5
5		421.5	421.5
6		449.5	449.5
7		421.5	421.5
8		456.5	456.5
9		422.5	422.5
10		415.4	415.5

[#] Basic structure: 

*Compound numbers (Comp. No.) correspond to those peaks in Figure 7.

library can be completed within 24 h. Furthermore, the library is ready for cell-based biological assay immediately after the completion of the synthesis and without further sample processing. The diversity of this library, however, is more limiting since we are restricting our synthesis to only one cross-linking reagent, 1,5-difluoro-2,4-dinitrobenzene, and "R" groups at two positions. In principle, other commercially

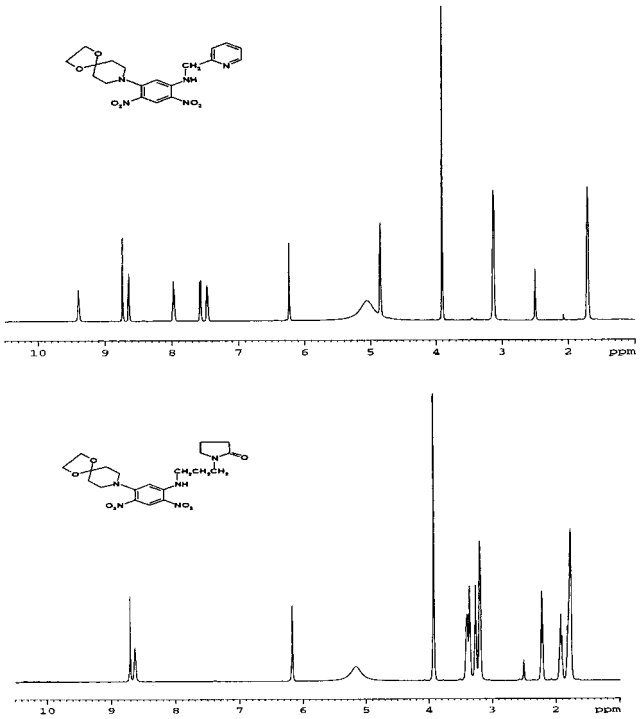


Figure 8. ^{1}H NMR spectra of compound **6** (top) and compound **10** (bottom) in DMSO at 500 MHz and 25 °C.

available homo- or heterobifunctional cross-linking reagents can also be used. To increase the diversity of the library, we envision that highly efficient cross-linkers with unique geometric configurations will be developed so that, in addition to randomizing the two "R" groups, we can also randomize the middle connector. Although we only used amines for the "R" groups in this report, in principle, thiol compounds as well as alcohols can also be used as building blocks. However, when alcohols are chosen, a base will be needed to facilitate the reaction, and the removal of the base prior to biological assay may be problematic and becomes the limiting factor. Work is currently underway in our laboratory to scale-up the synthesis of this small molecule solution-phase library by including more amines and thiol compounds and to screen these libraries for other biological targets.

Experimental Section

General Method. All of the amines and 1,5-difluoro-2,4-dinitrobenzene were purchased from Aldrich. HPLC grade *N*,*N*′-dimethylformide (DMF) was purchased from Burdick & Jackson. The HPLC was a Beckman System Gold with 125 solvent module pump, diode array detector 168, and autosampler 507. Electrospray ionization (ESI) mass spectrometer (Finnigan LCQ DECA) was used to determine the molecular mass.

Library Synthesis. A total of 5.0 g (24.5 mmol) of 1,5-difluoro-2,4-dinitrobenzene and 2.2 equiv of *N*,*N*-diisopropylethylamine (DIEPA) were dissolved in 35.0 mL of HPLC grade DMF and further distributed into 70 individual wells of a 96-deep well polypropylene plate (capacity of 1.2 mL/well). Seventy amines (Figure 2) in DMF were each (0.35 mL, 1.0 M) added into a well according to the amine code number and recorded. The plate was then sealed. After 3 h

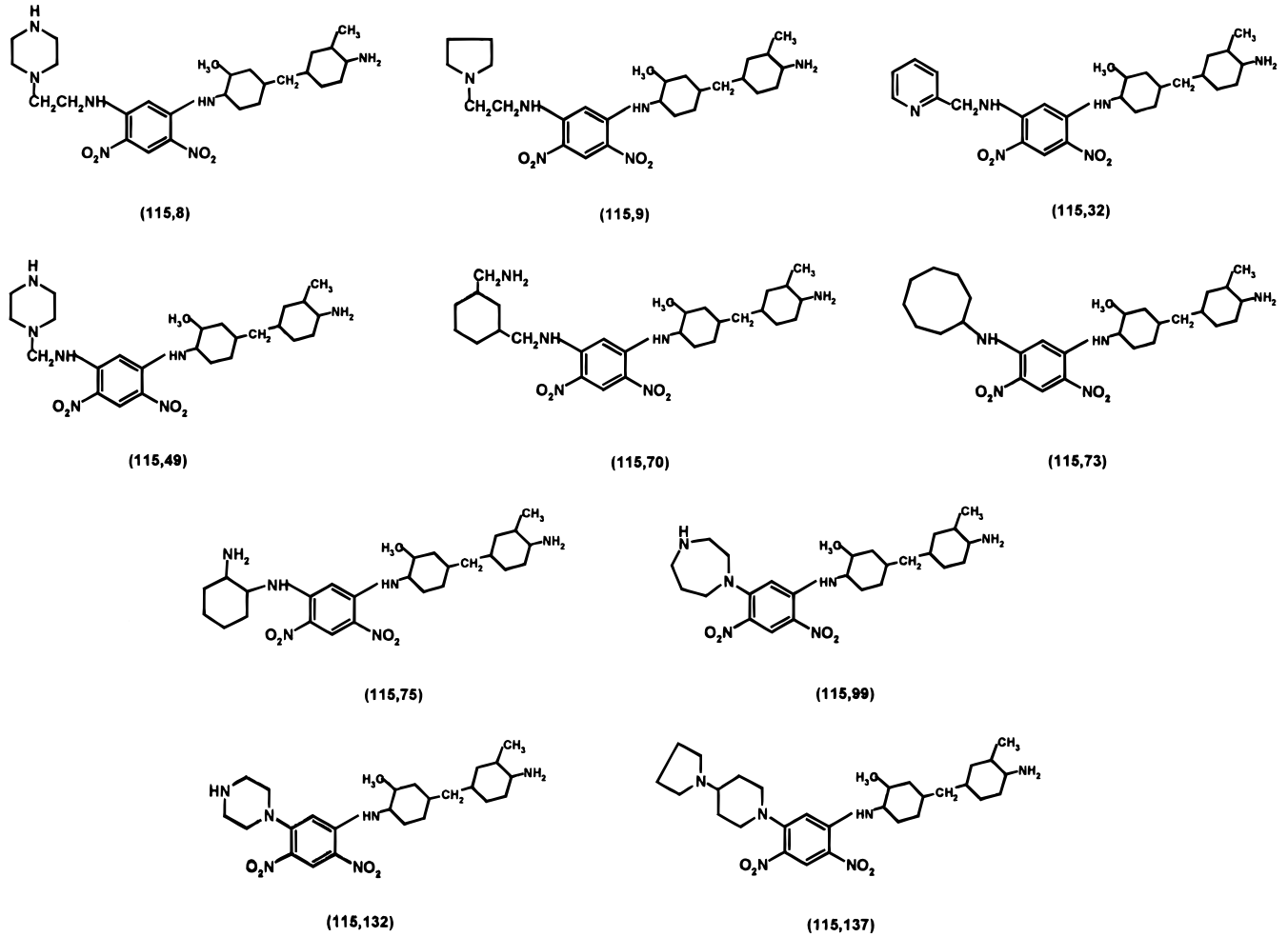


Figure 9. Identified compounds with antibacterial activities (see Table 2 for biological activities).

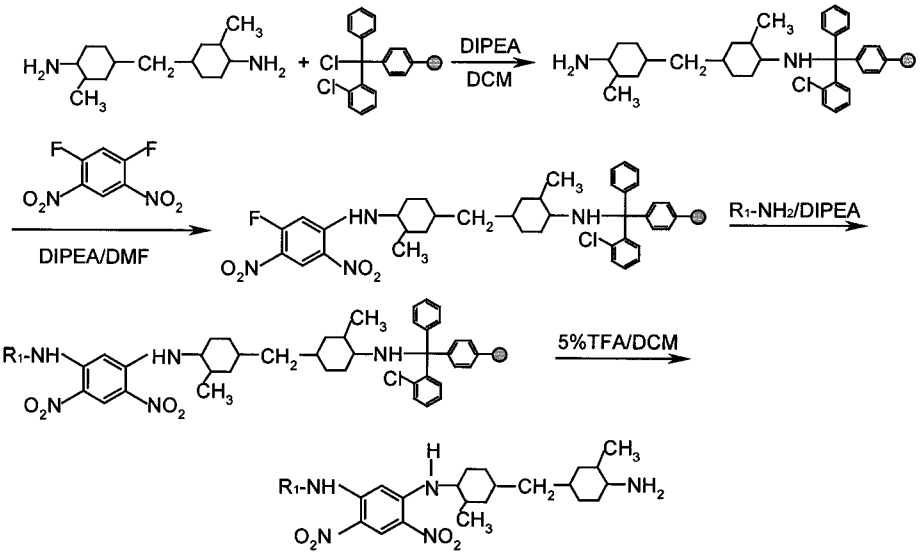


Figure 10. Solid-phase synthetic scheme for the antibacterial compounds.

of reaction at room temperature with strong agitation, the contents from each of the 10 wells were pooled (group I to VII, Figure 3). Each group was further diluted by DMF to 31.5 mL and then distributed at 0.45 mL/well into 70 individual wells of another 96-well plate. At this time there were a total of seven plates. The 70 amines (0.05 mL, 1.0 M) were each added to the corresponding wells of the seven plates. The plates were sealed, and the reaction proceeded overnight to completion with strong agitation. The final products were then stored in the same plates in the refrigerator at 4 °C for future use. The final concentration for each of the 10 compounds in each well was 0.01 M. The wells to which the amines were added were carefully tracked with the corresponding amine code numbers (Figure 2) so that

Table 2. Antibacterial Activities of 10 Novel Chemical Compounds Identified from the Library Screen[a]

	S. aureus (ATCC25923)		*E. faecalis* (ATCC51299)	
code	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)
115,8	85.0	170.0	42.5	85.0
115,9	31.45	>62.9	31.5	63.0
115,32	55.46	55.46	13.9	>27.8
115,49	51.18	102.4	25.6	51.2
115,70	34.5	69.0	15.9	>31.8
115,73	82.5	165.0	20.6	>41.2
115,75	11.09	22.18	5.5	11.0
115,99	18.36	36.72	9.2	18.4
115,132	26.25	>52.5	13.1	>27.8
115,137	97.8	97.8	24.5	49.0

[a] See Figure 9 for the chemical structure.

the content of the final products in each well was fully addressable.

Solution-Phase Synthesis of Individual Compound: A General Procedure. A total of 0.1 mmol of 1,5-difluoro-2,4-dinitrobenzene (20.4 mg) and 0.22 mmol of trimethylamine (30.6 μL) were dissolved in 2 mL of DMSO or DMF. Next, 0.1 mmol of 1,4-dioxa-8-azaspiro[4,5]-decane (12.8 μL) was added to start the first necleophilic substitution for 30 min at room temperature. Then, 14 μL of 1-(3-aminopropyl)-2-pyrrolidinone) (0.1 mmol) was subsequently added for an additional 24 h at room temperature. The final product was further diluted by HPLC grade acetonitrile to 5 mL. The crude compounds were analyzed on HPLC (Beckman) with a C_{18} reverse-phase column (Vydac). The purity was judged by three wavelengths (220, 254, and 280 nm). The following gradient was used: 0% to 100% buffer B over 25 min (buffer A: 0.1% TFA in water, buffer B: 0.1% TFA in acetonitrile). The purified sample was then collected from a semipreparative C_{18} (Vydac) column. The structure was confirmed by both mass spectrum and ^{1}H NMR spectrum (Bruker Avance DRX-500).

Solid-Phase Synthesis of Active Compounds. Dried 2-chloride trityl resin with substitution of 1.12 mmol/g was treated with 5-fold excess of 4,4′-methylenebis(2-methylcyclohexylamine) and 10-fold excess of DIEPA in dried DCM for 1 h at room temperature. The resin was then washed with a mixture of DCM/DIEPA/methanol (17/1/2 = v/v/v) to block the remaining active chloro group. The resin was washed five times with DMF. The resulting 4,4′-methylenebis(2-methylcyclohexylamine) resin was then treated with 2 equiv of 1,5-difluoro-2,4-dinitrobenzene (relative to the original resin substitution) and 4-fold excess of DIEPA in DMF for 1 h. After the resin was washed five times with DMF, three times with methanol, and five times again with DMF, a 5-fold excess of an amine in DMF and a 10-fold excess of DIEPA were added. The reaction vessel was rocked gently for 5 h at room temperature. The resin was then washed five times with DMF, three times with methanol, and five times with DCM and dried under vacuum for 1 h. The final products were released completely from the resin after treatment with 5% TFA/DCM for 1 h. DCM was then removed with a rotary evaporator under reduced pressure, and the crude compounds were analyzed on HPLC (Beckman) with a C_{18} reverse-phase column (Vydac) under the same gradient above. All the compounds showed over 95% purity with correct molecular weights as determined by electrospray ionization (ESI) mass spectrometry.

Antimicrobial Assay. Antibacterial growth assay was performed in 96-well round-bottom microtiter plates as previously described.[13] A low-level vancomycin-resistant *E. faecalis* (ATCC 51299) and a standard *S. aureus* (ATCC 25923) were used to screen this library. In a titration experiment, DMF (at 5% in Mueller Hinton broth (DIFCO)) did not affect bacterial growth. Test compounds were diluted to 1.0×10^{-3} M with DMF. To each of the 70 wells of the 96-well round-bottom microtiter plate, 180 μL of Mueller Hinton broth was first added followed by 10 μL of test chemical in DMF. Ten microliters of bacterial suspension was then added to a final concentration of 1.5×10^6 CFU/mL. The final concentration of each compound was estimated to be 5.0×10^{-5} M. The test plates were incubated at 35 °C for 18−24 h. The inhibitory concentration was determined by visual inspection of the microtiter plate for growth inhibition.

Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of the active individual compounds that were resynthesized by a solid-phase method (Figure 9) were determined as previously described.[13,14] These compounds were tested by a susceptibility assay as indicated above to determine MIC. Once the MIC was known, growth wells that demonstrated inhibition were subcultured to determine the percent reduction in colony forming units (CFU) compared to the initial inoculum. The concentration of test compound that achieved 99.9% reduction from the initial inoculum was determined to be the MBC.

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