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Brief Communication

Rapid Identification of Antibacterial Agents Effective against *Staphylococcus aureus* Using Small-Molecule Macroarrays

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SUMMARY

There is an urgent, global need for the development of new antibacterial agents. We have applied the small-molecule macroarray approach to the synthesis and screening of antibacterial compounds active against the Gram-positive pathogen *Staphylococcus aureus*. Several macroarrays of 1,3-diphenyl-2-propen-1-ones (chalcones), cyanopyridines, and pyrimidines were synthesized on a planar cellulose support system on the order of days. This support system was found to be highly compatible with antibacterial assay formats, including disk-diffusion and agar-overlay visualization methods. Further, sufficient compound was isolated from each spot of the macroarray for both compound characterization and minimum inhibitory concentration (MIC) estimation. Analysis of the small-molecule macroarrays in these assays uncovered a set of antibacterial agents with in vitro MIC values against methicillin-resistant *S. aureus* comparable to certain antibacterial drugs in use today.

INTRODUCTION

The continued emergence of bacterial strains resistant to antibacterial agents is a serious threat to human lives [1]. Each year, nearly two million patients in the United States acquire bacterial infections in hospitals, and 10% of these patients die as a result. Over 70% of these infections involve bacteria that are resistant to at least one antibacterial drug [2]. The Gram-positive pathogen *Staphylococcus aureus* is possibly most notorious as a cause of these infections due to the rapid appearance of multidrug-resistant strains, including strains impervious to methicillin and the last-line therapy vancomycin [3]. For example, only 1 year after U.S. Food and Drug Administration approval, *S. aureus* resistance emerged against oxazolidinones (e.g., linezolid), the newest class of synthetic antibacterials [1, 4]. Such a rapid growth of resistance underscores an urgent need for the continued development of new antibacterial agents. Here, we report the discovery of a suite of compounds that display potent antibacterial activities against methicillin-resistant *S. aureus*. In addition, we have identified, to our knowledge, a new antibacterial structure class, 2-methyl-3-cyanopyridines. These compounds were uncovered using the small-molecule macroarray approach and serve to demonstrate the utility of this technique for antibacterial research.

The continued need for new and structurally varied antibacterial agents strongly supports a combinatorial approach for their synthesis [1, 4], and this is evidenced by considerable research efforts in this area [5–7]. Our laboratory has been engaged in the development of the small-molecule macroarray as a tool for the rapid, parallel synthesis of libraries of organic molecules (ca. 50–200 compounds) [8]. This method involves the spatially addressed, solid-phase synthesis of discrete small molecules (mol wt $\leq$ 500 g/mol) on planar cellulose supports (spot size = 0.3 cm^2). Macroarrays provide several advantages relative to other combinatorial synthesis methods: the arrays are inexpensive to prepare, straightforward to manipulate, and yield sufficient compound per spot for numerous assays to be performed postsynthesis (100–200 nmol) [9–12]. We reasoned that these benefits could significantly streamline the antibacterial discovery process.

RESULTS AND DISCUSSION

Three complementary design criteria guided our synthesis of macroarrays for antibacterial screening. First, we sought to examine molecular scaffolds known to exhibit antibacterial activity against *S. aureus*, as macroarrays of such compounds would allow us to validate on- and off-support antibacterial array screening formats. Second, we also desired to study small-molecule classes that remain *unexplored* as antibacterials. Third, the synthetic routes needed to be compatible with our planar solid-support system [8]. We selected three compound classes that met these design criteria: 1,3-diphenyl-2-propen-1-ones (chalcones), pyrimidines, and 2-methyl-3-cyanopyridines. Certain lipophilic chalcones have been reported to exhibit antibacterial activities against *S. aureus* [13, 14], whereas

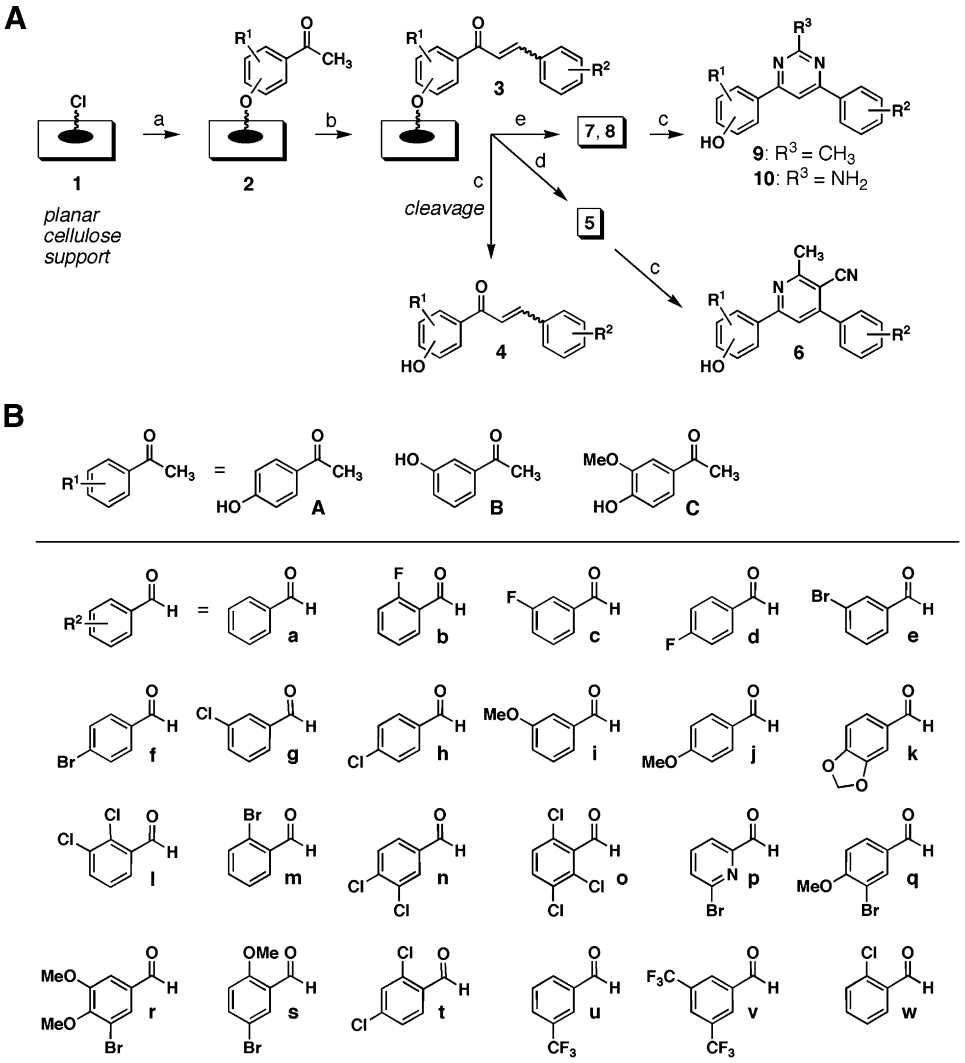

Figure 1. Small-Molecule Macroarray Construction

(A) Macroarray synthesis. Reaction conditions: (a) hydroxyacetophenones **A**–**C**, KO*t*Bu, DMF, 80°C, 10 min (3×); (b) benzaldehydes **a**–**w**, KOH, EtOH/H_2O (1:1), 80°C, 10 min (3×); (c) cleavage: TFA vapor, then neutralization with NH_3 vapor, room temperature, 2 hr; (d) 3-aminocrotononitrile, KOH, EtOH, room temperature, 26 hr; (e) acetamidine- or guanidine-HCl, KO*t*Bu, N,N-dimethyl acetamide, 80°C, 36 hr. All reactions were performed under air.

(B) Building blocks. Hydroxyacetophenones **A**–**C** (top) and benzaldehydes **a**–**w** (bottom) used in the construction of chalcone (**3**), 2-amino-3-cyanopyridine (**5**), and pyrimidine (**7**, **8**) macroarrays.

the latter two heterocycle classes have been largely unexplored as antibacterial agents [15, 16]. We built upon our previous macroarray synthesis methods and developed efficient synthetic routes to each structure class on planar supports (Figure 1A) [10, 11]. These routes were applied to the construction of 30- to 69-member macroarrays incorporating a broad range of functionality; the building blocks utilized in macroarray synthesis are shown in Figure 1B. Briefly, three hydroxyacetophenones (**A**–**C**) were coupled in a spatially addressed manner to planar cellulose derivatized with an acid-cleavable Wang linker (**1**) [8]. Claisen-Schmidt condensation of support **2** with various benzaldehydes (**a**–**w**) afforded chalcone macroarrays **3**. Further condensation of these arrays (**3**) with 3-aminocrotononitrile, acetamidine, or guanidine gave 2-methyl-3-cyanopyridine (**5**), methylpyrimidine (**7**), and aminopyrimidine (**8**) macroarrays, respectively [17].

Liquid chromatography-mass spectrometry analyses of a subset of the total compounds (70%) cleaved from the macroarrays indicated good to excellent purities (ca. 70%–98%). Certain chalcone products (**4**) were isolated as mixtures of *cis* and *trans* isomers, with the latter isomer predominating (ca. $\geq$ 4:1). As the double bond in certain chalcones are labile to photoisomerization [13, 14], we reasoned that separating these isomers prior to primary screening would be unproductive. For the small number of library members with lower purities, the major byproduct was starting material and therefore a known

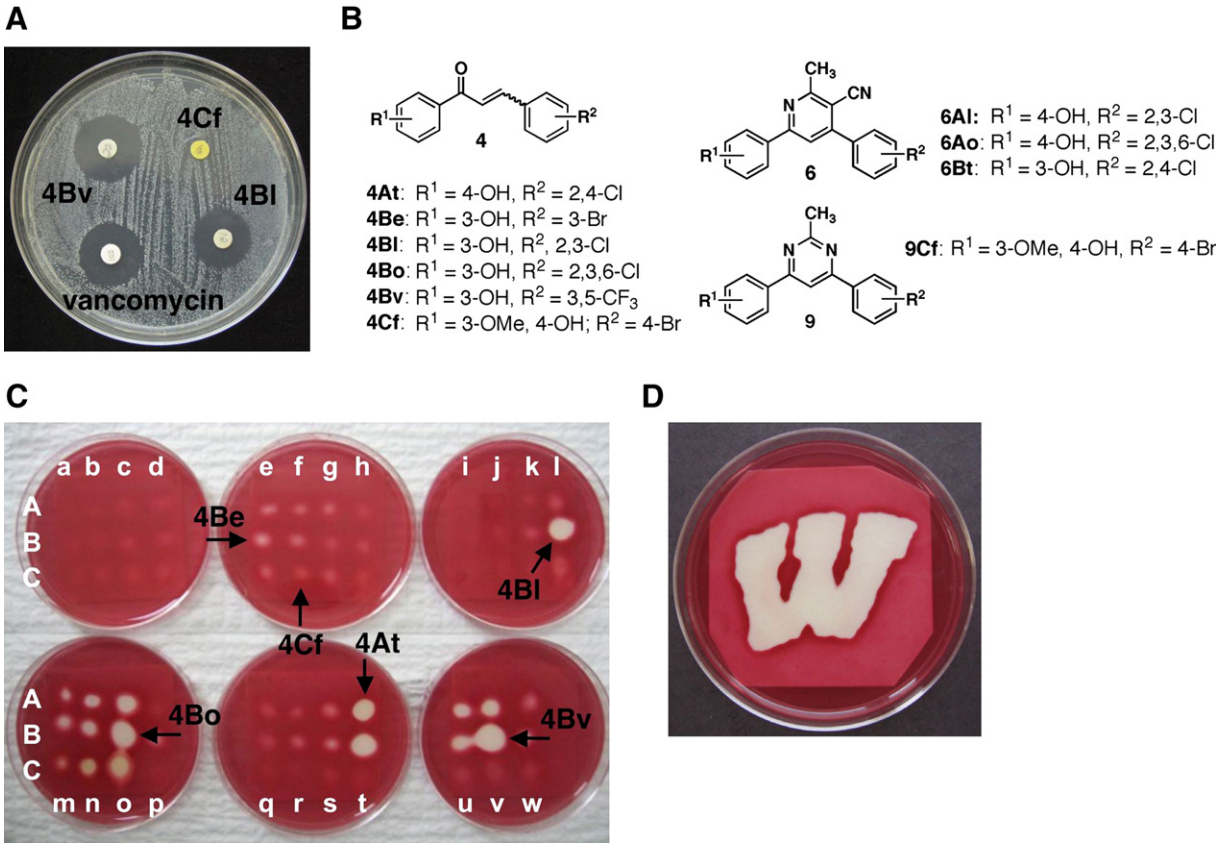

Figure 2. Representative Antibacterial Assays Performed on Macroarrays and Active Compounds Identified

The strain used was *S. aureus* ATCC 10390. Scale: Petri dish diameter = 9 cm.

(A) Disk-diffusion assay performed on compound spots punched out from a cleaved chalcone macroarray (**3**) and vancomycin standard (loadings = 30 μg/spot). Zones of inhibition (in mm): **4Bl** = 19; **4Bv** = 22; **4Cf** = <1; vancomycin = 20.

(B) Antibacterial compounds identified in assays conducted on macroarrays.

(C) Agar-overlay TTC assay of a cleaved 69-member chalcone macroarray (**3**) divided into six subarrays. The array building-block grid and compounds displaying a range of activities are indicated.

(D) Agar-overlay TTC assay of compound **6Ao** applied to planar cellulose in the shape of the federally registered University of Wisconsin, Madison "Motion W" logo.

compound. Consequently, we deemed the macroarray purity levels acceptable to proceed to antibacterial assays. The total time required for the synthesis of all four macroarray classes was less than 2 days, highlighting the efficiency of this synthetic approach for library construction [8].

We first evaluated test chalcone macroarrays (**3**) in antibacterial assays against *S. aureus*. Because the macroarrays were prepared on cellulose filter paper, we had convenient access to the compounds in disk form for standard disk-diffusion assays. Compound spots were punched out of the macroarrays, subjected to vapor-phase cleavage with acid (trifluoroacetic acid; TFA), and neutralized (NH_3) to afford disks containing ca. 30 μg of adsorbed compound [8]. These disks were manually placed on lawns of *S. aureus*, and zones of inhibition were measured after an 18 hr incubation period. Using this assay, we identified several chalcones (**4**) with antibacterial activities against *S. aureus*, including two compounds that were comparable on a per-microgram basis to vancomycin and had novel structures (**4Bv** and **4Bl**; Figures 2A and 2B) [13, 14]. However, this antibacterial assay format had drawbacks: (1) the entire sample of compound was consumed during the assay, and thus replicate arrays needed to be synthesized to confirm activity and purity, and (2) manipulating large numbers of compound disks was relatively labor intensive.

We found that an agar-overlay assay was more effective for the antibacterial screening of macroarrays relative to disk diffusion. This assay format also took advantage of the spatially addressed nature of the arrays (Figure 2C). Intact arrays were cleaved, overlaid with agar inoculated with *S. aureus*, and incubated for an 18 hr period; treatment thereafter with the redox indicator triphenyl tetrazolium chloride (TTC) allowed clear and reproducible visualization of areas of live (red) or dead (white) cells [18]. Antibacterial compounds generated an obvious white spot, with sizes reflecting their relative activities and solubilities. To reduce compound consumption in this assay, we developed a technique to transfer macroarray

Table 1. Antibacterial Activity Data of Selected Macroarray Compounds against *S. aureus*

Entry	Compound	Purity (%)[a,b]	*S. aureus* Estimated MIC Range (μM)[c,d]	*S. aureus* MIC (μM)[c,e]	MRSA MIC (μM)[e,f]
1	**4At**	94	25–50	21.2 ± 1.2	18.7 ± 1.2
2	**4Be**	88	25–50	21.0 ± 1.0	20.0 ± 1.0
3	**4Bl**	93	12.5–25	15.0 ± 0.5	15.0 ± 0.5
4	**4Bo**	91	<12.5	10.0 ± 1.0	10.0 ± 1.0
5	**4Bv**	88	<12.5	3.5 ± 0.5	3.0 ± 0.5
6	**4Cf**	86	>50	>250	>250
7	**6Al**	99	12.5–25	10.0 ± 0.5	10.0 ± 0.5
8	**6Ao**	99	<12.5	7.5 ± 1.2	7.5 ± 1.2
9	**6Bt**	99	<12.5	11.6 ± 0.9	13.4 ± 0.9
10	**9Cf**	86	25–50	32.5 ± 1.2	31.2 ± 1.2
11	Linezolid	–	–	10.0 ± 1.0	8.0 ± 1.0
12	Ciprofloxacin	–	–	0.6 ± 0.1	0.9 ± 0.1

[a] From HPLC analyses of crude macroarray compounds (UV detection at 254 nm).
[b] Certain chalcones **4** were mixtures of *cis* and *trans* isomers (on average 80% *trans*; see the Supplemental Data).
[c] *S. aureus* ATCC 10390.
[d] From serial dilution of spot stock solutions of crude macroarray compounds.
[e] From an authentic sample of the compound. Only *trans* isomers of chalcones **4** were evaluated. Error reflects step size in the serial dilutions.
[f] Methicillin-resistant *S. aureus* ATCC 33591 (MRSA).

members onto multiple sheets by simply sandwiching cleaved arrays between a solvent-saturated surface and dry cellulose sheets (to generate up to eight copies simultaneously). The copies were then subjected to replicate TTC assays or used for purity analysis.

Using this convenient transfer and overlay procedure, we identified antibacterial chalcones and cyanopyridines from test macroarrays **3** and **5** with a range of inhibitory activities (e.g., **4Be** and **4Bo**; Figure 2C). This straightforward assay also revealed preliminary structure-activity relationships, with chalcones (**4**) and cyanopyridines (**6**) bearing multiple halogens at the -R^2 position exhibiting the highest activities. Pyrimidines **9** and **10** displayed only low to moderate activities in this assay (see Figure S4 in the Supplemental Data available with this article online). In the course of these studies, we also found that the TTC assay is compatible with small-molecule macroarrays in formats other than spots, as exemplified in Figure 2D. We anticipate agar-overlay assays should find broad application in macroarray-based research due to their ease of use and versatility.

To acquire more quantitative antibacterial activity data about our compounds and verify our on-support assay results, we performed solution-phase absorbance assays that provided estimates of the minimum inhibitory concentration (MIC) of each macroarray member against *S. aureus*. Stock solutions (ca. 2 mM) were generated by cleaving and eluting 198 individual compounds from chalcone (**3**) and heterocyclic macroarrays (**5**, **7**, and **8**). The stock solutions provided sufficient compound for estimated MIC determination over a range of concentrations (50–12.5 μM) in quadruplicate. The good overall purities of the crude macroarray compounds allowed for reasonable estimations of MICs.

The preliminary MIC assays revealed two chalcones (**4Bo** and **4Bv**) and two cyanopyridines (**6Ao** and **6Bt**) with estimated MICs of less than 12.5 μM against *S. aureus* (Table 1). We resynthesized these active compounds in solution, along with several other compounds that exhibited varied levels of inhibition in these three assays for comparison, and determined their actual MIC values. (Note: all chalcones **4** were isolated and screened as the single *trans* isomer.) The absolute and relative MIC values compared favorably with our primary screening data (Table 1). Furthermore, this set of compounds exhibited analogous activities against a methicillin-resistant clinical strain of *S. aureus*. Notably, four compounds (**4Bo**, **4Bv**, **6Al**, and **6Ao**) were identified that displayed MIC values against methicillin-resistant *S. aureus* comparable to that of the current drug linezolid (entry 11), with chalcone **4Bv** closer in activity to ciprofloxacin (entry 12). Evaluation of these compounds in time-dependent bacterial killing assays indicated different modes of activity for the two structure classes, that is, chalcone **4Bv** was found to be bacteriostatic against *S. aureus* at its MIC, whereas cyanopyridine **6Ao** was bactericidal (see the Supplemental Data). Compounds with either mode of action are valuable as antibacterial strategies [4]. To our knowledge, chalcones **4Bo** and **4Bv** were previously unknown to have activity against *S. aureus*. Moreover, the discovery of **6Al**

Table 2. Antibacterial Activity Data of Lead Compounds against Selected Susceptible Bacterial Pathogens[a]

Entry	Compound	*S. epidermidis* MIC (μM)[b]	*B. subtilis* MIC (μM)[b]	*K. pneumoniae* MIC (μM)[b]
1	**4At**	27.5 ± 2.5	27.5 ± 2.5	>250
2	**4Be**	22.5 ± 2.5	22.5 ± 2.5	40 ± 10
3	**4Bl**	12.5 ± 2.5	22.5 ± 2.5	>250
4	**4Bo**	12.5 ± 2.5	8.8 ± 1.2	>250
5	**4Bv**	3.8 ± 1.2	8.8 ± 1.2	NA
6	**6Al**	12.5 ± 2.5	6.3 ± 1.2	NA
7	**6Ao**	6.3 ± 1.2	3.8 ± 1.2	NA
8	**6Bt**	17.5 ± 2.5	22.5 ± 2.5	NA
9	**9Cf**	>30	17.5 ± 2.5	NA

NA, not applicable.
[a] *S. epidermidis* ATCC 12228, *B. subtilis* subsp. *spizizenii* ATCC 6633, and *K. pneumoniae* ATCC 4352.
[b] From an authentic sample of the compound. Only *trans* isomers of chalcones **4** were evaluated. Error reflects step size in the serial dilutions.

and **6Ao** is significant, as 2-methyl-3-cyanopyridines represent, to our knowledge, a new antibacterial structure class.

To broaden the potential utility of these lead compounds as antibacterial agents, we examined their activities against a panel of bacterial strains. We selected the following six pathogens due to their clinical relevance: (1) Gram-negative: *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*, and (2) Gram-positive: *Staphylococcus epidermidis* and *Bacillus subtilis*. Previous reports have indicated that chalcones are most effective against Gram-positive bacteria [19], whereas the selectivities of 2-methyl-3-cyanopyridines and pyrimidines are for the most part unknown [15, 16]. To access rapidly the compounds' activities, we performed agar-overlay TTC assays against the panel of six strains. Briefly, we prepared arrays of our nine active compounds in Table 1 by spotting 30 nmol aliquots of purified compound onto individual filter paper sections, and subjected the arrays to TTC overlay assays against each strain as described above. These experiments revealed that the compounds were significantly more active against the Gram-positive strains relative to Gram-negative, with only *K. pneumoniae* being weakly susceptible to chalcones **4At**, **4Be**, **4Bl**, and **4Bo** (see Figure S5).

We determined MIC values for each active compound against *S. epidermidis*, *B. subtilis*, and *K. pneumoniae* (Table 2), and the MICs for the former two Gram-positive strains closely matched those determined for *S. aureus* (Table 1). Indeed, cyanopyridines **6Al** and **6Ao** and methylpyrimidine **9Cf** displayed increased activities against *B. subtilis* versus *S. aureus* (entries 6, 7, and 9, respectively; Table 2). In accordance with the agar-overlay assays, the MIC values against *K. pneumoniae* were considerably higher, however. Together, these data indicate that the lead compounds identified in this study represent both a potent and selective family of antibacterials.

In summary, we have demonstrated the utility of the small-molecule macroarray as a tool for the discovery of antibacterial agents. Three complementary assay protocols were developed that underscore the versatility of the macroarray platform for antibacterial research. Reasonably sized macroarrays were constructed *and* screened in these assays on the order of days. Through this work, we identified four compounds displaying in vitro MIC values against methicillin-resistant *S. aureus* that rival certain antibacterials in use today. Although these compounds are in an early stage of development, ongoing research is directed at determining their modes of action and preparing second-generation macroarrays to further optimize their potencies and pharmacological profiles.

SIGNIFICANCE

***Staphylococcus aureus* infections represent one of the largest health threats in hospital and community settings in the United States. The effectiveness of current antibacterial agents against *S. aureus* has become severely limited due to the rapid rise of bacterial resistance. As resistant strains will only continue to emerge, there is an urgent need for the development of new antibacterial compounds. Combinatorial synthesis approaches are poised to have an impact in this area. Toward this end, we have applied the small-molecule macroarray approach to the synthesis and screening of new antibacterial agents effective against *S. aureus*. Macroarrays of chalcones and heterocycles were constructed and subjected to a suite of antibacterial assays conducted either on or off the macroarray support. These studies revealed two chalcones and two 2-methyl-3-cyanopyridines with in vitro MIC values against *S. aureus* comparable to those of established antibacterial agents. Furthermore, these compounds displayed similar activities against a methicillin-resistant strain of *S. aureus* and**

selectivity for certain Gram-positive bacteria. These results are significant, as these lead compounds were identified through the synthesis and analysis of only 198 compounds in total. Further studies will be required to establish the efficacy of these compounds in vitro and in vivo. Overall, this work underscores the utility of the small-molecule macroarray as a tool for the identification of antibacterial agents.

EXPERIMENTAL PROCEDURES

Synthesis

Planar cellulose membranes were derivatized with a Wang-type linker as previously described (loading = ca. 1.5 μmol/cm^2) [11]. Macroarrays **3**, **5**, **7**, and **8** were synthesized according to modified procedures [10, 11]. Compounds displaying a range of antibacterial activities (Table 1) were resynthesized in solution using standard procedures and fully characterized (purities ≥ 98%); see the Supplemental Data for full synthetic details.

Bacteriological Assays

Bacteriological work was performed with strains obtained from the American Type Culture Collection (ATCC). Luria-Bertani (LB) medium was used, as directed, for all bacterial work and was solidified with agar as needed. Overnight cultures were grown at 37°C with shaking (*B. subtilis* was grown at 30°C).

Disk-Diffusion Assay

Compound spots were cleaved with TFA and neutralized with NH_3 as described in Supplemental Data. A 200 μl portion of diluted *S. aureus* 10390 (10^6 CFU/ml) was spread homogeneously across an agar plate. Compound spots were placed onto the agar, the plate was incubated at 37°C for 18 hr, and the diameters of the zones of inhibition were measured.

Agar-Overlay TTC Assay

Macroarray copies were generated using the array transfer protocol described in the Supplemental Data. Warm agar (15 ml) containing 10^6 CFU/ml bacteria was poured into a Petri dish (9 cm diameter). The dish was swirled to eliminate air bubbles, and a macroarray copy (6 × 6 cm) was fully submerged in the agar. Following an 18 hr incubation at 37°C, the plates were flooded with 0.1% (w/v) TTC in LB and allowed to develop for 1 hr to visualize the zones of inhibition. Red zones indicated healthy bacteria, while white zones indicated that a compound on the macroarray inhibited growth of the bacterial strain [18].

MIC Determination

For estimated MIC determination, dimethyl sulfoxide was added to the dried compound residue obtained from a single spot to afford ca. 100 μl of a 2 mM stock solution. Aliquots (5 μl) of these solutions were added to a 96-well plate, followed by 195 μl of diluted *S. aureus* 10390 (10^6 CFU/ml) to yield ca. 50 μM final concentrations. The plates were swirled for 1 hr to ensure compound dissolution, incubated for 12 hr at 37°C, and the absorbance at 595 nm was recorded using a plate reader [5]. Compounds that showed complete growth inhibition at ca. 50 μM (abs = 0.04) were subjected to further testing at lower concentrations (ca. 25 and 12.5 μM). Actual MIC values were determined for lead compounds resynthesized in solution using an analogous procedure with solutions of known concentration (see the Supplemental Data).

Supplemental Data

Supplemental data include eight figures and full details of all synthetic and biological work and can be found with this article online at http://www.chembiol.com/cgi/content/full/14/4/351/DC1/.

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