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RESEARCH ARTICLE

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Improved Small-Molecule Macroarray Platform for the Rapid Synthesis and Discovery of Antibacterial Chalcones

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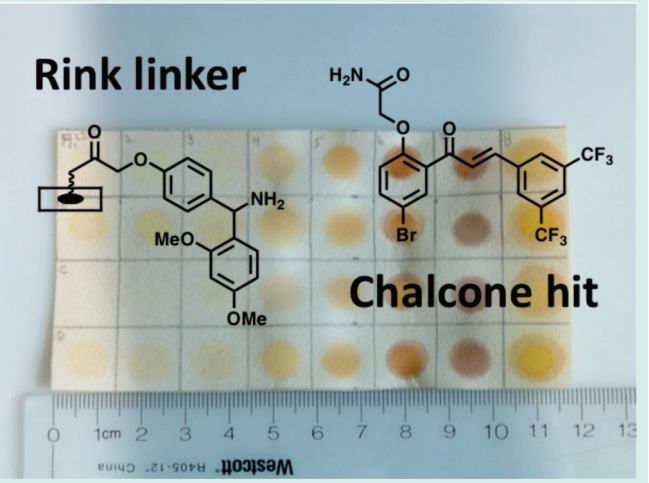
Supporting Information

ABSTRACT: Bacterial resistance to current antibiotics is a major global health threat. Consequently, there is an urgent need for the identification of new antibacterial agents. We are applying the small-molecule macroarray platform to rapidly synthesize and screen compounds for activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Herein, we report the synthesis of a 1,3-diphenyl-2-propen-1-one (chalcone) macroarray using a Rink-amide linker-derivatized cellulose support. The Rink linker allowed for the incorporation of a broader array of library building blocks relative to our previous syntheses because milder reaction conditions could be utilized; significantly higher compound loadings were also achieved (~80% vs ~15%). Analysis of the 174-member chalcone macroarray in off-support antibacterial screening assays revealed three chalcones with minimum inhibitory concentration (MIC) values against MRSA comparable to currently used antibacterial drugs and low hemolytic activities. These results serve to further showcase and extend the utility of the small molecule macroarray for antibacterial discovery.



KEYWORDS: cellulose, chalcone, MRSA, rink linker, small molecular macroarray, SPOT-synthesis

INTRODUCTION

The growing resistance of bacteria to antibiotics constitutes a global health emergency.[1,2] Methicillin-resistant *Staphylococcus aureus* (MRSA) has received considerable recent attention because of its dramatically increasing prevalence in both hospital and community settings.[3−8] There is an immense and growing need for the identification of new antibacterial agents active against MRSA.[9] One approach toward the discovery such compounds entails the synthesis and screening of structurally diverse combinatorial libraries of small molecules.[10,11]

Our laboratory has developed methods for the efficient parallel synthesis of chemical libraries using small-molecule macroarrays.[12−20] The small-molecule macroarray approach offers certain advantages over traditional solid-phase supports.[21] The macroarray support (cellulose filter paper) is inexpensive, mechanically robust, easy to manipulate, and compatible with a variety of on- and off-support biological screening assays.[17,19] These attributes make the small-molecule macroarray a versatile tool for the synthesis and screening of compound libraries for the discovery of agents that can modulate biological processes.

We recently reported the synthesis and antibacterial screening of several 1, 3-diphenyl-2-propen-1-one (chalcone), pyrimidine, and 2-methyl-3-cyanopyridine libraries using our small-molecule macroarray platform.[17] The antibacterial screening assays revealed four compounds, most notably two chalcones, with minimum inhibitory concentration (MIC) against MRSA in the low micromolar range (≤10 μM). While this study demonstrated the compatibility of the macroarray platform for new antibacterial discovery, we sought to improve certain aspects of the library synthesis route, expand the size of the possible chalcone libraries, and thereby, uncover additional compounds with antibacterial activity. For example, attachment of the initial hydroxyacetophenone building blocks to the Wang linker-derivatized cellulose support via nucleophilic substitution proceeded with low conversion rates in this previous work (~15%).[17] Additionally, certain commercially available hydroxyacetophenones were insoluble in the KO*t*Bu/DMF reagent solution required to couple the hydroxyacetophenones to the Wang-support, which limited the size and resulting structural diversity of the libraries.

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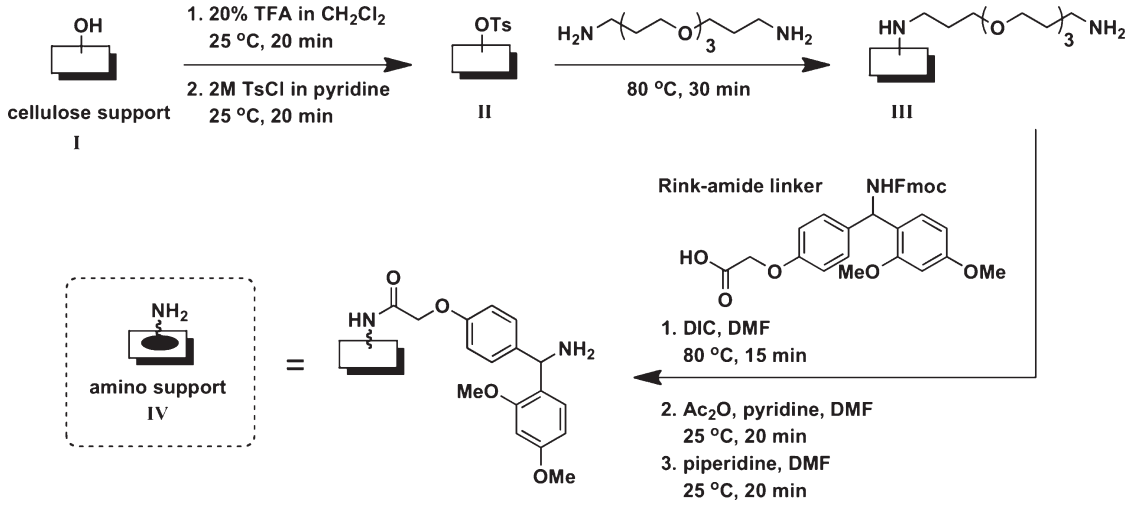
Scheme 1. Synthesis of Rink-Amide Linker Derivatized Cellulose Support IV



In an effort to improve the coupling efficiencies of the acetophenone building blocks and increase the number of compatible acetophenones available for chalcone and heterocycle macroarray construction, we explored the use of an alternative chemical linker for macroarray synthesis. We reasoned that the well-characterized Rink-amide linker[22,23] would allow efficient attachment of carboxylic acid-derivatized acetophenones to the cellulose support via an amide bond using mild coupling reagents, such as *N*,*N*′-diisopropylcarbodiimide (DIC). We therefore sought to develop a Rink-amide linker variant of our cellulose support platform and test this platform through the synthesis of a new chalcone macroarray. Herein, we report the results of these studies. We were able to generate a 174-member chalcone library in high purities using mild reaction conditions on the Rink-linker support system. Moreover, off-support antibacterial assays of the macroarray revealed three previously unreported chalcones with potent antibacterial activities against MRSA and low hemolytic activities.

RESULTS AND DISCUSSION

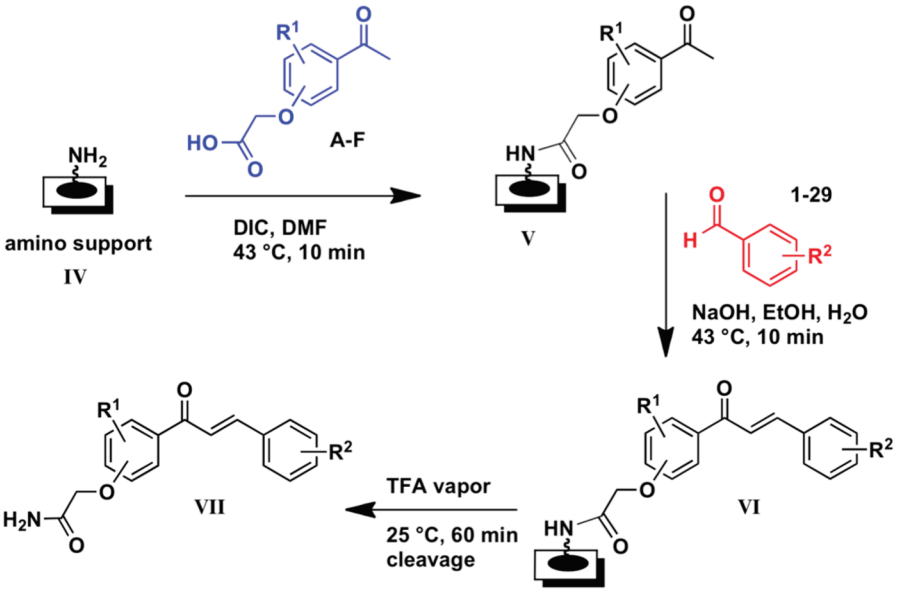
Amino Cellulose Functionalization. Derivatization of cellulose supports with the acid-cleavable Rink-amide linker has previously been described, primarily for the SPOT-synthesis of peptide libraries.[20,21,24] We used a modified version of these reported protocols in the current study that incorporated features from our past cellulose support syntheses (Scheme 1).[12−15,17] First, planar cellulose support **I** (Whatman 1CHR filter paper) was tosylated to generate support **II**, and then reacted with a flexible diamine spacer to yield amine-derivatized support **III**. The Fmoc-protected Rink-amide linker was spotted onto support **III** in a spatially addressed fashion using a micropipet (to minimize use of this relatively expensive reagent), followed by a blanket acetylation step to cap any unreacted spacer amines on the support. Fmoc deprotection of the linker-functionalized support using piperidine afforded the amino support **IV**. Using this method, we reproducibly obtained Rink linker loadings of ∼500 nmol/cm^2, as determined by standard UV Fmoc quantification at 296 nm (see Supporting Information).[25]

Chalcone Macroarray Construction. Synthesis of the chalcone macroarray first required covalent attachment of an acetophenone building block to the amino support **IV**. We reasoned that this coupling could be achieved by amide bond formation between the amino support **IV** and an acetophenone derivatized with a carboxylic acid "handle" using a standard carbodiimide (DIC) coupling reaction. This envisaged reaction is shown in Scheme 2. We chose to test six different acetyl phenoxyacetic acid building blocks (A−F) containing various substitution patterns for incorporation into the chalcone macroarray (Figure 1). These acetyl phenoxyacetic acids were readily synthesized in solution in good yields and purities by reaction of an appropriate hydroxyacetophenone with methyl bromoacetate, followed by basic ester hydrolysis (see Supporting Information).

The acetyl phenoxyacetic acids A−F were efficiently coupled to amino support **IV** in a spatially addressed manner using standard DIC coupling reactions at 43 °C (Scheme 2). Through a series of optimization experiments, we found that performing this reaction at 43 °C (on a sand bath) afforded the highest product purities relative to higher and lower temperatures. Loading efficiencies for acids A−F were calculated by cleaving a subset of the acids, analysis by HPLC, and comparison to calibration curves generated from authentic samples of the resulting primary amide acetophenone derivatives (see Supporting Information). Using this DIC coupling method, we routinely achieved acetyl phenoxyacetic acid loadings of ∼400 nmol/cm^2 (∼80% coupling efficiency to amino support **IV**). Notably, this is a significant improvement over our previous synthetic method of attaching acetophenone building blocks to a Wang linker-support using nucleophilic substitution chemistry, which gave coupling efficiencies of ∼15%.[17]

With the support-bound acetophenone macroarray **V** in hand, completion of the chalcone macroarray **VI** was accomplished by Claisen−Schmidt condensations with various benzaldehydes (**1**−**29**) (Scheme 2). We chose to incorporate an expanded set of structurally diverse benzaldehydes relative to our previous chalcone library in order to explore a broader range of functional group substitution patterns in the chalcone scaffold. We utilized our previously reported conditions for the Claisen−Schmidt reaction on planar supports.[17] These reactions yielded a 174-member

Scheme 2. Chalcone Macroarray Synthesis[a]



[a] DIC = *N*,*N*′-diisopropylcarbodiimide, DMF = *N*,*N*′-dimethylformamide, TFA = trifluoroacetic acid.

chalcone macroarray (**VI**) by combining the six acetyl-phenoxy-acetic acids and 29 variably substituted benzaldehydes (Figure 1).

Compound Cleavage and Analysis. We subjected the chalcone macroarray (**VI**) to acid-mediated cleavage to investigate product purities. Individual compound spots of chalcone array **VI** were punched out of the array, placed in glass vials, and cleaved with TFA vapor in a sealed desiccators (according to our previously reported method).[17] The cleaved chalcone products (**VII**) were then eluted from the support spots with acetonitrile, the solvent was removed in vacuo, and DMSO was added to each sample to generate individual compound stock solutions (~2 mM based on initial acetophenone loading). We randomly selected 35 chalcone products (**VII**) (20% of the library) and analyzed their purities by HPLC with UV detection at 254 nm. This subset of chalcones displayed moderate to excellent purities (72−99%), with an average purity of 87% (see Supporting Information Table S-1). The amount of chalcone product obtained (~113 nmol/spot) was sufficient for postsynthesis antibacterial assays and analytical characterization.

Antibacterial Screening of Chalcone Macroarrays. We sought to determine if any of the new chalcones (**VII**) had antibacterial activity against MRSA. We tested the antibacterial activities of the entire 174-member chalcone library against a MRSA strain (ATCC 33591) using a standard solution-phase absorbance assay that measures bacterial growth.[26] We tested aliquots of the chalcone stock solutions to obtain estimates of MIC values over a range of concentrations (3.1−50 μM) according to our previously reported method for off-support macroarray antibacterial assays of products.[17] Notably, we discovered three chalcones (**B19**, **F17**, and **F19**) that displayed estimated MIC values against MRSA below 6.3 μM (Figure 2 and Table 1). We selected these lead compounds for further study and quantitative MIC measurements.

We determined the actual MIC values for chalcones **B19**, **F17**, and **F19** against MRSA using stock solutions of authentic chalcone samples (Table 1). A straightforward two-step, solution-phase synthesis procedure was employed to generate pure samples of chalcones **B19**, **F17**, and **F19** (see Supporting Information). We were pleased to observe that the actual and estimated MIC values for **B19**, **F17**, and **F19** were in close agreement (<3−6 μM vs 3−4 μM, respectively; Table 1), indicating that our estimates of the quantities of cleaved products from the macroarray were accurate. For comparison, we obtained the MIC values for the two antibacterial drugs linezolid and ciprofloxacin against MRSA. Notably, the three chalcones (**B19**, **F17**, and **F19**) displayed MIC values that were only ~3−4× higher than ciprofloxacin (1 μM) and comparable to linezolid in this assay (4 μM; Table 1).

To extend our characterization of the most active chalcones, we measured the hemolytic activities of **B19**, **F17**, and **F19** using human red blood cells. Hemolysis assays are a standard technique to assess the toxicity of antibacterial agents against human cells. **F19** showed <10% hemolytic activity at 10 μM, which is ~3× its effective MIC value (Table 1). Both **B19** and **F17** exhibited <10% hemolysis at a concentration of 20 μM, which is 4× their effective MIC values (see Supporting Information for data). Such low hemolytic activities are advantageous properties for antibacterial agents, and ongoing studies in our laboratory are focused on determining the mechanisms of antibacterial action of these and related chalcones.

SUMMARY

In summary, we have demonstrated the compatibility of the Rink-amide linker with chalcone macroarray synthesis and have applied this linker system in the synthesis of a 174-member chalcone library (**VI**). The use of the Rink linker allowed us to efficiently construct the chalcone macroarray using milder reaction conditions relative to our previous syntheses. Off-support antibacterial assays of the resulting chalcone products (**VII**) uncovered three previously unreported chalcones that display potent MIC values against MRSA. These studies serve to expand the utility of the small molecule macroarray approach for the synthesis and discovery of biologically active compounds. In addition, our results provide

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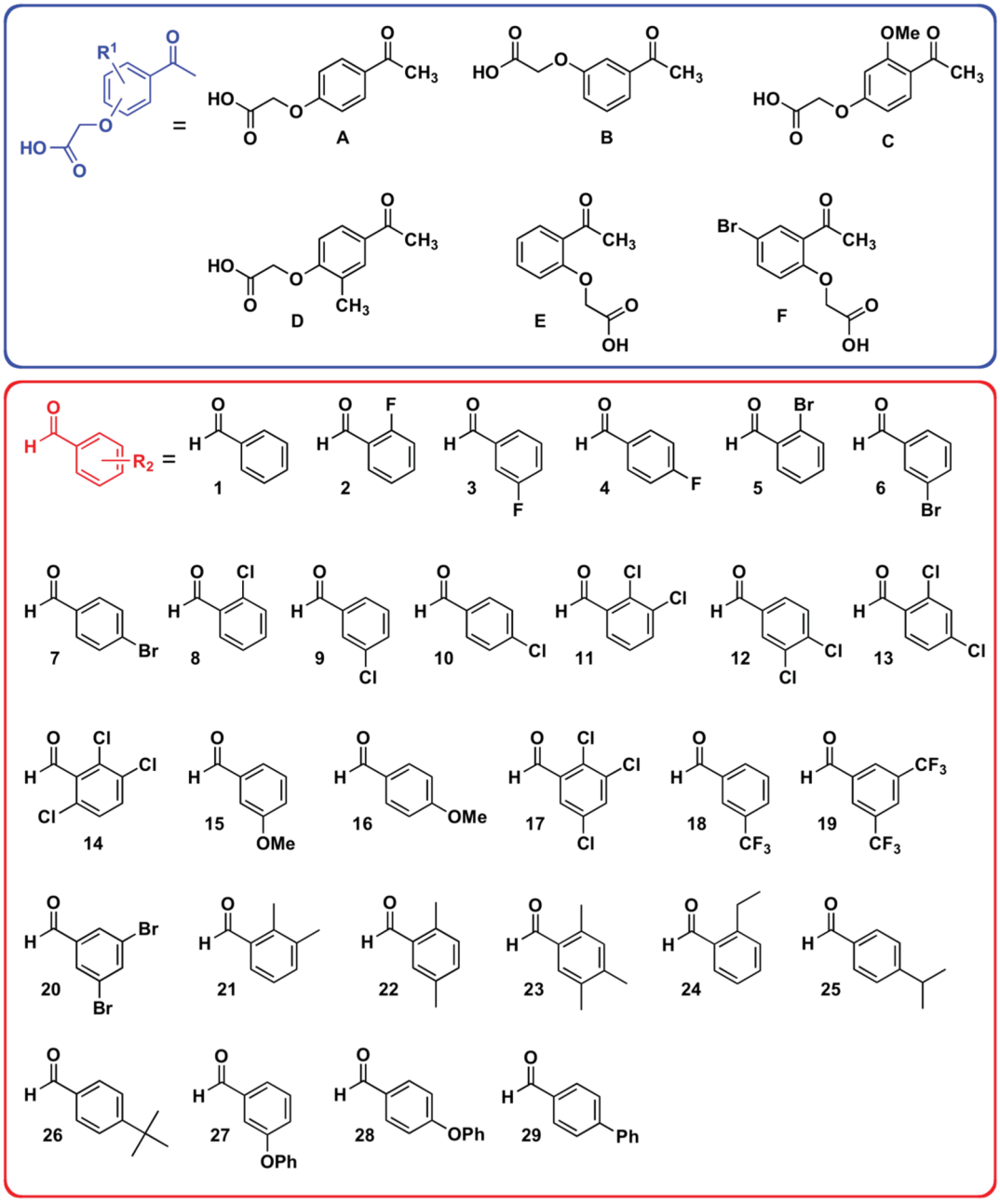

Figure 1. Two sets of building blocks used in the synthesis of the chalcone macroarray. (Top) Acetyl phenoxyacetic acids A−F. (Bottom) Benzaldehydes 1−29.

support for further study of chalcone scaffolds[27−29] in antibacterial development.

EXPERIMENTAL PROCEDURES

General Experimental Information. All chemical reagents and solvents were purchased from commercial sources (Alfa-Aesar, Aldrich, and Acros) and used without further purification with the exception of dichloromethane (CH_2Cl_2), which was distilled over calcium hydride immediately prior to use. Planar cellulose membranes (Whatman 1CHR) was purchased from Fisher Scientific and stored in a desiccator at room temperature until ready for use. Dots were marked on 15 cm ×18 cm cellulose sheets at distances 1.4 cm apart using a No. 2 lead pencil to designate spot locations. In this format, 120 compound spots (area/spot = 0.3 cm^2) could be accommodated on a single sheet without any detectable cross contamination. A calibrated micropipet was used to deliver all spotted reagents.

Macroarray Synthesis. Planar cellulose membranes (Whatman 1CHR filter paper) were derivatized with a Rink-amide linker using a modified version of previously reported protocols to yield amino support **IV** (loading ∼500 nmol/cm^2).[20,21,24] A 1.0 M solution of acetyl phenoxyacetic acid in 1.0 M DIC in anhydrous DMF was prepared. After standing at room temperature for 2 min, aliquots (6.0 μL) of this solution were applied to amino support **IV** using a micropipet. The support was heated at 43 °C on a sand bath and then washed by immersion and swirling in 150 mL portions of 1 N NaOH, EtOH (2×), and CH_2Cl_2 (2×). The resulting acetophenone

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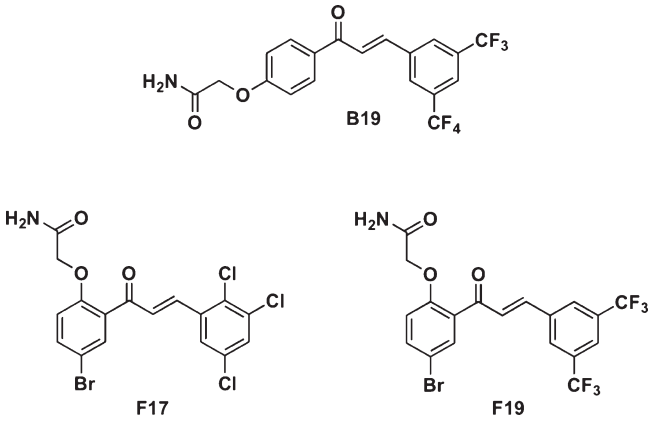

Figure 2. Lead antibacterial chalcones uncovered in this study (**B19**, **F17**, and **F19**).

Table 1. Purity and Antibacterial Activity Data for Lead Chalcones and Controls against MRSA

compound	purity (%)[a,b]	estimated MIC range (μM)[c,d]	actual MIC (μM)[c,e]
B19	84	<6.3	4.0 ± 0.5
F17	87	<3.1	4.0 ± 0.5
F19	92	<6.3	3.2 ± 0.2
linezolid			4.0 ± 1.0
ciprofloxacin			1.0 ± 0.2

[a] From HPLC analyses of crude macroarray compounds (UV detection at 254 nm). [b] Certain chalcones were mixtures of cis and trans isomers (see Supporting Information for details). [c] Methicillin-resistant *S. aureus* ATCC 33591 (MRSA). [d] From serial dilution of spot stock solutions of crude macroarray compounds. [e] From authentic sample of the compound. Only trans isomers of chalcones were evaluated. Error reflects step size in the serial dilution.

macroarray **V** was dried under a stream of N_2 gas for 15 min. Thereafter, a 1.0 M solution of benzaldehyde in 1 N NaOH in 50% aq. ethanol was prepared, and aliquots (6.0 μL) of this solution were applied (3×) to acetophenone macroarray **V**. The support was washed by adding and then decanting 150 mL portions of 1% AcOH, DMSO, EtOH (2×), and CH_2Cl_2. The chalcone macroarray **VI** was dried under a stream of N_2 gas for 15 min. Individual spots were punched out into 4 mL glass vials using a desktop hole punch. TFA vapor-phase cleavage of the spots (60 min, 25 °C)[17] yielded chalcones **VII** (see Supporting Information for full synthetic details).

Antibacterial Assays. Bacteriological work was performed with *S. aureus* (ATCC 33591) obtained from the American Type Culture Collection (ATCC). Antibacterial assays were performed according to our previously reported methods.[17] Actual MIC values were determined for lead compounds resynthesized in solution using an analogous procedure (see Supporting Information for full bacteriological assay details).

Hemolysis Assay. The hemolysis assay was based on an established procedure.[30,31] Briefly, freshly drawn human red blood cells (hRBC, blood type O) were washed 3× with Tris-buffered saline (pH 7.2, 0.01 M Tris-HCl, 0.155 M NaCl) and centrifuged at 3500 rpm until the supernatant was clear. 2-fold serial dilutions of chalcone in Tris-buffered saline (containing 2% (v/v) DMSO for compound solubility) were added to each well in a sterile 96-well plate (BD Falcon 353072 tissue culture plates), for a total volume of 50 μL in each well. A 2% (v/v) hRBC suspension (50 μL in Tris buffer) was added to each well. The plate was incubated at 37 °C for 1 h, and the cells were then pelleted by centrifugation at 3500 rpm for 5 min. The supernatant (80 μL) was transferred to a fresh 96-well plate, and hemoglobin was detected by measuring the optical density (OD) at 405 nm. The OD of cells incubated with an excess concentration of Triton-X100 defined 100% hemolysis; the OD of cells incubated in Tris buffer (containing 2% (v/v) DMSO) defined 0% hemolysis (see Supporting Information for assay data).

■ ASSOCIATED CONTENT

Supporting Information. Full details of instrumentation, macroarray construction, macroarray library purity, solution-phase synthesis of building blocks and lead compounds, compound characterization, and biological assay protocols and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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