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Combinatorial Synthesis and in Vitro Evaluation of a Biaryl Hydroxyketone Library as Antivirulence Agents against MRSA

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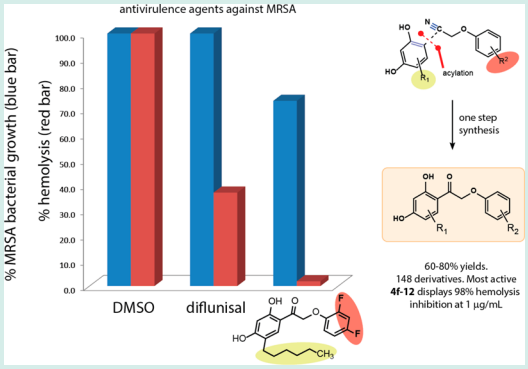
Supporting Information

ABSTRACT: Antibiotic resistance coupled with decreased development of new antibiotics necessitates the search for novel antibacterial agents. Antivirulence agents offer an alternative to conventional antibiotics. In this work, we report on a family of small-molecule antivirulence agents against methicillin-resistant *Staphylococcus aureus* (MRSA), the most widespread bacterial pathogen. Structure−activity relationship studies led to the development of a concise synthesis of a 148-member biarylhydroxyketone library. An acylation bond-forming process afforded resorcinols (**1**) and aryloxy acetonitriles (**2**) as synthons. A Lewis-acid-activated Friedel−Crafts' acylation step involving a nitrile functionality of **2** by $ZnCl_2$, followed by nucleophilic attack by **1** was executed to obtain biaryl hydroxyketones in excellent yields. A large number of products crystallized. This strategy affords a range of biarylhydroxyketones in a single step. This is the first collective synthetic study documenting access to this class of compounds through a single synthetic operation. In vitro efficacy of compounds in this library was evaluated by a rabbit erythrocyte hemolysis assay. The most efficacious compound, **4f-12**, inhibits hemolysis by 98.1 ± 0.1% compared to control in the absence of the compound.



KEYWORDS: *biaryl hydroxyketone library, MRSA, antivirulence agents, antibiotic resistance*

INTRODUCTION

Antivirulence Agents against MRSA. Antibiotic resistance coupled with the decline in the development of new antibiotics necessitates the search for novel agents to prevent and treat serious bacterial infections. Herein, we report a family of antivirulence agents effective against methicillin-resistant *Staphylococcus aureus* (MRSA), the most widespread bacterial pathogen in the United States[1] and in the developed world.[2] MRSA causes a wide range of infections ranging from skin and soft tissue to more invasive infections, such as pneumonia, endocarditis, meningitis, bacteremia, and sepsis. The increase in *S. aureus* infections has been associated with hospitalization, affecting preferentially immunocompromised individuals. Recently, such infections also increasingly occur in the community in healthy individuals, such as athletes, students, prisoners, etc. These community-associated infections (CA-MRSA) are generally more virulent than hospital-associated infections (HA-MRSA).[2] Treatment of *S. aureus* infections is hampered by the steady increase of resistance against conventional antibiotics. Over two-thirds of *S. aureus* infections are now resistant to methicillin, a second-generation β-lactam antibiotic.[3] Vancomycin, linezolid, and daptomycin are the antibiotics of last resort against MRSA. Alarmingly, strains recently have emerged that are resistant to vancomycin.[4] Therefore, the development of new therapeutic solutions against MRSA represents an urgent medical need.

Antivirulence agents present alternatives to conventional antibiotics.[5] In contrast to antibiotics, antivirulence agents are not bactericidal and, generally, are not even bacteriostatic. Since the survival of the bacteria is not threatened there is less pressure to develop resistance against an antivirulence agent. The mechanism of action is based upon curtailing the pathogen's ability to elicit toxins against the host's immune system. An unimpaired immune system may be able to fight off the infection on its own. Alternatively, a boost in the form of a low-dose conventional antibiotic in combination with an antivirulence agent may become a successful strategy against more invasive infections. Antivirulence therapy offers the attractive prospect of bringing back conventional and affordable antibiotics into the clinic. In previous work, a series of simple aromatic compounds were identified as effective antivirulence candidates that included diflunisal, an FDA-approved anti-inflammatory drug.[6] In this study, we report a concise synthetic route that affords a larger library of biaryl hydroxyketones and show that the antivirulence activity displayed be these analogs is much higher than diflunisal.

Biaryl Hydroxyketones as Antivirulence Compounds. Recently, we reported the discovery of a small molecular library

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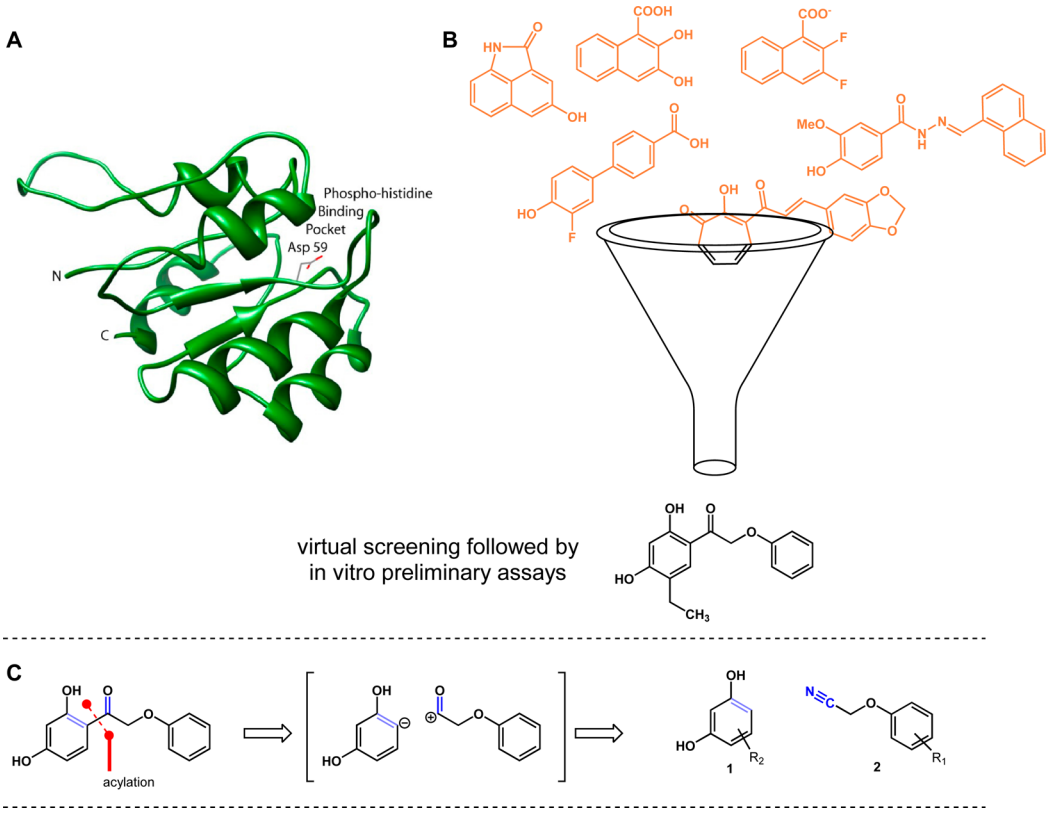
Scheme 1. (A) Ribbon Diagram of the Homology-Built Model of the N-Terminal Domain of AgrA (AgrA_N), (B) Structures of a Few Representative Scaffolds Identified As Privileged Motifs for Inhibiting AgrA Activation and Thereby Toxin Synthesis by MRSA, and (C) Library Design through Retrosynthetic Analysis of Biaryl Hydroxyketone Scaffold to Afford Resorcinols and Aryloxyacetonitriles As Precursors



and showed that a privileged subset of chemical entities elicited antivirulence activity against MRSA causing diminished production of the staphylococcal toxins, α-hemolysin, also known as α-toxin (Hla) and phenol-soluble modulin α (PSM-α) in a dose-dependent manner.[6] Virtual screening was employed to discover small molecules that block the phosphorylation site on the regulatory domain of AgrA, a response regulatory protein integral to the toxin biosynthetic pathway in MRSA (Scheme 1A,B). Initial results indicated that the small molecules inhibited the transcription of Hla and PSM-α.[6] One of the compounds discovered as an initial candidate, (referred to as compound VI),[6] the parent compound (PC) in this study, inhibited rabbit erythrocyte hemolysis by 98% and 9% at 10 and 1 μg/mL, respectively.[6] On the basis of the biaryl hydroxyketone structural scaffold of this candidate we envisioned applying chemical synthesis to derivatize the aromatic rings in order to discover compounds with more potent antivirulence activity.

Herein, we describe a concise synthesis of 148 individual chemical entities through a robust acylation method that affords a range of biarylhydroxyketones in high yields and in a single step. Furthermore, the in vitro efficacy of this library was evaluated by a rabbit blood hemolysis assay affording a subset of efficacious antivirulence agents for further in vivo studies. The underlying bond disconnection, resulting in the generation of a library of derivatives, is the acylation bond-forming process affording resorcinols (**1**) as the nucleophilic synthon and aryloxy acetonitriles (**2**) as the electrophilic synthon as shown in Scheme 1C. This strategy led to the discovery of new compounds with considerably higher quorum sensing inhibitory activity than the parent compound.

RESULTS AND DISCUSSION

Synthesis of Biarylhydroxyketone Library. The retrosynthetic analysis of biaryl hydroxyketone moiety, as shown in Scheme 1C allowed for resorcinol derivatives (represented by **1**) and aryloxy acetonitriles (represented by **2**) as convenient starting points for the assembly of the targeted library. Scheme 2A shows the individual variations selected based on availability of substituted resorcinols and aryloxyacetonitrile precursors. All of the resorcinol derivatives were commercially available except for 4-methyl and 4-propyl substitutions represented by **1d** and **1e** (necessary for synthesis of **4d** and **4e** series respectively). These were synthesized using reduction reactions of corresponding carbonyl compounds (see Supporting Information (SI)). All the substitutions of aryloxyacetonitrile compounds desired for the construction of targeted library needed to be synthesized. Alkylation reaction of phenols with α-bromoacetonitriles reported by McManus et al.[7] served as a template for the synthesis of these precursors. Individual protocols were adapted based on existing methods to provide precursors in excellent yields (see SI).

As shown in Scheme 2B, a Lewis-acid catalyzed Friedel–Crafts' acylation step involving the activation of the nitrile functionality of **2** by $ZnCl_2$, followed by nucleophilic attack by **1** was executed in the presence of gaseous hydrogen chloride in benzene-diethyl ether mixture to yield the iminium hydrochlorides **3** as the intermediate. Upon hydrolysis of **3** the biaryl

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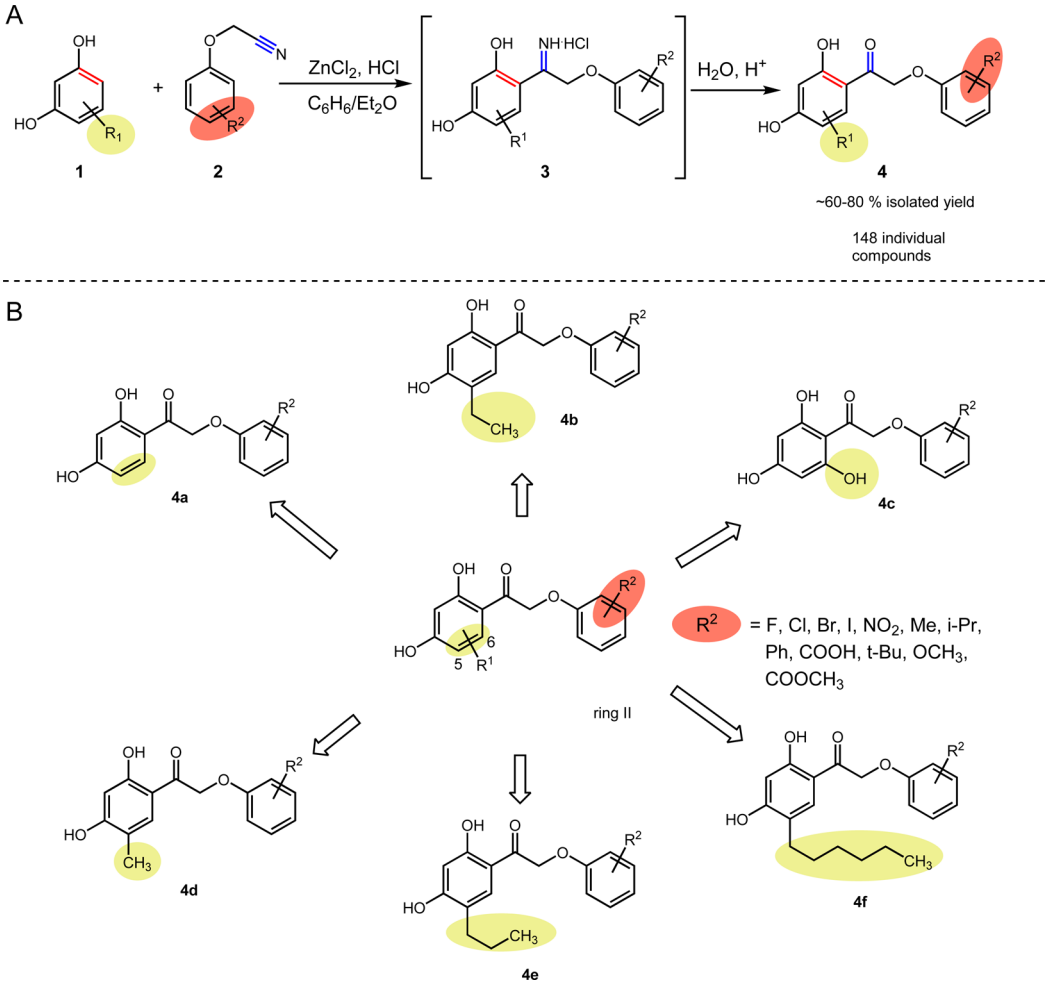
Scheme 2. (A) Synthesis of Biarylhydroxyketone Library through Friedel−Crafts Acylation between 1 and 2 and (B) Structural Variations of Biarylhydroxyketone Library



hydroxy ketone library (represented by **4**) was obtained in moderate to excellent yields depending on individual substitution pattern. Each derivatization reaction afforded product **4** in moderate to high yields averaging about 70%. Purification of the compounds was relatively simple as a large number of products crystallized, thereby not requiring chromatography. Though a few of these biaryl hydroxyketones were reported in the literature, this is the first collective synthetic study documenting this class of compounds directly in a single operation. Structural characterization of each member of the library was performed through ^{1}H, ^{13}C NMR analyses (See SI). Compounds **4a-1** and **4a-11** yielded X-ray diffraction data (See SI). Table 1 lists the individual substitutions on each derivative that was synthesized using this one-step method. Charts S1−S6 (SI) provide chemical structures of each member of this family of molecules.

Efficacy of the Biarylhydroxyketone Library against MRSA-Triggered Hemolysis. The antivirulence activity of these compounds against MRSA strain USA 300 was measured by the extent of hemolysis inhibition in rabbit erythrocytes in vitro. MRSA secretes the cytotoxin Hla, which creates holes in red blood cells of rabbits and other organisms, causing hemolysis.[8] The assay measures the level of hemoglobin released from the erythrocytes through this rupture. In addition to hemolysis, we measured the extent to which the family of biaryl hydroxyketones **4** inhibited bacterial growth. A high magnitude of hemolysis inhibition (indicated by a lower % for hemolysis) concomitantly with no or low inhibition of bacterial growth is essential for a good antivirulence agent. Molecules conferring such effect are desirable because they are good candidates for eliminating virulence and low potential for resistance development. The hemolysis data for all 148 compounds is shown as grouped sections in the SI (Tables S5−1−S5−3) along with data on bacterial growth. By screening this relatively large library for efficacy, we identified a subset of 24 analogs displaying the most efficacious hemolysis inhibition at a concentration of 1 μg/mL. These results are plotted in Figure 1. DMSO was used as a control, assigned 100% hemolysis and 100% bacterial growth. The biaryl parent compound (PC) is the control candidate having the same chemical constituency as compound VI in prior work.[6] Therefore PC served as a starting point for comparison of hemolysis inhibitory effect of newer analogs. Diflunisal, an FDA-approved anti-inflammatory was used as a comparative standard. In general, among the 148 compounds tested, compounds in the **4a** and **4c** series containing no substitution on the resorcinol portion of the compound or containing a hydroxyl functionality elicited much less hemolysis inhibition when compared to compounds in the **4d**, **4e**, and **4f** series. This trend points to the beneficial effect of having sterically larger substitutions on the resorcinol portion. In the presence of 1 μg/mL of the most efficacious compound, **4f-12**, rabbit

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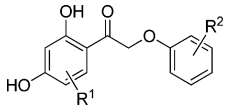
Table 1. Individual Structure of Biaryl Hydroxyketone Library Synthesized According to Scheme 2



4a R^1= 5-H		**4b** R^1= 5-Ethyl		**4c** R^1= 6-OH		**4d** R^1= 5-methyl		**4e** R^1= 5-propyl		**4f** R^1= 5-hexyl	
compd	R^2	compd	R^2	compd	R^2	compd	R^2	compd	R^2	compd	R^2
4a-0[a]	H	**4b-0**[a]	H	**4c-0**[f]	H	**4d-0**	H	**4e-0**	H	**4f-0**	H
4a-1	*m*-F	**4b-1**	*m*-F	**4c-1**	*m*-F	**4d-1**	*m*-F	**4e-1**	*m*-F	**4f-1**	*m*-F
4a-2[g]	*o*-Cl	**4b-2**	*o*-Cl	**4c-2**	*o*-Cl	**4d-2**	*o*-Cl	**4e-2**	*o*-Cl	**4f-12**	2,4-F_2
4a-3	*m*-Cl	**4b-3**	*m*-Cl	**4c-3**	*m*-Cl	**4d-3**	*m*-Cl	**4e-4**	*p*-Cl	**4f-14**	3,4-F_2
4a-4[b]	*p*-Cl	**4b-4**[g]	*p*-Cl	**4c-4**[f]	*p*-Cl	**4d-4**	*p*-Cl	**4e-5**	*o*-F	**4f-19**	*p*-*i*Pr
4a-5[b]	*o*-F	**4b-5**[e]	*o*-F	**4c-5**	*o*-F	**4d-5**	*o*-F	**4e-6**	*p*-F	**4f-22**	*p*-NO_2
4a-6[b]	*p*-F	**4b-6**[g]	*p*-F	**4c-6**[f]	*p*-F	**4d-6**[g]	*p*-F	**4e-7**	*p*-Br	**4f-26**	*p*-OCH_3
4a-7[a]	*p*-Br	**4b-7**	*p*-Br	**4c-7**[f]	*p*-Br	**4d-7**	*p*-Br	**4e-8**	*p*-I	**4f-27**	*o*-$COOCH_3$
4a-8[g]	*p*-I	**4b-8**[g]	*p*-I	**4c-8**[f]	*p*-I	**4d-8**	*p*-I	**4e-9**	*o*-Me	**4f-28**	*m*-$COOCH_3$
4a-9[b]	*o*-Me	**4b-9**	*o*-Me	**4c-9**	*o*-Me	**4d-9**	*o*-Me	**4e-10**	*m*-Me	**4f-29**	*p*-$COOCH_3$
4a-10[b]	*m*-Me	**4b-10**	*m*-Me	**4c-10**	*m*-Me	**4d-10**	*m*-Me	**4e-11**	*p*-Me		
4a-11[c]	*p*-Me	**4b-11**	*p*-Me	**4c-11**[g]	*p*-Me	**4d-11**[g]	*p*-Me	**4e-12**	2,4-F_2		
4a-12	2,4-F_2	**4b-12**	2,4-F_2	**4c-12**	2,4-F_2	**4d-12**	2,4-F_2	**4e-13**	2,6-F_2		
4a-13	2,6-F_2	**4b-13**	2,6-F_2	**4c-13**	2,6-F_2	**4d-13**	2,6-F_2	**4e-14**	3,4-F_2		
4a-14	3,4-F_2	**4b-14**	3,4-F_2	**4c-14**	3,4-F_2	**4d-14**	3,4-F_2	**4e-15**	3,5-F_2		
4a-15	3,5-F_2	**4b-15**	3,5-F_2	**4c-15**	3,5-F_2	**4d-15**	3,5-F_2	**4e-17**	3,4,5-F_3		
4a-16	2,4,5-F_3	**4b-16**	2,4,5-F_3	**4c-16**	2,4,5-F_3	**4d-16**	2,4,5-F_3	**4e-18**	Pentafluoro		
4a-17	3,4,5-F_3	**4b-17**	3,4,5-F_3	**4c-17**	3,4,5-F_3	**4d-17**	3,4,5-F_3	**4e-19**	*p*-*i*Pr		
4a-18	pentafluoro	**4b-18**	pentafluoro	**4c-18**	pentafluoro	**4d-18**	pentafluoro	**4e-20**	*p*-tBu		
4a-19[g]	*p*-*i*Pr	**4b-19**[g]	*p*-*i*Pr	**4c-19**[g]	*p*-*i*Pr	**4d-19**	*p*-*i*Pr	**4e-21**	*p*-Ph		
4a-20[g]	*p*-tBu	**4b-20**	*p*-tBu	**4c-20**	*p*-tBu	**4d-20**	*p*-tBu	**4e-22**	*p*-NO_2		
4a-21[g]	*p*-Ph	**4b-21**[g]	*p*-Ph	**4c-21**	*p*-Ph	**4d-21**	*p*-Ph	**4e-25**	*p*-COOH		
4a-22[b]	*p*-NO_2	**4b-22**[g]	*p*-NO_2	**4c-22**[f]	*p*-NO_2	**4d-22**	*p*-NO_2	**4e-26**	*p*-OCH_3		
4a-23[d]	o-COOH	**4b-24**	*m*-COOH	**4c-26**[f]	*p*-OCH_3	**4d-25**	*p*-COOH	**4e-27**	*o*-$COOCH_3$		
4a-24	*m*-COOH	**4b-26**	*p*-OCH_3	**4c-27**[d]	*o*-$COOCH_3$	**4d-26**	*p*-OCH_3	**4e-28**	*m*-$COOCH_3$		
4a-26[b]	*p*-OCH_3	**4b-27**	*o*-$COOCH_3$	**4c-28**	*m*-$COOCH_3$	**4d-27**	*o*-$COOCH_3$	**4e-29**	*p*-$COOCH_3$		
4a-27[d]	*o*-$COOCH_3$			**4c-29**[f]	*p*-$COOCH_3$	**4d-28**	*m*-$COOCH_3$				
4a-28	*m*-$COOCH_3$	**4b-28**	*m*-$COOCH_3$			**4d-29**	*p*-$COOCH_3$				
4a-29[b]	*p*-$COOCH_3$	**4b-29**	*p*-$COOCH_3$								

[a]Ref 17. [b]Ref 18. [c]Ref 19. [d]Ref 20. [e]Ref 22. [f]Ref 21. [g]These compounds have a CAS Number, but no reference report.

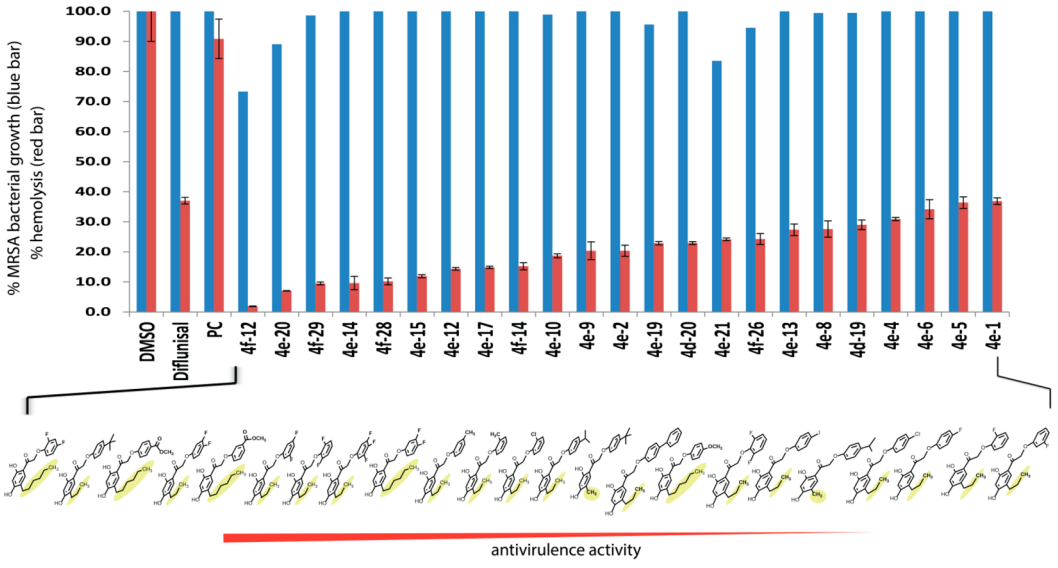


Figure 1. Rabbit erythrocyte hemolysis (red bars) and bacterial growth (blue bars) of the most efficacious compounds at 1 μg/mL.

erythrocyte hemolysis was only 1.9 ± 0.1% compared 100% in the absence of the compound. However, this compound inhibited bacterial growth by 26.7% at a concentration of 1 μg/mL, as shown in Supporting Information (Table S5−3). Thus,

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some decrease in hemolysis can be attributed to a lesser amount of bacteria present. However, **4f-12** has been shown to inhibit binding of AgrA to its cognate DNA by an electrophoretic mobility shift assay (data not shown), thereby inhibiting the production of the α-toxin, the agent causing hemolysis. Thus, **4f-12** has dual activity. It is a bacteriostatic agent in addition to being an antivirulence compound.

The presence of a larger hexyl side chain on the 5-position of resorcinol ring of **4f-12** in addition to the presence of two fluorine atoms on the aryloxy portion of this molecules are noteworthy and seem to confer a significant hemolysis inhibitory effect. This trend is across the rest of the 23 derivatives mapped in Figure 1 revealing the effects of hydrophobic groups present on either one or both aromatic rings. The exact nature of substitution seems only moderately specific for rendering hemolysis inhibitory activity. For example, the fluorine atoms (that adds favorable drug-like properties)[9−11] could be replaced by methyl groups or isopropyl groups and the activity is maintained with minor differences. The compounds described herein are novel anti-MRSA agents. There are no other known efficacious anti-MRSA agents except for antibiotics. Diflunisal and PC (the parent compound of the combinatorial synthesis) are included in the hemolysis graph to show that some of the synthesized compounds are much better inhibitors than these two previously discovered antivirulence agents in our laboratory.[6] The nonsteroidal anti-inflammatory drug diflunisal contains two aromatic fluorine atoms. Interestingly, diflunisal was discovered in our preliminary screening to have antivirulence activity against MRSA,[6] demonstrating a new use for an old drug.

Mechanism of Action of Antivirulence Compounds. Toxins secreted by *S. aureus* cause damage to host cells and impair the ability of host defense mechanisms to fight the infection. The exact nature of the toxins varies between species. α-hemolysin (Hla) and phenol-soluble modulin (PSMα) are virulence factors transcriptionally regulated by the *agr* operon, the most important *S. aureus* operon for the expression of toxin biosynthesis and pathogenicity.[6] The *agr* system is activated by a quorum sensing mechanism. The autoinducing peptide serves as the signaling molecule to activate the histidine kinase AgrC, which in turn activates the response regulator AgrA to bind to its cognate DNA to drive the expression of a series of toxins and virulence factors.[12] Multiple accounts of *agr* inhibition have recently been reported. AgrC was the target in a synthetic approach to inhibit *agr* activation by cyclic peptide mimics of the autoinducing peptide (AIP).[13] Sequestration of the autoinducing peptide by designed inhibitory antibodies was another approach to quench *agr* expression.[14] Depsipeptides isolated from a marine bacterium and ambuic acid isolated from a fungus have been shown to inhibit expression of the *agr* system, apparently by competition with the AIPs.[15,16]

In a complementary study, on biaryl compounds sharing structural features to those reported herein, we unambiguously showed that antivirulence mechanism is effected through disruption of AgrA binding to its cognate DNA counterpart.[6] In a subsequent recent attempt, we have found that **4f-12** inhibits binding of AgrA to its cognate DNA by an electrophoretic mobility shift assay (data not shown), thereby inhibiting the production of the α-toxin agent that causes hemolysis. Toxins secreted by *S. aureus* cause damage to host cells, such as red blood cells and cells of the immune system.[6] Blocking toxin expression by antivirulence agents might therefore enable host defense factors to contain the infection. If proven to be efficacious and nontoxic in animal models some compounds discovered in this work could potentially be used as adjuvants in conventional antibiotic therapy or perhaps in monotherapy for topical applications. Further delineation of the mechanism of action with the series of efficacious compounds reported in Figure 1 is underway.

CONCLUSION

In summary, we report the first collective synthetic study documenting access to a biarylhydroxyketone library. Discovery of a privileged subset led to the development of a concise synthesis of a 148-member biarylhydroxyketone library. An acylation bond-forming process afforded resorcinols (**1**) and aryloxy acetonitriles (**2**) as synthons. A Lewis-acid activated Friedel−Crafts' acylation step involving a nitrile functionality of **2** by $ZnCl_2$, followed by nucleophilic attack by **1** was executed to obtain **4** in moderate to excellent yields. Each derivatization reaction afforded **4** in yields averaging about 70%. A large number of products crystallized. This strategy affords a range of biarylhydroxyketones in a single step. The in vitro efficacy of this library was evaluated by a hemolysis assay. Compounds listed in this manuscript have advanced to in vivo efficacy studies in an MRSA insect larvae model (data not shown). The three most efficacious compounds from the insect larvae study have been subjected to a murine MRSA wound infection model with encouraging results. These in vivo results will be published in a subsequent report.

EXPERIMENTAL PROCEDURE

General Methods. All commercially available reagents and solvents were used as analytically pure substances as received. Reactions were monitored by thin-layer chromatography (TLC) on silica gel plates (60 F_{254}) with a fluorescent indicator, and independently visualized with UV light. Target molecules **4a−4f** were recrystallized from 2-propanol. Yields refer to crystallized compounds. All separations of intermediates (**1d**, **1e**, **2a-2aa**) were carried out under flash chromatography (Silica gel grade: 200−400 mesh, 40−63 μm) at medium pressure (20 psi). All new compounds gave satisfactory spectroscopic analyses (^{1}H NMR, ^{13}C NMR). NMR spectra were recorded at 400 MHz in $CDCl_3$, acetone-d_6 or DMSO-d_6 and chemical shift values (δ) are given in parts per million. ^{1}H NMR spectra are reported in parts per million (δ) relative to the residual (indicated) solvent peak. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, ddd = double double doublet, m = multiplet, cm = complex multiplet), integration, and coupling constants in hertz. ^{13}C NMR spectra were obtained on 400 MHz spectrometers (100 MHz actual frequency) and are reported in parts per million (δ) relative to the residual (indicated) solvent peak. ^{19}F NMR spectra were obtained on 400 MHz spectrometers (376 MHz actual frequency) and are reported in parts per million (δ). High-resolution mass spectrometry (HRMS) data were obtained on spectrometer with a quadrupole analyzer. All melting points for solids are reported uncorrected.

General Procedure for Synthesis of α-Aryloxy-2,4-dihydroxy-Substituted Acetophenones 4a−4f.[18] Dry hydrogen chloride was passed for 1 h into a solution of **2** (1 mmol, 1.0 equiv) in dry benzene (1.5 mL) at 0 °C. A solution

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of the substituted-resorcinol **1** (1.2 mmol, 1.2 equiv.) and $ZnCl_2$ (0.136 g, 1 mmol, 1.0 equiv.) in 1.5 mL dry ether were then added. Dry hydrogen chloride was bubbled for an additional 2 h and the reaction mixture was left overnight. The liquid was decanted from the solid, hot water (8 mL) was added to the residue, and the mixture was boiled at 80−100 °C for 2−3 h. After the mixture was cooled, the solid that formed was filtered off, washed with water until pH reached 7, and recrystallized from 2-propanol to yield **4**.

Growth and Hemolysis Assay. All reagents and culture media were purchased from Fisher Scientific except rabbit blood (HemoStat Lab., Dixon, CA, U.S.A.) and MRSA strain USA300, which was obtained from Dr. Robert Bonomo at the Louis Stokes Cleveland VA Medical Center. OD_{600} readings were recorded on a BioPhotometer; OD_{541} readings were recorded on a UV-1700 Pharmaspec instrument. MRSA strain USA300 was cultured overnight at 37 °C in 1.5 mL of Tryptic Soy (TS) broth. The overnight culture was diluted 1 to 100 and 2 mL was added to designated incubation tubes. DMSO solutions of compound 4 were subsequently added to yield a final concentration of either 1 or 10 μg/mL compound in 2% DMSO. 100% DMSO was added to a control incubation tube. The tubes were placed in a shaker and incubated at 37 °C for 6 h. OD_{600} was measured every hour to generate a growth curve. After 6 h the bacterial samples were filtered through a 0.22 μm syringe filter (Fisher Scientific). Bacterial filtrate (100 μL) was added to 1 mL hemolysin buffer (0.145 M NaCl, 0.02 M $CaCl_2$). Twenty-five microliters of defibrinated rabbit blood was added and incubated for 15 min at 37 °C. The unlysed blood cells were pelleted by centrifugation (5500 × *g*, room temperature, 2 min). The hemolytic activity of the supernatant was determined by measuring the optical density of hemoglobin at 541 nm. Defibrinated rabbit blood without bacterial filtrate served as blank (D^b), 2% DMSO supernatant without antivirulence agent served as control culture (D^c). The percent hemolysis was calculated by the formula $H = (D^t - D^b)/(D^c - D^b) \times 100$, where the D^t is the OD_{541} reading for supernatant with antivirulence compound.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectroscopic data and copies of ^1H and ^{13}C NMR spectra for all new compounds and X-ray crystallography data for **4a-1** and **4a-11** (including the CIF files). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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