

# Potent Small-Molecule Suppression of Oxacillin Resistance in Methicillin-Resistant *Staphylococcus aureus*\*\*

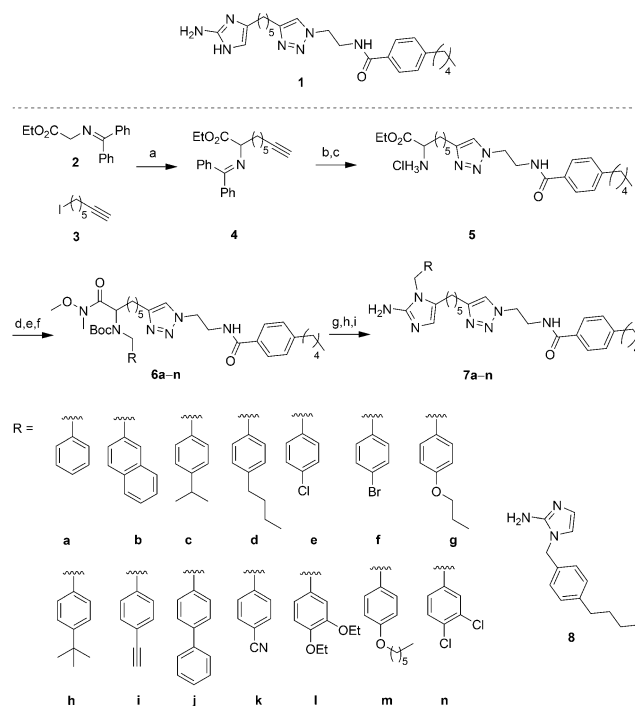
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The emergence of resistance to multiple antimicrobial agents in pathogenic bacteria is a significant global public health threat and causes considerable patient mortality and morbidity. In the United States, methicillin-resistant *Staphylococcus aureus* (MRSA) accounts for 80 % of all hospital-acquired *S. aureus* infections.<sup>[1]</sup> In 2005, almost 95 000 people acquired MRSA infections in the United States, of which nearly 19 000 people died — more than die annually from HIV/AIDS, emphysema, Parkinson's disease, and homicide combined.<sup>[2]</sup> Furthermore, MRSA infections, which are traditionally only observed among hospitalized patients, have now become prevalent outside of the hospital setting, with the emergence of community-associated MRSA (CA-MRSA).<sup>[3]</sup> In the USA, the USA300 clone is the most prevalent CA-MRSA clone.<sup>[3]</sup>  $\beta$ -Lactam antibiotics have typically been the most effective drugs for the treatment of infections caused by staphylococci; however, increasing occurrence of resistance means that they are often no longer efficacious.

Although the design of new antibiotics could address the threat of multi-drug resistance, the sobering fact is that there have been very few novel classes of antibiotics marketed in the last four decades.<sup>[4]</sup> Furthermore, bacteria inevitably develop resistance to all microbicidal agents that are introduced into the clinic.<sup>[5]</sup> An orthogonal approach to the development of new antibiotic entities is the use of small-molecule adjuvants.<sup>[6]</sup> Recently, our group and others, have been exploring the use of small molecules that are able to render multi-drug resistant bacteria sensitive to the effects of conventional antibiotics.<sup>[7–10]</sup> One of the key features of this approach is the targeting of pathways within the bacteria that, by themselves, are not essential for bacterial growth, by which the rate of resistance acquisition may be significantly reduced.

We have developed a class of 2-aminoimidazole/triazole conjugates (2-AIT) that are able to inhibit and disperse

biofilms of several pathogenic bacteria.<sup>[11,12]</sup> Some members of this class of compounds also possess the ability to suppress the resistance of planktonic bacteria to  $\beta$ -lactam antibiotics.<sup>[7]</sup> We recently reported 2-AIT **1** (Scheme 1), which lowers the minimum inhibitory concentration (MIC) of oxacillin against



**Scheme 1.** Synthesis of N1-substituted 2-AITs. Reagents and conditions: a) *t*BuOK, THF,  $-78^{\circ}\text{C}$ –RT 72 h; b) *N*-(2-azidoethyl)-4-pentylbenzamide,  $\text{CuSO}_4$ , sodium ascorbate,  $\text{H}_2\text{O}/\text{EtOH}/\text{CH}_2\text{Cl}_2$ , RT, 3 h; c) 2 M HCl/ $\text{Et}_2\text{O}$ , RT, 2 h; d) 1)  $\text{RCHO}$ ,  $\text{LiOH}\cdot\text{H}_2\text{O}$ ,  $\text{MeOH}$ , RT, 2 h, 2)  $\text{NaBH}_4$ , RT, 1 h; e)  $\text{Boc}_2\text{O}$ ,  $\text{Et}_3\text{N}$ , 1:1 dioxane/water, RT, 16 h; f)  $\text{HN}(\text{OMe})\text{Me}\cdot\text{HCl}$ ,  $i\text{PrMgCl}$ , THF,  $-20^{\circ}\text{C}$ –RT, 18 h; g) DIBAL-H, THF,  $-78^{\circ}\text{C}$ , 2 h; h) 9:1  $\text{CH}_2\text{Cl}_2/\text{TFA}$ , RT, 15 min; i)  $\text{H}_2\text{O}/\text{EtOH}$ , pH 4.3,  $\text{H}_2\text{NCN}$ ,  $95^{\circ}\text{C}$ , 3 h. Boc = *tert*-butoxycarbonyl, DIBAL-H = diisobutylaluminum hydride, TFA = trifluoroacetic acid.

an Iberian MRSA clone when co-dosed at sub-MIC levels.<sup>[13]</sup> Herein, we report that this compound exhibits similar activity against a USA300 MRSA clone. Analogue synthesis resulted in the identification of a significantly more active derivative that reduced oxacillin MICs up to 512-fold, thus taking the MIC significantly below the breakpoint for clinical resistance. Screening this compound against a number of USA300 MRSA mutant strains indicates that the *VraSR* two-component system (TCS) plays a role in the activity of this compound.

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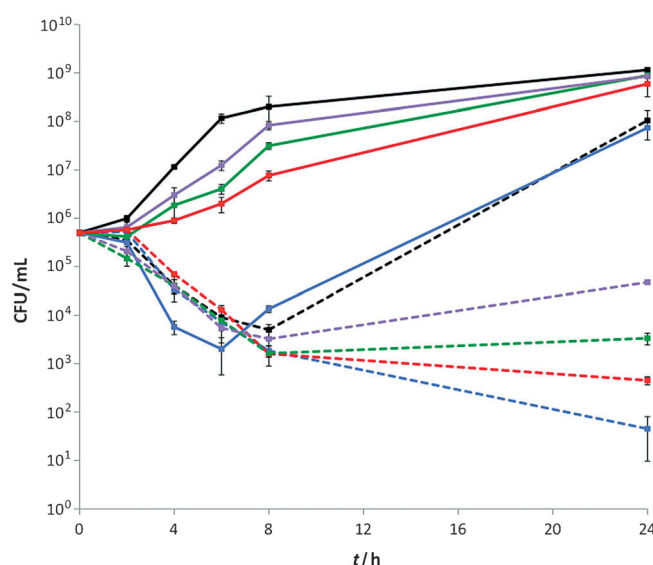
The MIC of compound **1** against a USA300 MRSA strain (ATCC BAA-1556) was determined to be 50  $\mu\text{M}$  ( $23.7 \mu\text{g mL}^{-1}$ ) using the CLSI broth microdilution protocol.<sup>[14]</sup> We subsequently determined the oxacillin MIC in the absence and presence of 40% of the MIC of compound **1** (20  $\mu\text{M}$ ,  $9.5 \mu\text{g mL}^{-1}$ ). Compound **1** lowered the oxacillin MIC by eight-fold at this concentration, from 32  $\mu\text{g mL}^{-1}$  to 4  $\mu\text{g mL}^{-1}$ . With the aim of developing a compound with increased activity, a series of analogues of compound **1**, in which substituents were placed at the N1-position of the 2-aminoimidazole, were synthesized using an approach that we had previously developed.<sup>[15]</sup>

The reaction of  $\alpha$ -amino ester **4**<sup>[8]</sup> with *N*-(2-azidoethyl)-4-pentylbenzamide<sup>[13]</sup> under Huisgen cycloaddition conditions followed by deprotection delivered key intermediate **5** (Scheme 1). Diversity was introduced through reductive amination of **5** with a variety of commercially available benzaldehydes. Boc-protection (Boc = *tert*-butoxycarbonyl) of the resulting secondary amines and subsequent conversion of the ester to the corresponding Weinreb amide generated intermediates **6a–n**. Finally, each Weinreb amide was reduced to the corresponding  $\alpha$ -amino aldehyde with diisobutylaluminum hydride (DIBAL-H) which, following Boc-deprotection and cyclization with cyanamide, afforded the 1,5-substituted 2-aminoimidazole/triazole conjugates **7a–n**. After purification, each compound was converted to the corresponding HCl salt for biological screening.

As with 2-AIT **1**, the MIC of each compound against MRSA ATCC BAA-1556 was first established. The MIC of oxacillin in the presence of 40% of the MIC of each compound was then determined (Table 1). Previously, we had shown that the introduction of an N1-substituent resulted in increased antibiotic activity relative to the parent compound.<sup>[15]</sup> As expected, this trend was followed in the generation of this library. Most compounds exhibited a significantly reduced or abrogated ability to lower the oxacillin MIC. Three compounds however (**7d**, **7k**, and **7l**), displayed

a marked increase in activity, lowering the oxacillin MIC by 128-fold, 64-fold, and 64-fold, respectively. The ability of the pilot library to suppress oxacillin resistance in another CA-MRSA USA300 strain (JE2) was next investigated (Table 1) and activity for each compound against this strain was virtually identical to that of BAA-1556.

Compound **7d**, which possesses a 4-butylbenzyl substituent, was selected as the lead compound for further study, owing to the considerably lower concentration at which it displayed activity compared to **7k** and **7l** (5  $\mu\text{M}$  compared to 40  $\mu\text{M}$  and 20  $\mu\text{M}$  respectively). A control compound, **8**, which possesses only the N1 substituent of compound **7d** was synthesized in the same manner as the pilot library and shown to be devoid of both bactericidal activity (MIC > 200  $\mu\text{M}$ ) and resistance suppression activity at concentrations as high as 50  $\mu\text{M}$ . Time-kill curves were constructed for strain JE2 cultured in the presence of combinations of oxacillin and compound **7d** (Figure 1). Compound **7d**, when dosed alone at



**Figure 1.** Time-kill curves for USA300 MRSA strain JE2. Solid lines: no 2-AIT, broken lines: 5  $\mu\text{M}$  **7d**. Black: no oxacillin, blue: 64  $\mu\text{g mL}^{-1}$  oxacillin, red: 16  $\mu\text{g mL}^{-1}$  oxacillin, green: 4  $\mu\text{g mL}^{-1}$  oxacillin, purple: 1  $\mu\text{g mL}^{-1}$  oxacillin.

5  $\mu\text{M}$  ( $3.1 \mu\text{g mL}^{-1}$ ), is bactericidal at early time points (up to 8 h); however, bacterial growth is similar to that of the control by the 24 h time point. When bacteria are cultured in the presence of combinations of oxacillin and compound **7d**, a large reduction in the number of colony forming units (CFU) is observed, as compared to treatment with oxacillin alone. A considerable synergistic effect can be observed at the 24 h time point. At 5  $\mu\text{M}$  concentration, compound **7d** alone effected a 1.08 log reduction in CFU after 24 h, and oxacillin effected less than 0.4 log reduction at concentrations of 16  $\mu\text{g mL}^{-1}$  and below. Combining **7d** (5  $\mu\text{M}$ ) and oxacillin resulted in log CFU reductions of 6.41, 5.54, and 4.38 for oxacillin concentrations of 16, 4, and 1  $\mu\text{g mL}^{-1}$  respectively. Finally, we tested the ability of lead compound **7d** at 5  $\mu\text{M}$  to suppress oxacillin resistance in eight additional MRSA

**Table 1:** MIC values and oxacillin resistance suppression activity against MRSA USA300 strains.

Entry	Compound	2-AIT conc. [ $\mu\text{M}$ ] <sup>[a]</sup>	Oxacillin MIC [ $\mu\text{g mL}^{-1}$ ]	
			ATCC BAA-1556	NARSA JE2
1	–	–	32	32
2	<b>1</b>	20	4	4
3	<b>7a</b>	10	32	32
4	<b>7b</b>	5	32	16
5	<b>7c</b>	5	32	32
6	<b>7d</b>	5	0.25	0.5
7	<b>7e</b>	5	32	32
8	<b>7f</b>	5	16	16
9	<b>7g</b>	5	32	32
10	<b>7h</b>	5	4	16
11	<b>7i</b>	5	16	32
12	<b>7j</b>	5	4	8
13	<b>7k</b>	40	0.5	0.5
14	<b>7l</b>	20	0.5	0.5
15	<b>7m</b>	10	32	16
16	<b>7n</b>	20	4	2
17	<b>8</b>	50	32	32

[a] 2-AIT concentration is 40% of the MIC, up to a limit of 50  $\mu\text{M}$ .

isolates obtained from the ATCC (see the Supporting Information) and observed reduction in MIC values of 4-fold to 512-fold.

As the synthesized molecules are amphipathic, we first investigated the effect of the compounds on cell-membrane integrity. The ability of compound **7d** to permeabilize the bacterial cell membrane was quantified using the BacLight assay.<sup>[16]</sup> After exposure of strain JE2 to compound **7d** for one hour, the ratio of intact/damaged cells was measured and compared to control (treated with DMSO only) bacteria. At 4 × the MIC, 96% of cells were damaged, whereas at 1 ×, 0.4 ×, and 0.25 × the MIC, only 33%, 21%, and 9% of cells were damaged, respectively. An inactive compound (**7e**) was found to be comparable, with 83%, 24%, 23%, and 16% of cells damaged at 4 ×, 1 ×, 0.4 ×, and 0.25 × the MIC, respectively; this suggests that cell-membrane permeabilization is not the mechanism by which compound **7d** suppresses resistance to oxacillin. Importantly for a potential antibiotic adjuvant, **7d** exhibited little effect on eukaryotic cell membranes, as determined by measuring the hemolytic activity against mechanically defibrinated sheep blood.<sup>[9]</sup> At its active resistance suppression concentration (5 μM), less than 1% lysis was observed compared to triton × positive control, while only 5.6% lysis was observed at as high as 50 μM.

To further delineate the mechanism by which these 2-AIT conjugates are able to lower the oxacillin MIC against MRSA, we obtained a number of mutant strains belonging to the Nebraska Transposon Mutant Library from the Network on Antimicrobial Resistance in *S. aureus* (NARSA). These mutants are all derived from JE2, thus allowing us to probe non-essential pathways that may be involved in suppression of oxacillin resistance. For this screen, we focused largely on mutants of non-essential TCS. Bacterial TCS, consisting of a membrane-bound histidine kinase and a response regulator, regulate adaptation to environmental changes and have been shown to play a role in resistance to certain antibiotics<sup>[17–19]</sup> as well as being master regulators of biofilm formation.<sup>[20,21]</sup> Furthermore, biotinylated analogues of related 2-AI anti-biofilm compounds employed in pull down assays bind to response regulators involved in biofilm formation (unpublished work). Therefore, we posited that these 2-AIT derivatives, which also have anti-biofilm activity, might also target other response regulators involved in antibiotic resistance.

We first established the MICs of oxacillin and **7d** against each mutant strain (Table 2; see the Supporting Information for gene descriptions). As expected, the MIC of **7d** was fairly consistent against all mutant strains (either 6.25 or 12.5 μM), whereas a majority of the strains examined, including several response regulator mutants (strains NE958, NE481, NE262, and NE49), histidine kinase mutants (strains NE218, NE147, NE618, NE873, NE820, NE116, and NE423), and a MecR1 regulatory protein mutant (strain NE839), did not exhibit a greater than two-fold difference in oxacillin MIC compared to the parent strain. However, three of the strains tested exhibited considerably lower oxacillin MIC values: NE481 (an unidentified DNA-binding response regulator mutant), NE554 (a *vraR* mutant), and NE823 (a *vraS* mutant) exhibited oxacillin MICs that were reduced 16-fold, eight-fold, and eight-fold, respectively. These results are in line with

**Table 2:** MIC values and oxacillin resistance suppression activity of compound **7d** against strains from the Nebraska Transposon Mutant Library.

Entry	Strain	Concentration of <b>7d</b> [μM]	Oxacillin MIC [μg mL <sup>-1</sup> ]	Oxacillin MIC with <b>7d</b> [μg mL <sup>-1</sup> ] <sup>[a]</sup>
1	JE2	5	32	0.5
2	NE218	2.5	32	8
3	NE147	2.5	32	0.25
4	NE958	5	32	0.25
5	NE481	5	2	0.25
6	NE262	5	32	0.5
7	NE618	5	32	1
8	NE554	2.5	4	4
9	NE823	2.5	4	4
10	NE873	5	32	0.25
11	NE210	5	32	0.5
12	NE820	5	32	0.25
13	NE839	5	32	0.5
14	NE49	2.5	32	16
15	NE116	2.5	32	32
16	NE95	5	16	0.5
17	NE423	2.5	16	4

[a] Oxacillin MIC values recorded in the presence of 40% MIC of **7d**.

previous studies which show that expression of *VraSR* contributes to oxacillin resistance.<sup>[22]</sup> The ability of **7d** to lower the oxacillin MIC against the mutant strains was then examined in an identical manner to that used for the parent strain (at 40% of the MIC). Of the mutants that exhibited altered oxacillin MIC values versus the parent, compound **7d** failed to lower the MIC of both the *VraSR* two-component system mutant strains NE554 and NE823, which suggests that the mode of action of the oxacillin resistance suppression activity of compound **7d** involves *VraSR*. Compound **7d** also failed to lower the oxacillin MIC by more than two-fold against strains NE116 and NE49, which suggests that compound **7d** may have some interaction with the pathways controlled by the disrupted genes of these two mutants. NE116 is a putative histidine kinase mutant, whereas NE49 is an *AraC* family response regulator mutant. *AraC* family proteins are known to play a role in antibiotic resistance and stress responses,<sup>[23]</sup> however, as these two mutant strains did not exhibit oxacillin MICs that differed from the parent strain, these pathways most likely do not relate to the mechanism of oxacillin resistance suppression by compound **7d**.

As compound **7d** exhibited a lower MIC against a number of mutant strains than the parent strain (and was therefore screened for resistance suppression at a lower concentration, 2.5 μM, 1.55 μg mL<sup>-1</sup>), we wanted to ensure that the lack of resistance suppression activity was not simply a result of lower bactericidal activity of the compound. A time-kill curve was therefore constructed for NE554 in the presence of 2.5 μM **7d** (40% MIC) and compared to the time-kill curve of JE2 in the presence of 5 μM **7d** (see the Supporting Information). The bactericidal activity of compound **7d**, was in fact slightly higher against strain NE554 than JE2 at the concentrations used in the resistance suppression assay, which suggests that the lack of activity against NE554 is due to the absence of *VraR*, rather than altered bactericidal activity.

VraSR has been proposed to be a “sentinel” system that is capable of sensing perturbation of cell wall synthesis and coordinating a response involving expression of a number of genes involved in antibiotic resistance.<sup>[22]</sup> The expression of VraSR is induced upon exposure to cell-wall-acting antibiotics, including  $\beta$ -lactams, glycopeptides, daptomycin, and bacitracin,<sup>[24,25]</sup> and it has been shown that VraSR mutants are treatable with an oxacillin regimen in vivo.<sup>[26]</sup> To further establish the disruption of VraSR signaling in the mechanism of **7d**, we tested the ability of **7d** to lower the MIC of vancomycin. The MIC of vancomycin against JE2 was established as  $1\ \mu\text{g mL}^{-1}$ , but in the presence of  $5\ \mu\text{M}$  **7d** this is lowered to  $0.25\ \mu\text{g mL}^{-1}$ . The MIC of vancomycin is  $0.5\ \mu\text{g mL}^{-1}$  against both NE554 and NE823, and this remained unchanged in the presence of  $2.5\ \mu\text{M}$  **7d**. Furthermore, compound **7d** had little or no effect on the MICs of streptomycin or chloramphenicol (see the Supporting Information), which are non-cell-wall-acting antibiotics that do not activate the VraSR TCS. The fact that compound **7d** did not lower the MIC of these latter antibiotics against the parent strain further suggests that the reduction in oxacillin MIC brought about by this compound is not simply due to a combined microbicidal effect, but is rather due to disruption of the VraSR TCS pathway.

In conclusion, we have developed a compound that is able to suppress resistance to oxacillin in diverse MRSA strains, achieving MIC suppressions upwards of 512-fold. We have shown that this activity is not dependent upon membrane disruption, and preliminary screening of USA300 mutants indicates that VraSR plays an important role in the activity of this compound. Given the pressing need for new strategies to deal with the threat of multi-drug resistant pathogenic bacteria, the identification of molecules that restore the efficacy of approved antibiotics by interfering with bacterial TCS represents a potential avenue for the development of antibiotic adjuvants.

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