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## Second-generation aryl isonitrile compounds targeting multidrug-resistant *Staphylococcus aureus*

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### Abstract

Antibiotic resistance remains a major global public health threat that requires sustained discovery of novel antibacterial agents with unexploited scaffolds. Structure-activity relationship of the first-generation aryl isonitrile compounds we synthesized led to an initial lead molecule that informed the synthesis of a second-generation of aryl isonitriles. From this new series of 20 compounds, three analogues inhibited growth of methicillin-resistant *Staphylococcus aureus* (MRSA) (from 1 – 4  $\mu$ M) and were safe to human keratinocytes. Compound **19**, with an additional isonitrile group exhibited improved activity against MRSA compared to the first-generation lead compound. This compound emerged as a candidate worthy of further investigation and further reinforced the importance of the isonitrile functionality in the compounds' anti-MRSA activity. In a murine skin wound model, **19** significantly reduced the burden of MRSA, similar to the antibiotic fusidic acid. In summary, **19** was identified as a new lead aryl isonitrile compound effective against MRSA.

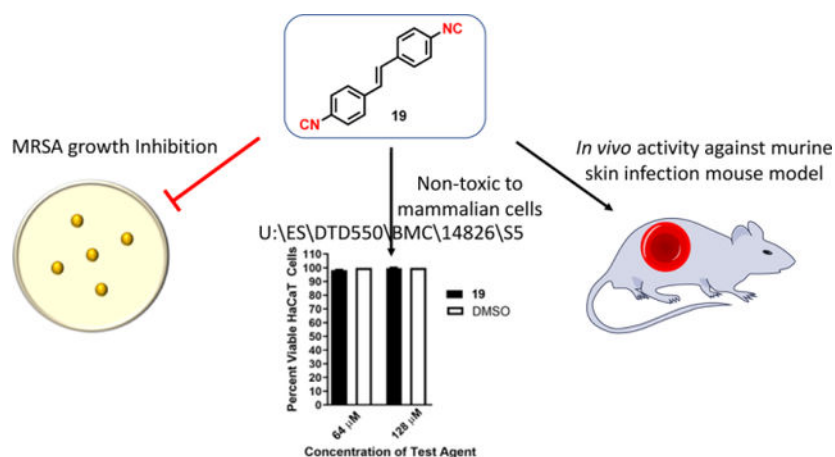
### Graphical Abstract

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## INTRODUCTION

Bacterial infections resistant to currently available antibiotics continue to pose a major global public health threat that requires the constant identification and development of new antibacterial agents. Though extensive research efforts and funding have recently been focused to identify new agents to treat Gram-negative bacterial infections, particularly those caused by carbapenem-resistant Enterobacteriaceae (CRE), the reality remains that Gram-positive bacteria (particularly methicillin-resistant *S. aureus* (MRSA)) are more prevalent sources of community-acquired and nosocomial infections, particularly in the United States of America.<sup>1–3</sup> In its landmark report in 2013, the US Centers for Disease Control and Prevention noted that serious infections caused by MRSA (>80,000) alone outnumbered infections attributed to extended-spectrum  $\beta$ -lactamase producing Enterobacteriaceae (26,000), CRE (9,000), multidrug-resistant *Acinetobacter* (7,300), and multidrug-resistant *Pseudomonas aeruginosa* (6,700) combined.<sup>4</sup> Furthermore, significantly more fatalities were attributed to MRSA infections (11,285 deaths) relative to infections caused by the aforementioned drug-resistant Gram-negative pathogens (3,240 deaths).<sup>4</sup> Given MRSA infections continue to persist both in healthcare and community settings, and resistant isolates to key antibiotics (vancomycin and linezolid) used to treat MRSA infections have emerged,<sup>3, 5–8</sup> new antibacterial agents are still needed.

Current antibacterial discovery programs often focus on optimizing existing antibiotic scaffolds (i.e.  $\beta$ -lactams, quinolones, glycopeptides, oxazolidinones) to develop new therapeutics. This approach has yielded regulatory approval for several new antibacterial agents to treat MRSA infections, including delafloxacin, dalbavancin, oritavancin, and tedizolid phosphate with improved potency relative to other antibiotics in the same drug class.<sup>9</sup> However, bacterial resistance to these newer agents is most probably inevitable, particularly given the similarity in structure to existing antibiotics, which will further hinder clinicians' ability to effectively treat drug-resistant bacterial infections. Identifying novel antibacterial agents with unique scaffolds or mechanisms of action is critical to circumvent the growing challenge of treating drug-resistant bacterial infections.

As part of efforts to identify compounds with unique functionalities and novel structural moieties capable of targeting multidrug-resistant bacterial infections, we recently identified aryl isonitriles as a unique class of compounds with anti-MRSA activity.<sup>10</sup> Though extensive efforts in isolating naturally occurring isonitriles from marine sponges and cyanobacteria have been fruitful, these and other naturally occurring isonitriles with antimicrobial activity have been precluded from extensive medicinal chemistry and structure-activity-relationship (SAR) studies.<sup>11–14</sup> This is primarily because their complex structural motifs make them very difficult to access. Therefore, the isonitrile functionality and the aryl isonitrile scaffold remain one of the least extensively studied scaffolds. Previous SAR studies on the first series of over 40 aryl isonitriles revealed the isonitrile functionality as the most essential structural component that contributed to the antibacterial activity of the compounds. The presence of a second non-isonitrile-bearing aromatic ring and an alkene bridge/linker were also shown to be important. However, substituents incorporated on this bridge did not result in improved antibacterial activity (Figure 1).

With this in mind, the current work aims to further explore the structural-anti-MRSA activity of a second generation of aryl isonitriles, characterize their spectrum of antibacterial activity, and evaluate the most promising analogue's activity in a mouse model of MRSA infection. This exploration takes into consideration a novel group of stilbene bis-isonitriles. New stilbene aryl isonitriles with medicinally-relevant structural molecules and heterocyclic moieties, and those with a saturated linker have also been evaluated (Figure 2). From this effort, compound **19** emerged as a new lead compound with antibacterial activity *in vitro* and *in vivo* against MRSA (Figure 3).

## RESULTS

### Synthesis of new aryl isonitrile analogues

Following the identification of **1** as the initial hit aryl isonitrile and subsequent synthetic exploration resulting in **2** as the lead molecule (Figure 3), our initial synthetic efforts were focused on incorporating medicinally-relevant groups to the aryl isonitrile core. These groups included electron donating and electron withdrawing small molecules such as fluoro, chloro, trifluoromethyl, and methoxy groups.<sup>15–17</sup> To access these novel stilbene isonitriles, Michaelis-Arbuzov reaction involving the commercially available nitrobenzyl bromide starting material (**3**) was used to form nitrobenzyl phosphonate (**4**).<sup>18</sup> Subsequent reduction of the nitro group, followed by Hofmann isonitrile conversion of the resulting amine, led to the formation of the isonitrile phosphonate (**5**).<sup>19</sup> Serving as the divergent point, the isonitrile phosphonate was then subjected to Horner-Wadsworth-Emmons (HWE)<sup>20</sup> reaction to produce isonitrile compounds **6**, **7**, **8**, **9**, **10**, **11**, and **12** from the corresponding aldehydes (Scheme 1). The aforementioned synthetic procedure was also used to synthesize aryl isonitriles to explore the importance of incorporating heterocyclic moieties as the second aromatic ring. These analogues included furan (**12**) and quinoline (**13** and **14**) groups.

As part of SAR studies on the first-generation molecules, the isonitrile functionality was identified as the integral component of the stilbene aryl isonitriles (Figure 1). Bis-isonitriles **17**, **18**, and **19** were therefore synthesized to explore the influence of an additional isonitrile group on the antibacterial activity of the lead molecule **2**. Similarly, using **4** as the divergent

point, the stilbene bis-isonitriles **17**, **18**, and **19** were synthesized (Scheme 2). HWE reaction involving **4** and the corresponding nitro substituted benzaldehydes was employed as the first step in the formation of dinitrostilbenes. The selective reduction of the nitro group followed by Hoffmann isonitrile synthesis led to compounds **17** and **18**. Bis-isonitrile **19** was prepared by a stepwise formylation and  $\text{POCl}_3$ -promoted dehydration of the corresponding diamine.

Finally, after the synthesis of the stilbene isonitriles and the bis-isonitriles, the question of the impact of an alkane bridge on the biological activity arose. This led to the synthesis of compounds **21–28** (Scheme 3). In the synthesis of aryl isonitriles with an alkane linker, HWE reaction involving **4** and various aldehydes was first employed to form the stilbene compounds. A sequence of reduction and Hofmann isonitrile synthesis afforded the corresponding aryl isonitriles in good yields.

### Initial screening and structure-activity relationship of new aryl isonitrile analogues against MRSA NRS123 (USA400)

In order to investigate the antibacterial activity of the newly synthesized isonitrile analogues, the minimum inhibitory concentration (MIC) against MRSA NRS123 was determined using the broth microdilution assay (Table 1). The newly synthesized stilbene bis-isonitriles **17** (MIC = 16  $\mu\text{M}$ ), **18** (MIC = 4  $\mu\text{M}$ ), and **19** (MIC = 8  $\mu\text{M}$ ) all exhibited moderate to good anti-MRSA activity. The most potent analogues were compounds where the isonitrile moiety was located in the meta- (**18**) or para- position (**19**). The moderate to good activity exhibited by the stilbene bis-isonitriles further emphasized the importance of the isonitrile group for antibacterial activity against MRSA. An eight-fold reduction in activity was observed when the most potent stilbene bis-isonitrile **18** (MIC = 4  $\mu\text{M}$ ) was transformed to its saturated bis-isonitrile analogue **22** (MIC = 32  $\mu\text{M}$ ). The observance of a similar pattern for bis-isonitriles **17** (MIC = 16  $\mu\text{M}$ ) and **19** (MIC = 8  $\mu\text{M}$ ) with respect to the saturated bis-isonitrile analogues **21** and **23** (MIC > 64  $\mu\text{M}$ ) further emphasized the importance of the alkene linker.

Intrigued by the importance of the non-isonitrile-bearing aromatic ring resulting from the previous SAR studies, heterocyclic moieties with established therapeutic properties and widely known in medicinal chemistry to improve the physicochemical and pharmacokinetic properties of lead molecules were investigated.<sup>21, 22</sup> While none of these heterocyclic-containing aryl isonitriles positively improved the activity of the lead compound, the quinoline-containing compound **13** (MIC = 8  $\mu\text{M}$ ) was more potent than the quinoline analogue **14** (MIC > 64  $\mu\text{M}$ ) and furan analogue **12** (MIC = 32  $\mu\text{M}$ ).

With the knowledge of the medicinal importance of F,  $-\text{OCH}_3$ , Cl and  $\text{CF}_3$  and their value in the enhancement of the pharmacokinetic and physiochemical activity of several therapeutic small molecule candidates, installing these species on the aryl isonitrile compounds was investigated.<sup>15–17</sup> Of the methoxy substituted derivatives, the meta-substituted methoxy **7** (MIC = 2  $\mu\text{M}$ ) was the most potent analogue, but adding the second methoxy group (**8**) diminished the compound's anti-MRSA activity (MIC = 64  $\mu\text{M}$ ). Interestingly, none of the analogues with electron-withdrawing fluoro, chloro, and trifluoromethyl groups exhibited a major improvement in anti-MRSA activity. This reveals that having the right electronic environment around the second aromatic ring is important.

### Evaluation of most potent analogues against additional strains of *S. aureus*

Based upon the initial screening results, the four most potent analogues from this series (**7**, **13**, **18**, and **19**) were further evaluated against additional multidrug-resistant *S. aureus* clinical isolates (Table 2). The *S. aureus* clinical isolates included strains resistant to linezolid (NRS119) and vancomycin (VRS10, VRS11a), two antibiotics frequently prescribed to treat MRSA infections. Against methicillin-sensitive *S. aureus*, the compounds inhibited growth from 2 – 16  $\mu\text{M}$ . When evaluated against MRSA NRS384 (USA300), all four aryl isonitrile compounds inhibited growth at concentrations ranging from 1 – 4  $\mu\text{M}$ . Methoxy analog **7** (MIC = 1  $\mu\text{M}$ ) and bis-isonitrile **19** (MIC = 2  $\mu\text{M}$ ) were more potent than first generation lead **2** (MIC = 4  $\mu\text{M}$ ) against MRSA NRS384. However, quinoline isonitrile **13** (MIC = 4  $\mu\text{M}$ ) and bis-isonitrile **18** (MIC = 4  $\mu\text{M}$ ) exhibited similar potency to **2**. Against MRSA NRS119, **7** and **19** were the most potent analogues (MIC = 1  $\mu\text{M}$ ), followed by **13** (MIC = 4  $\mu\text{M}$ ), and **18** (MIC = 4  $\mu\text{M}$ ). Once again, the second-generation analogues **7** and **19** were more potent than the first-generation lead **2** (MIC = 4  $\mu\text{M}$ ). All four compounds were more potent than linezolid (MIC = 32  $\mu\text{M}$ ) against MRSA NRS119. The MIC results obtained against MRSA NRS384 (USA300) and MRSA NRS119 (linezolid-resistant *S. aureus*) demonstrate that incorporating a second isonitrile functionality (as in compound **19**) improved the antibacterial activity of the first-generation lead molecule **2**.

Against two *S. aureus* strains (VRS10 and VRS11a) resistant to vancomycin (MIC > 64  $\mu\text{M}$ ), compounds **7** and **19** retained their antibacterial activity (MIC ranged from 2 – 8  $\mu\text{M}$ ). Interestingly, **13** (MIC = 32  $\mu\text{M}$ ) exhibited very poor antibacterial activity against VRS10. Thus, this compound was eliminated from further biological evaluation.

### Investigation of the antibacterial spectrum of activity of the aryl isonitrile compounds

To examine their spectrum of antibacterial activity, compounds **7**, **18**, and **19** were examined against clinical isolates from the ESKAPE pathogens (*Enterococcus faecium* (E), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii* (A), *Pseudomonas aeruginosa* (P), and *Enterobacter* species (E)) (Table 3). Collectively, these six pathogens are a significant source of hospital-acquired bacterial infections and exhibit resistance to most clinically available antibiotics.<sup>23, 24</sup> All four compounds were significantly less potent or inactive (MIC  $\geq$  64  $\mu\text{M}$ ) against vancomycin-resistant *E. faecium* (VRE), *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae*, similar to the antibiotics erythromycin, linezolid, and vancomycin. The compounds were also evaluated against two additional clinically-relevant Gram-positive bacterial pathogens, *Staphylococcus epidermidis* and *Streptococcus pneumoniae*. All three compounds exhibited potent activity against methicillin-resistant *S. epidermidis* (MIC = 1  $\mu\text{M}$ ). Against, penicillin-resistant *S. pneumoniae*, **7** was the most potent compound (MIC = 4  $\mu\text{M}$ ) followed by **19** (MIC = 8  $\mu\text{M}$ ). Compound **18** was the least potent analogue against *S. pneumoniae* (MIC = 16  $\mu\text{M}$ ).

### Effect of the outer membrane and efflux pump on antibacterial activity against Gram-negative bacterial pathogens

We hypothesized that the lack of antibacterial activity against Gram-negative bacteria observed for the aryl isonitrile compounds was due either to the presence of the outer

membrane (OM) or due to expulsion via efflux pumps, two common resistance mechanisms utilized by Gram-negative bacterial pathogens.<sup>25</sup> The OM has been known to prevent numerous antibiotics (including erythromycin) from gaining entry into the bacterial cell at sufficient concentrations to bind to/inhibit the molecular target. To examine if the OM was impeding the antibacterial activity of the aryl isonitrile compounds, compound **19** and control antibiotics were incubated with the same Gram-negative bacterial species presented in Table 2 in the presence of a subinhibitory concentration of the membrane-permeabilizing agent colistin. As presented in Table 4, a noticeable improvement in the antibacterial activity of compound **19** was observed against *A. baumannii* (MIC = 16  $\mu$ M) and *E. cloacae* (MIC = 4  $\mu$ M) indicating the OM was having a deleterious effect on the antibacterial activity of this compound. Interestingly, no improvement in antibacterial activity for **19**, in the presence of colistin, was observed against *K. pneumoniae* and *P. aeruginosa*, (similar to linezolid) suggesting the presence of the outer membrane was not solely responsible for the lack of activity observed in these particular pathogens. The antibacterial activity of the antibiotic erythromycin, in the presence of colistin, improved against *A. baumannii* (MIC = 4  $\mu$ M), *E. cloacae* (MIC = 1  $\mu$ M), *K. pneumoniae* (MIC = 16  $\mu$ M), and *P. aeruginosa* (MIC = 4  $\mu$ M). Erythromycin is thought to be capable of diffusing across the OM but at a very slow rate.<sup>26</sup> Thus permeabilizing the OM is expected to enhance penetration of the antibiotic into bacterial cells.

We next examined if the presence of efflux pumps may be responsible for the lack of antibacterial activity observed for the aryl isonitrile compounds against Gram-negative bacteria (Table 4). These pathogens express different efflux pumps that confer resistance to numerous antibiotics including the AdeIJK efflux pump in *A. baumannii*, AcrAB-TolC efflux pump (present in *E. coli*, *E. cloacae*, and *K. pneumoniae*) and its homologue MexAB-OprM (expressed by *P. aeruginosa*).<sup>26, 27</sup> We evaluated the antibacterial activity of **19** against an *Escherichia coli* strain (JW25113) deficient in the AcrAB-TolC efflux pump responsible for excluding many xenobiotics from accumulating inside *E. coli* cells.<sup>25</sup> No discernible improvement in antibacterial activity of **19** was observed against the mutant *E. coli* strain relative to the wild-type strain (BW25113). In contrast, there was noticeable improvement in the antibacterial activity of erythromycin (MIC = 2  $\mu$ M) and linezolid (MIC = 16  $\mu$ M), two substrates of the AcrAB-TolC efflux pump.<sup>26, 28</sup> This suggests that the presence of efflux pumps alone on the surface of the OM on Gram-negative bacteria may not be responsible for conferring resistance to the aryl isonitrile compounds. We next investigated if the combination of the outer membrane and efflux pumps may impede the antibacterial activity of the aryl isonitrile compounds. The compound and control antibiotics were incubated with a subinhibitory concentration of colistin against *E. coli* BW25113 and *E. coli* JW25113. No improvement in the antibacterial activity of **19** (MIC > 64  $\mu$ M) was observed in the presence of colistin against *E. coli* JW25113. Similarly, no improvement in the MIC of linezolid was observed against *E. coli* JW25113 in the absence and presence of colistin, indicating the lack of antibacterial activity of linezolid observed against *E. coli* is due primarily to efflux. The MIC of erythromycin against *E. coli* JW25113, in contrast, improved one-fold in the presence of colistin indicating both the OM and efflux pumps interfere with the effect of this antibiotic against Gram-negative bacteria, in agreement with previous reports.<sup>26, 29</sup> Due to the lack of antibacterial activity observed against VRE and

Gram-negative bacterial pathogens, we moved to evaluate the aryl isonitrile compounds antibacterial activity *in vivo* against MRSA.

### ***In silico* pharmacokinetic evaluation of compound 19**

MRSA is a source of infection for both superficial skin lesions and complicated systemic infections. To determine a suitable animal model of MRSA infection to evaluate the aryl isonitrile compounds, the pharmacokinetic profile of compound **19** and linezolid were simulated utilizing a dose of 600 mg (as is commonly administered for linezolid in adult human patients). As shown in Table 5, the results indicate that compound **19** would not be suitable for oral administration for the treatment of systemic MRSA infections as it is not expected to attain a concentration in plasma/blood sufficient to inhibit bacterial growth. The maximum plasma concentration ( $C_{max}$ ) predicted for compound **19** is 0.17  $\mu\text{g/mL}$  (0.73  $\mu\text{M}$ ), whereas the MIC against MRSA ranges from 1 to 8  $\mu\text{M}$ . Intravenous administration of compound **19** is predicted to result in a slow rate of clearance (12.58 mL/min-kg) correlating with a long half-life (22.90 hours) which could alleviate the need for multiple daily dosing. The high values obtained for the volume of distribution at steady-state for **19** (24.94 L/kg compared to 1.11 L/kg for linezolid) indicate this compound is expected to distribute extensively into tissues. This may be due to the high degree of lipophilicity ( $c\text{LogP} = 3.88$ ) present with the compound. These pharmacokinetic simulations suggest that though intravenous administration of **19** may be possible for treatment of systemic MRSA infections, the extensive distribution of the compound into tissues would necessitate a higher dose be administered/continuous infusion of compound (to ensure the concentration remained above the MIC to inhibit MRSA growth). The values obtained for linezolid via the *in silico* pharmacokinetic simulation overall were in close proximity to experimental values obtained by Stalker, *et al.* from healthy human subjects administered a single 625 mg dose of linezolid either orally or intravenously.<sup>30</sup> However, the simulation underestimated the  $C_{max}$  for linezolid and overestimated the half-life and volume of distribution compared to the experimental values. Based upon the *in silico* pharmacokinetic simulation, **19** appears most suitable for evaluation topically to treat localized/uncomplicated MRSA skin infections.

### **The active aryl isonitrile compounds are safe to mammalian keratinocytes**

*S. aureus* is a leading source of skin and soft tissue infections, both uncomplicated and invasive, globally.<sup>31–34</sup> As such, based upon the *in silico* pharmacokinetic data, we decided to investigate the antibacterial activity of the second-generation aryl isonitriles as topical antibacterial agents in a MRSA murine skin infection mouse model. Before exposing mice to the compounds, we evaluated the safety profile of **7**, **18**, and **19** against keratinocytes (HaCaT). All three compounds were safe up to the maximum tested concentration of 128  $\mu\text{M}$  (more than 90% of HaCaT cells remained viable after 24 hours of exposure to the compounds) (Figure 5).

### **Compound 19 significantly reduces the burden of MRSA in a skin infection mouse model**

After confirming the aryl isonitrile compounds are safe to keratinocytes, we moved to investigate the antibacterial activity of the newly synthesized analogues in a mouse model of MRSA skin infection. Given the safety profile against keratinocytes was identical for all

three aryl isonitrile compounds, **19** was selected for this experiment. Given **18** and **19** are structurally similar, the more potent compound *in vitro* against MRSA USA300 was selected for evaluation. After the formation of an abscess at the site of infection, mice were treated twice daily for five days. As the skin wounds were uncomplicated and localized, treatment was administered topically directly onto the surface of the abscesses. Mice were euthanized 12 hours after the last dose was administered and the abscesses were harvested to enumerate MRSA CFU. As presented in Figure 6, **19** (79.02% reduction) was as effective as the control antibiotic fusidic acid (77.78% reduction) in reducing the burden of MRSA in the wounds of infected mice after five days of treatment. Importantly, no excess inflammation or toxicity was observed in wounds after exposure to the compounds or fusidic acid.

## DISCUSSION

Antibiotics have played a critical role in resolving bacterial infections throughout the world since their initial discovery. However, bacteria have proven to be shrewd microorganisms capable of acquiring resistance to multiple antibiotics utilizing an array of clever mechanisms (e.g. through modification of the drug target, degradation of the antibiotic, and efflux of the antibiotic). As such there is a continuous need to identify and develop novel antibacterial agents capable of treating drug-resistant bacterial infections. MRSA remains a significant cause of superficial and invasive antibiotic-resistant infections globally. Though several antibacterial agents effective against MRSA are currently in clinical trials, the remarkable ability of this organism to develop resistance to different antibiotics necessitates new antibacterial agents, particularly from unexploited scaffolds, be identified and developed.

We previously synthesized and evaluated over 40 aryl isonitrile compounds for antibacterial activity against MRSA.<sup>10</sup> The first-generation compounds inhibited MRSA growth at concentrations ranging from 2 – 64  $\mu\text{M}$ <sup>10</sup> but none of the compounds possessed a suitable physicochemical profile to evaluate their effectiveness in an animal model of MRSA infection. The present study aimed to expand the library of aryl isonitrile compounds to better understand these limitations and further characterize the compounds' structure-antibacterial activity relationship. To this end, 20 second-generation aryl isonitrile compounds consisting of stilbene bis-isonitriles, stilbene aryl isonitriles with medicinally-relevant molecules and heterocyclic moieties, and those with a saturated linker were synthesized and evaluated for antibacterial activity.

The newly synthesized analogues were initially evaluated against a clinical isolate of MRSA USA400. From the initial screening, inclusion of an additional isonitrile moiety, the stilbene core, and the non-isonitrile-bearing aromatic ring were all found to be key for anti-MRSA activity. Four compounds (**7**, **13**, **18**, and **19**) inhibited growth of MRSA at a concentration ranging from 2 – 8  $\mu\text{M}$ , similar to the lead compound (**2**) from the first-generation aryl isonitriles. Bis-isonitrile **19** was more potent than the first-generation lead **2** against MRSA NRS384 (USA300) and MRSA NRS119 (LRSA) which further confirmed the importance of the isonitrile functionality.



Importantly, three compounds retained potent antibacterial activity against MRSA isolates exhibiting resistance to vancomycin and linezolid, antibiotics frequently used as agents of last resort for treatment of MRSA infections.<sup>35,36</sup> Though not prevalent clinically, the identification of *S. aureus* strains exhibiting resistance to vancomycin and linezolid, two key therapeutic options for treatment of MRSA infections, is a noteworthy problem. The lack of cross-resistance observed between the aryl isonitrile compounds and vancomycin and linezolid is important therapeutically as it provides a potential alternative source of treatment against linezolid-resistant and vancomycin-resistant *S. aureus* infections.

Previously, the first-generation aryl isonitrile analogues were only evaluated against clinical isolates of *S. aureus*. We were curious to investigate the spectrum of antibacterial activity of the second-generation aryl isonitrile compounds. Thus, three of the most potent analogues against MRSA (**7**, **18**, and **19**) were evaluated against a panel of clinically-relevant pathogens, including members of the ESKAPE group, *S. epidermidis*, and *S. pneumoniae*. The compounds exhibited potent antibacterial activity against the Gram-positive pathogens *S. epidermidis* and *S. pneumoniae*. Interestingly, the compounds were inactive against vancomycin-resistant *E. faecium*. The lack of antibacterial activity against VRE (a Gram-positive pathogen similar to MRSA) is not entirely surprising given certain antibiotics that are active against *S. aureus* (such as ampicillin and vancomycin) are inactive against vancomycin-resistant *E. faecium*.<sup>37</sup> The aryl isonitrile compounds also lacked antibacterial activity against five Gram-negative bacterial pathogens tested (*A. baumannii*, *K. pneumoniae*, *E. cloacae*, *E. coli*, and *P. aeruginosa*). The lack of antibacterial activity against Gram-negative pathogens is not surprising given most small molecules are unable to permeate the complex outer membrane (OM) present in Gram-negative bacteria. Those that are capable of diffusing across the OM may be susceptible to expulsion (decreasing the intracellular concentration of compound/drug) by a number of different efflux pumps expressed by Gram-negative bacteria, including the AcrAB-TolC pump present in *E. coli*.<sup>25</sup> Thus we evaluated the antibacterial activity of the aryl isonitrile compounds after permeabilizing the OM (using a subinhibitory concentration of colistin) and against an *E. coli* strain deficient in the AcrAB-TolC efflux pump. Interestingly, there was improvement in the MIC for compound **19** observed in the presence of colistin against *A. baumannii* and *E. cloacae* but not against *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. Against a mutant *E. coli* strain deficient in AcrAB-TolC, no improvement in the MIC of **19** was observed indicating that this compound may not be a substrate for efflux. This suggests that though the outer membrane may contribute to the lack of antibacterial activity for the aryl isonitrile compounds observed against specific Gram-negative bacterial pathogens, additional resistance mechanism(s) may play a role as well. Alternatively, the compounds may have a weaker affinity for the molecular target in Gram-negative bacteria as opposed to against MRSA, *S. epidermidis*, and *S. pneumoniae*. Though the molecular target of the aryl isonitrile compounds is currently unknown, this question is being intensely investigated using both genetic and phenotypic approaches. Identification of the molecular target may provide key insight into the difference in potency observed for the aryl isonitrile compounds against *S. aureus* relative to other bacterial pathogens.

The aryl isonitrile compounds possessed good *in vitro* activity against drug-resistant *S. aureus* isolates and were safe to mammalian cells (non-toxic to human keratinocytes at 128  $\mu\text{M}$ , more than 30-fold higher than the concentration where they inhibited MRSA growth *in vitro*). However, an *in silico* pharmacokinetic evaluation of **19** revealed this compound may not be effective in treatment of systemic MRSA infections (as the maximum predicted concentration of the compound in plasma was lower than the MIC of the compound against MRSA). As a means to address this problem while still retaining antibacterial activity, the alkene core of the current lead **19** is undergoing further structural diversification with molecular entities we hypothesize will help improve the physicochemical profile of the lead compound.

Based upon the *in vitro* antibacterial activity assay results and *in silico* pharmacokinetic simulation, we proceeded to evaluate the effectiveness of **19** as a topical antibacterial to treat MRSA skin infection. MRSA remains a major source of skin and soft tissue infections (SSTIs) throughout the world.<sup>31–34, 38, 39</sup> Treatment of SSTIs can be challenging given the emergence of resistance to several antibiotics frequently used in the clinic. One example is the antibiotic fusidic acid, which is prescribed for use topically in Europe for the treatment of SSTIs. Extensive use of fusidic acid has resulted in the emergence of resistant isolates<sup>40</sup> that necessitates new therapeutic agents to treat uncomplicated, localized *S. aureus* SSTIs. In the United States of America, MRSA USA300 is the predominant strain linked to community-acquired SSTIs.<sup>41</sup> As such, we moved to evaluate the efficacy of compound **19** administered topically in a MRSA USA300 skin wound mouse model. Compound **19** significantly reduced the burden of MRSA USA300 in infected abscesses by over 70% (after only five days of treatment) similar to fusidic acid.<sup>42</sup>

## CONCLUSIONS

In conclusion, the present study identified four new aryl isonitrile compounds bearing potent antibacterial activity against MRSA *in vitro*. The analogues were inactive against important Gram-negative bacterial pathogens, and their activity appeared to be negatively impacted by the presence of the outer membrane. The newly synthesized analogues were safe to keratinocytes at concentrations up to 128  $\mu\text{M}$  and significantly reduced the burden of MRSA in infected wounds in a mouse model of MRSA skin infection. However, *in silico* pharmacokinetic simulation revealed compound **19** would not effectively permeate across the GI tract and may extensively distribute into tissues thus precluding its evaluation in a systemic animal model of MRSA infection. Addressing these limitations and deducing the molecular target are necessary questions to resolve to further develop aryl isonitrile compounds as a novel class of antibacterial agents to treat drug-resistant *S. aureus* infections.

## EXPERIMENTAL SECTION

### Biological Methods

**Bacterial strains and reagents**—Relevant information pertaining to all bacterial isolates used in this study are presented in Supplementary Table 1. Clinical isolates of *S. aureus* and *S. epidermidis* were obtained through the Network of Antimicrobial Resistance

in *Staphylococcus aureus* (NARSA) program. Isolates of *S. pneumoniae*, *E. faecium*, *A. baumannii*, *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa* were obtained from the American Type Culture Collection (ATCC). *E. coli* strains BW25113 and JW25113 were obtained from The Coli Genetic Stock Center (CGSC), Yale University. Antibiotics were purchased commercially and dissolved in DMSO (for linezolid), ethanol (for erythromycin), or sterile deionized water (for colistin and vancomycin). Stock 10 mM solutions were prepared for all antibiotics. Brain heart infusion broth (BHI), Tryptic soy broth (TSB), Tryptic soy agar (TSA), phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 96-well plates were all purchased from commercial vendors.

**Evaluation of antibacterial activity of compounds and control antibiotics**—The minimum inhibitory concentration of the aryl isonitrile analogues and control antibiotics was determined using the broth microdilution assay following the Clinical and Laboratory Standards Institute guidelines, with the following modifications.<sup>43</sup> Bacteria were cultured either in TSB or BHI (for *E. faecium*) and exposed to either compounds or control antibiotics, in triplicate, in 96-well plates. For permeabilization of the outer membrane, Gram-negative bacterial pathogens were exposed to a subinhibitory concentration of colistin equivalent to either  $\frac{1}{4} \times \text{MIC}$  (for *A. baumannii*, *E. cloacae*, *E. coli*, and *P. aeruginosa*) or  $\frac{1}{2} \times \text{MIC}$  (for *K. pneumoniae*). Plates were incubated aerobically at 37 °C for 18 – 24 hours before the MIC values were recorded. *S. pneumoniae* was incubated with compounds at 37 °C + 5% CO<sub>2</sub>. The MICs reported represent the lowest concentration of each compound/drug necessary to inhibit visual bacterial growth.

**In silico pharmacokinetic evaluation of 19**—Compound **19**'s pharmacokinetic profile was examined *in silico*, using chemPK version 2.0 (Cyprotex, Inc.) simulating a dose of 600 mg administered both orally and intravenously.

**Toxicity assessment of aryl isonitrile analogues against human keratinocytes**—Compounds **7**, **18**, and **19** were assayed (at concentrations of 16, 32, 64, and 128 μM) against a human keratinocyte (HaCaT) cell line (AddexBio, San Diego, CA, USA) to determine the potential toxic effect to mammalian skin cells *in vitro*, as previously described.<sup>44–48</sup> In brief, cells were cultured in DMEM supplemented with 10% FBS at 37 °C with CO<sub>2</sub> (5%). Control cells received DMSO alone at a concentration equal to that in drug-treated cell samples. The cells were incubated with the compounds (in triplicate) in a 96-well plate at 37 °C with CO<sub>2</sub> (5%) for 24 hours. The assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Madison, WI, USA) was subsequently added and the plate was incubated for four hours. Absorbance readings (at OD<sub>490</sub>) were taken using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound was expressed as a percentage of the viability of DMSO-treated control cells (average of triplicate wells ± standard deviation). The toxicity data was analyzed via a one-way ANOVA, with post hoc Dunnett's multiple comparisons test ( $P < 0.05$ ), utilizing GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

**Evaluation of 19 in a murine model of MRSA skin infection**—The mice study was conducted under the guidelines of the Purdue University Animal Care and Use Committee (PACUC) and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The method used for this study was similar to one described in previous reports, with slight modifications. 44, 49–53 Three groups (n = 5) of eight-week old female Balb/c mice (obtained from Envigo, Indianapolis, IN, USA) received an intradermal injection (40 µL) containing  $1.32 \times 10^9$  CFU/mL MRSA USA300. Following formation of an abscess at the injection site (~48 hours post-infection), topical treatment was initiated subsequently with each group of mice receiving a 2% suspension (formulated in petroleum jelly) of fusidic acid or **19**. One group of mice received the vehicle alone (negative control). Each group of mice was housed separately in a ventilated cage with appropriate bedding, food, and water. Mice were checked at least four times daily during infection and treatment to ensure no adverse reactions were observed. Mice were treated twice daily for five days, before they were humanely euthanized via CO<sub>2</sub> asphyxiation 12 hours after the last dose was administered. Wounds were aseptically extracted and subsequently homogenized in PBS (2 mL). The homogenized tissue was then serially diluted in PBS before plating onto mannitol salt agar plates. Plates were incubated for at least 16 hours at 37 °C before viable CFU were counted and MRSA reduction in the skin wound (relative to the negative control) post-treatment was determined for each group.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements:

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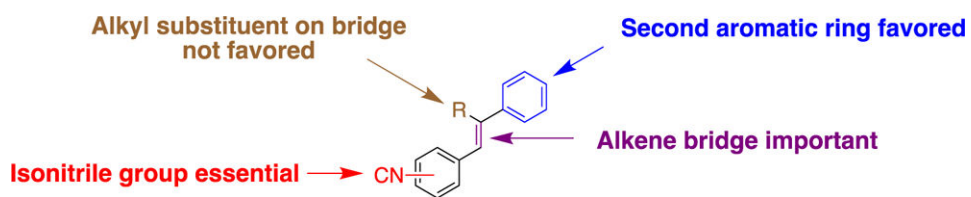
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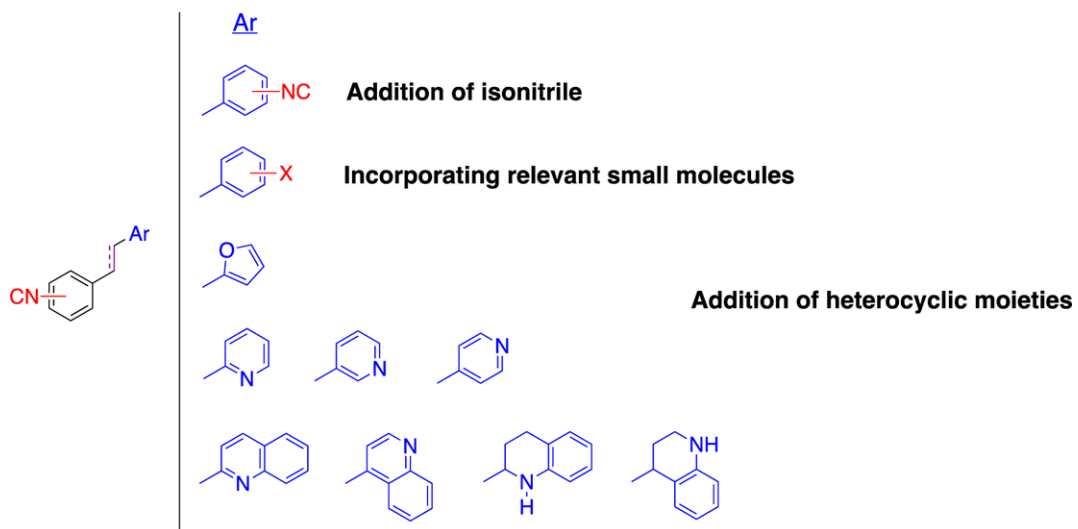
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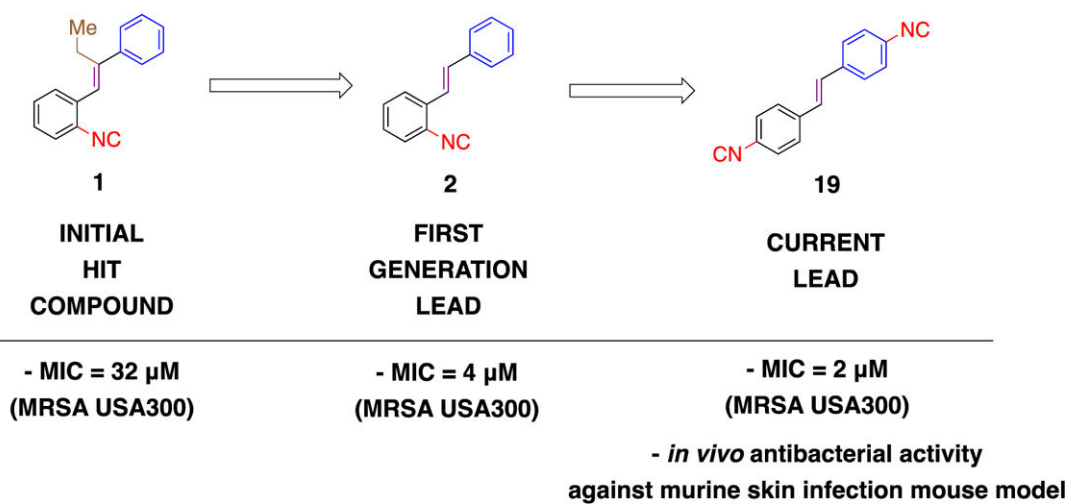


**Figure 1.**  
First generation SAR.

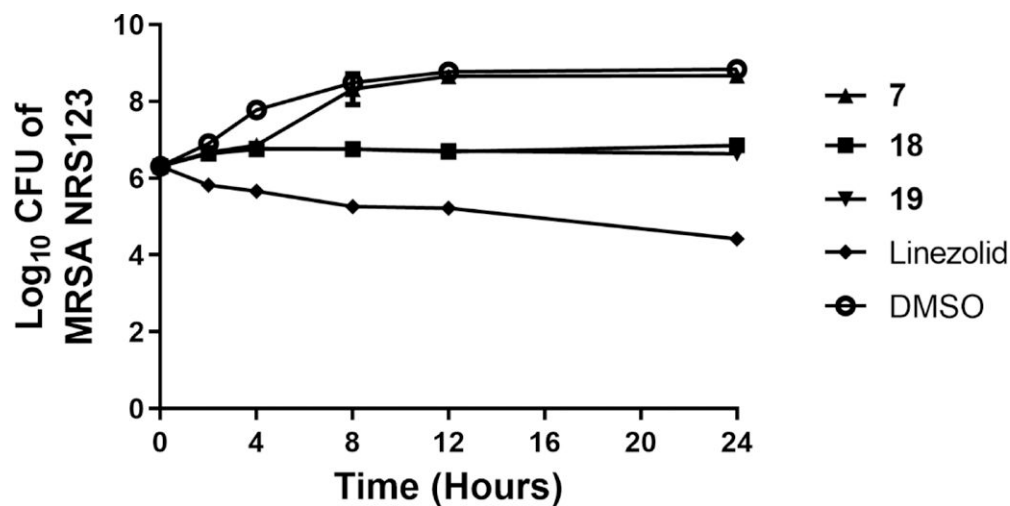




**Figure 2.**  
Design strategy for the second-generation aryl isonitrile compounds.

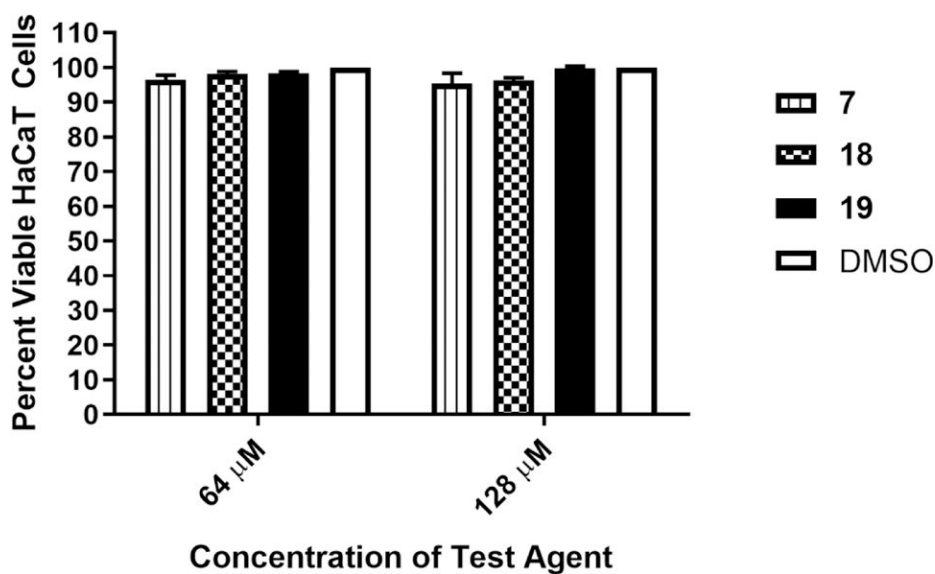


**Figure 3.** Structural evolution leading to current lead aryl isonitrile compound and minimum inhibitory concentration (MIC) values against methicillin-resistant *S. aureus* (MRSA).

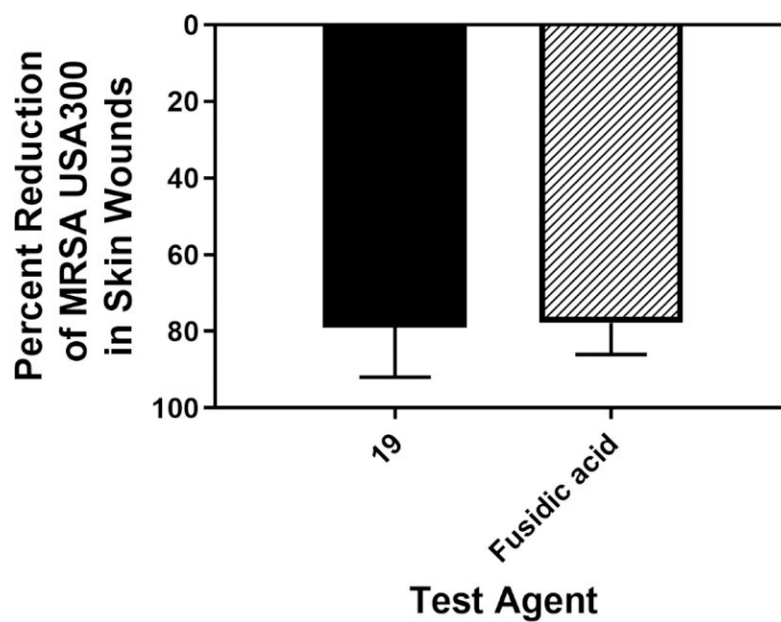


**Figure 4.** Time-kill analysis of aryl isonitrile compounds 7, 18, 19, and linezolid (all at  $4 \times \text{MIC}$ ) against methicillin-resistant *Staphylococcus aureus* (MRSA NRS123) over a 24-hour incubation period at  $37^\circ\text{C}$ .

DMSO served as a negative control. The error bars represent standard deviation values obtained from triplicate samples used for each compound/antibiotic studied.

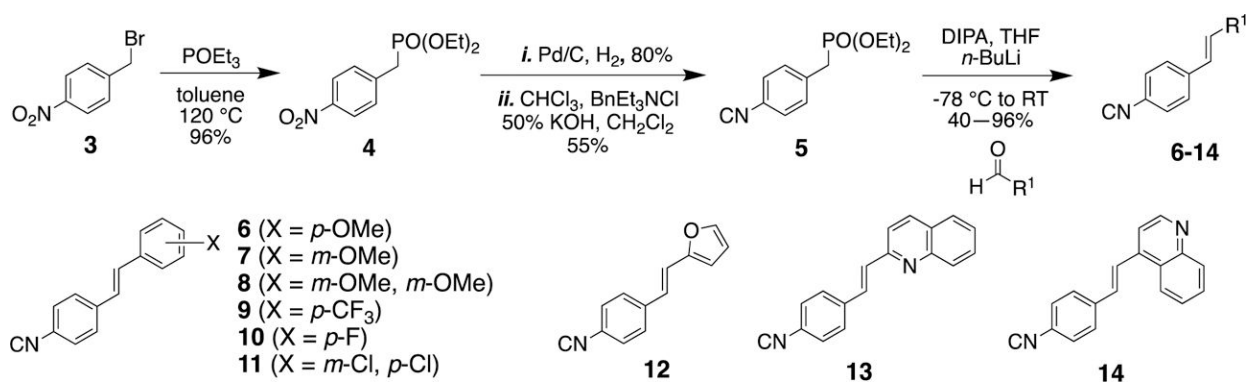


**Figure 5. Toxicity analysis of aryl isonitrile compounds against human keratinocytes (HaCaT).** Percent viable mammalian cells (measured as average absorbance ratio (test agent relative to DMSO)) for cytotoxicity analysis of compounds **7**, **18**, and **19** (tested in triplicate) at 64 and 128 μM against HaCaT cells over a 24 hour period using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Dimethyl sulfoxide (DMSO) was used as a negative control to determine a baseline measurement for the cytotoxic impact of each compound. The absorbance values represent an average of a minimum of three samples analyzed for each compound. Error bars represent standard deviation values. A one-way ANOVA, with post hoc Dunnet's multiple comparisons test, determined statistical difference between the values obtained for each compound and DMSO ( $P < 0.05$ ).

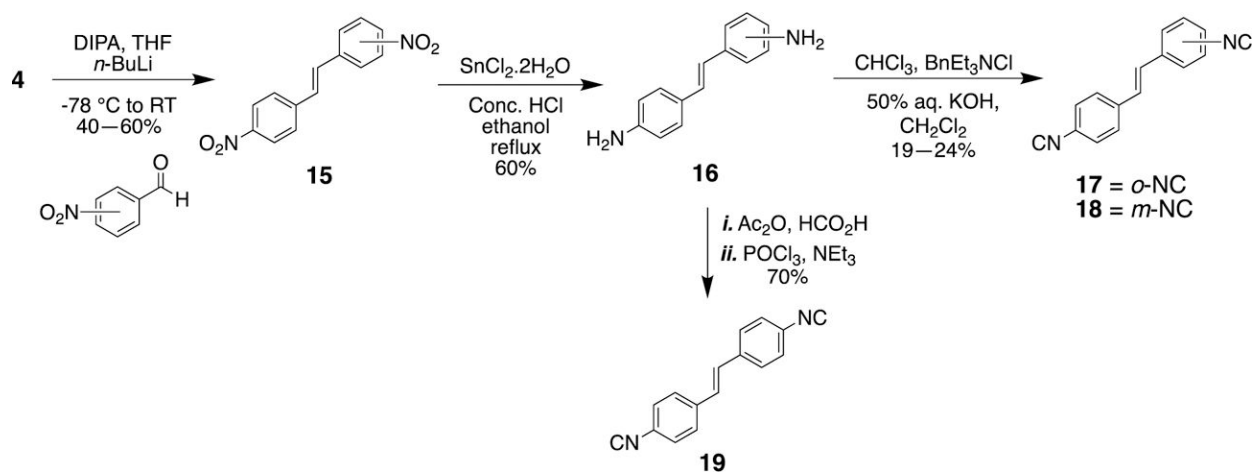


**Figure 6. Reduction of MRSA USA300 in infected lesions of mice.**

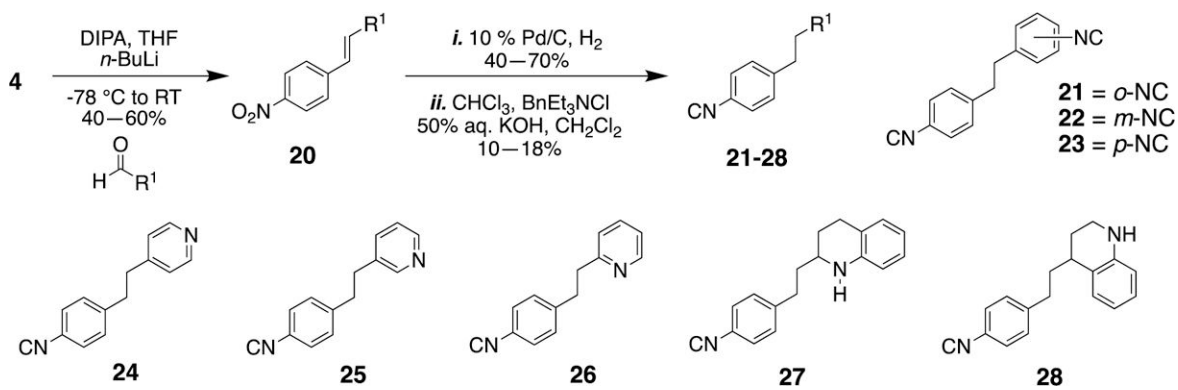
Average percent reduction of MRSA CFU/mL in murine skin lesions after treatment with **19** or fusidic acid. A one-way ANOVA with post-hoc Dunnet's multiple comparisons found no statistical difference between mice treated with fusidic acid and mice treated with compound **19**.



**Scheme 1.**  
Synthesis of stilbene aryl isonitrile compounds.



**Scheme 2.**  
Synthesis of stilbene bis-isonitriles.



**Scheme 3.**  
Synthesis of isonitriles with an alkane bridge.



**Table 1:**

Minimum inhibitory concentration (MIC, in  $\mu\text{M}$ ) of second-generation aryl isonitrile analogues and control antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) NRS123 (USA400).

Compound/Drug Name	MRSA NRS123 (USA 400)
	MIC
6	32
7	2
8	64
9	32
10	64
11	>64
12	32
13	8
14	>64
17	16
18	4
19	8
21	>64
22	32
23	>64
24	64
25	>64
26	>64
27	16
28	32
Linezolid	1
Vancomycin	1

<sup>1</sup>N.D. = Not determined

**Table 2:**

Minimum inhibitory concentration (MIC, in  $\mu\text{M}$ ) of the four most potent aryl isonitrile compounds and control antibiotics against methicillin-sensitive (MSSA), methicillin-resistant (MRSA), linezolid-resistant (LRSA), and vancomycin-resistant *S. aureus* (VRSA).

Compound Name	<i>S. aureus</i> NRS107 (MSSA)	MRSA NRS119 (LRSA)	MRSA NRS384 (USA300)	MRSA NRS385 (USA500)	VRS10 (VRSA)	VRS11a (VRSA)
<b>7</b>	2	1	1	4	2	4
<b>13</b>	N.D. <sup>1</sup>	4	4	N.D.	32	N.D.
<b>18</b>	16	4	4	16	8	16
<b>19</b>	4	1	2	8	8	8
<b>Linezolid</b>	2	32	2	4	2	2
<b>Vancomycin</b>	1	1	1	1	>64	>64

<sup>1</sup>N.D. = Not determined

**Table 3:**

Minimum inhibitory concentration (MIC, in  $\mu\text{M}$ ) of the three most potent aryl isonitrile compounds and control antibiotics against clinically-relevant Gram-positive and Gram-negative bacterial pathogens.

Test Agent	Bacterial Strain							
	<i>S. epidermidis</i> NRS101	<i>S. pneumoniae</i> ATCC 700677	<i>E. faecium</i> ATCC700221	<i>A. baumannii</i> BAA-1605	<i>E. cloacae</i> BAA-1134	<i>K. pneumoniae</i> ATCC BAA-1705	<i>Pseudomonas aeruginosa</i> ATCC 15442	
7	1	4	>64	>64	>64	>64	>64	
18	1	16	>64	>64	>64	>64	>64	
19	1	8	>64	>64	>64	>64	>64	
Erythromycin	N.D. <sup>1</sup>	N.D.	N.D.	16	>64	>64	>64	
Linezolid	2	2	0.50	>64	>64	>64	>64	
Vancomycin	4	1	>64	32	>64	>64	>64	

<sup>1</sup>N.D. = Not determined

**Table 4:**

Minimum inhibitory concentration (MIC, in  $\mu\text{M}$ ) of 19 and control antibiotics against Gram-negative bacterial pathogens in the presence of a subinhibitory concentration of colistin (COL).

Test Agent	Bacterial Strain							
	<i>A. baumannii</i> BAA-1605	<i>E. cloacae</i> BAA-1134	<i>K. pneumoniae</i> ATCC BAA-1705	<i>P. aeruginosa</i> ATCC 15442	<i>E. coli</i> BW25113		<i>E. coli</i> JW25113 ( <i>tolC</i> )	
					(-COL) <sup>I</sup>	(+COL)	(-COL)	(+COL)
<b>19</b>	16	4	>64	>64	>64	>64	>64	>64
Erythromycin	4	1	16	4	64	1	2	0.50
Linezolid	>64	16	>64	>64	>64	>64	16	16

<sup>I</sup>No colistin added to the media

**Table 5:** *In silico* pharmacokinetic analysis for compound **19** and linezolid (simulated at 600 mg).

	Oral		Linezolid (Experimental) <sup>1</sup>	Intravenous	
	19 (Simulated)	Linezolid (Simulated)		19 (Simulated)	Linezolid (Simulated)
$C_{max}$ <sup>2</sup> (µg/mL)	0.17	5.33	12.7	-	13.4
$t_{max}$ <sup>3</sup> (hours)	3	2.75	1.33	-	0.5
Fraction absorbed (FA <sub>abs</sub> )	0.80	0.92	1.03	-	-
CL <sup>4</sup> (mL/min-kg)	-	-	-	12.58	1.74 <sup>9</sup>
$t_{1/2}$ <sup>5</sup> (hours)	-	-	-	22.90	4.40
MRT <sup>6</sup> (hours)	-	-	-	12.82	-
$V_d$ <sup>7</sup> (L/kg)	-	-	-	24.94	0.58 <sup>10</sup>
$V_{ss}$ <sup>8</sup> (L/kg)	-	-	-	9.68	-

<sup>1</sup> (adapted from Stalker DJ *et al. J Antimicrob Chemother* **2003**, 51: 1239–46 (Table 2, 625 mg dose))

<sup>2</sup>  $C_{max}$  = maximum concentration of drug in plasma/blood

<sup>3</sup>  $t_{max}$  = time required to reach  $C_{max}$

<sup>4</sup> CL = rate of clearance

<sup>5</sup>  $t_{1/2}$  = half-life

<sup>6</sup> MRT = mean residence time

<sup>7</sup>  $V_d$  = volume of distribution

<sup>8</sup>  $V_{ss}$  = volume of distribution at steady-state

<sup>9</sup> Clearance for linezolid (experimental) obtained by dividing the mean clearance value by the mean weight of patients

<sup>10</sup> Volume of distribution for linezolid (experimental) obtained by dividing the mean  $V_d$  by the mean weight of patients