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Isothiazolone–Nitroxide Hybrids with Activity against Antibiotic-Resistant *Staphylococcus aureus* Biofilms

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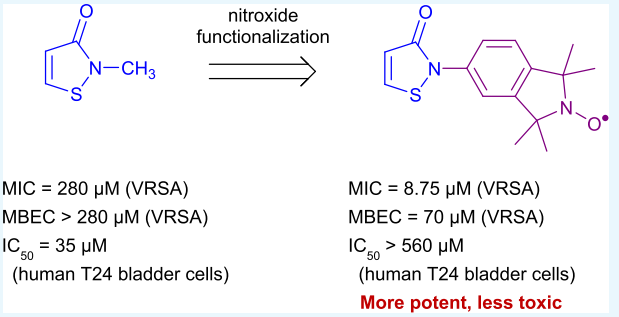
ABSTRACT: Isothiazolones are widely used as biocides in industrial processing systems and personal care products, but their use to treat infections in humans has been hampered by their inherent cytotoxicity. Herein, we report a strategy to alleviate isothiazolone toxicity and improve antibacterial and antibiofilm potency by functionalization with a nitroxide moiety. Isothiazolone–nitroxide hybrids **6** and **22** were prepared over three steps in moderate yields (58 and 36%, respectively) from (*Z*)-3-(benzylsulfanyl)-propenoic acid. Hybrid **22** displayed better activity (minimum inhibitory concentration (MIC) = 35 μM) than the widely used methylisothiazolinone (MIT **1**, MIC = 280 μM) against methicillin-susceptible *Staphylococcus aureus* (MSSA). Hybrid **22** was even more active against drug-resistant strains, such as vancomycin-resistant *Staphylococcus aureus* (VRSA, MIC = 8.75 μM) over MIT **1** (MIC = 280 μM). The enhanced antibacterial activity of hybrid **22** over MIT **1** was retained against established MSSA and VRSA biofilms, with minimum biofilm eradication concentration (MBEC) values of 35 and 70 μM, respectively, for **22** (the MBEC value for MIT **1** against both strains was ≥280 μM). No toxicity was observed in human epithelial T24 cells treated with hybrid **22** in concentrations up to 560 μM using a lactate dehydrogenase assay.



INTRODUCTION

The global emergence of infectious pathogens resistant to many first-line medicines is of great concern and could have serious health, economic, and social implications. Accordingly, the World Health Organization (WHO) has prioritized antibiotic-resistant pathogens for the research and development of new antibiotics, classifying *Staphylococcus aureus* as a high-priority antibiotic-resistant pathogen.[1] *S. aureus* is a Gram-positive bacterium commonly associated with persistent, chronic, and recurrent infections, particularly of the skin and soft tissue[2,3] and indwelling medical devices.[4,5] The incidence of *S. aureus* ranges from 20 to 50 cases/100,000 population per year, with a 10–30% mortality rate (a greater number of deaths than for AIDS, tuberculosis, and viral hepatitis combined).[6] Commonly evolved resistance mechanisms in *S. aureus* make infections difficult to treat with generally prescribed antibiotics such as penicillin and vancomycin, because of the prevalence of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA).[7,8] A major factor contributing to the propensity of *S. aureus* infections persisting despite in principal adequate antibiotic therapy is the formation of biofilms, which are surface-aggregated communities of bacteria enveloped in a self-produced extracellular matrix.[9] In an established biofilm, bacteria are less susceptible to antibiotics and can detach, or aggregates may slough off, causing the spread of infection in the host.[10] Thus, there is a huge demand for the development of novel antibiofilm strategies against which *S. aureus* does not show resistance.[11]

One recent approach to target biofilms is to utilize agents that trigger the dispersal of biofilm-residing cells back into their planktonic and antibiotic susceptible state. A number of molecules that disperse biofilms have been reported including quorum-sensing inhibitors such as *N*-acyl homoserine lactones,[12] amino acids,[13,14] nitric oxide,[15] and nitroxides.[16] While biofilm dispersal is a promising strategy toward biofilm eradication, most biofilm dispersal agents are inherently nonantimicrobial and should ideally be coadministered with an antimicrobial agent to limit the spread of infection. Accordingly, biofilm eradication agents[17] have been developed by directly linking the dispersal agent to the antibiotic.[18−20] Alternatively, antibiofilm activity has been demonstrated by agents that specifically target cells within biofilms such as quaternary ammonium compounds,[21] antibiofilm peptides,[22−24] and macromolecular agents.[25]

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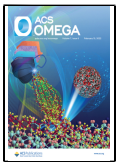


In our recent work, we developed antibiofilm agents by incorporating nitroxides into the structure of antibiotics.[18,19,26,27] Nitroxides are stable free radical species that contain a disubstituted nitrogen atom linked to a univalent oxygen.[28] They are widely used as potent antioxidants in biological systems,[29−32] as a result of their ability to undergo redox and radical trapping reactions, thereby reducing the levels of oxidative stress in cellular systems. In the context of bacterial biofilms, we have demonstrated that nitroxides can act as dispersal agents[33,34] and can be tethered to fluoroquinolone antibiotics to completely eradicate *Pseudomonas aeruginosa*, *Escherichia coli*, and *S. aureus* biofilms.[18,19,26,27] For *S. aureus*, this was the first demonstration of nitroxide-mediated dispersal of a Gram-positive biofilm, and our hybrid approach resulted in better biofilm eradication than coadministration of the individual components.[11]

In this work, we aimed to extend our nitroxide hybridization approach to isothiazolone-based compounds. Isothiazolones[35] such as MIT **1** and CMIT **2** (Figure 1) have long been

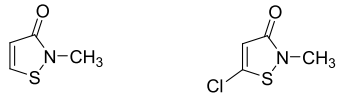


Figure 1. Chemical structures of methylisothiazolone (MIT) **1** and methylchloroisothiazolone (CMIT) **2**.

exploited in industrial and household settings as preservatives with antibacterial and antifungal activity. They have been widely used for more than 30 years as biocides in wastewater treatment processes, cosmetics, liquid soaps, shampoos, paints, and detergents. For example, the commercial biocide Kathon CG consists of a 1:3 mixture of the isothiazolones **1** and **2**.

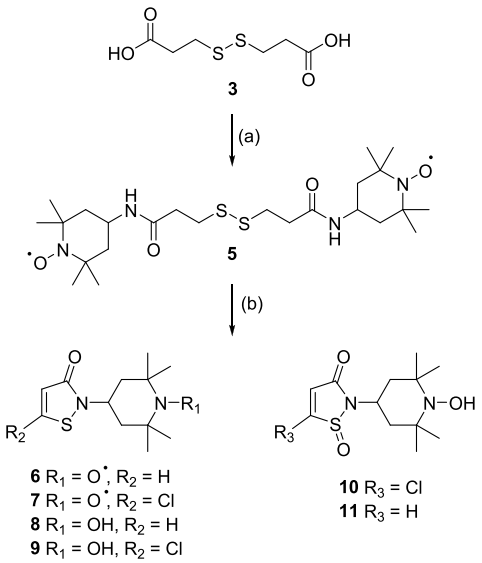
Despite the effectiveness of isothiazolones against Gram-positive and Gram-negative bacteria, yeast, and fungi, they are not currently approved by the Food and Drug Administration as antibiotics as their use for humans has been hindered by their inherent cytotoxicity and their propensity to cause hypersensitivity.[36−38] Isothiazolones are known to gain intracellular access by diffusing across the cell membrane of bacteria or the cell wall of fungi.[39] Once inside the cell, the electron-deficient sulfur atom of the isothiazolone moiety can react with cellular components, which contain nucleophilic groups, such as the thiol moieties of cysteine units, subsequently impairing their activity and causing cell death.[35,39,40] The low propensity of pathogens to develop resistance to these drugs makes them ideal antibiotic candidates, provided the human toxicity issues can be addressed. In this work, we sought to develop potent isothiazolone-based antibiofilm agents with minimal human cytotoxicity.

We hypothesized that covalently linking isothiazolones with nitroxides would provide a hybrid structure that could eradicate biofilms (through biofilm dispersal and eradication of the resulting planktonic cells), but that the hybrid also had the potential to facilitate bacterial cell entry and reduce human cytotoxicity. We have previously documented the ability of cyclic α-tetrasubstituted nitroxides to facilitate the entry of nitroxide-functionalized antibiotics into the cytoplasm of *S. aureus* cells.[41] Furthermore, nitroxides have previously demonstrated protective properties toward human cells[42,43] and they have well-recognized low toxicity at biologically relevant concentrations.[44] Hence, we sought to exploit these findings and herein report the synthesis and evaluation of isothiazolone−nitroxide hybrids with good planktonic and antibiofilm activity against *S. aureus* and minimal human cytotoxicity.

RESULTS AND DISCUSSION

Chemistry. In the design of our isothiazolone−nitroxide hybrids, we considered the various positions surrounding the isothiazolone core for nitroxide functionalization. However, functionalization at the nitrogen atom, to generate *N*-substituted isothiazolones, is one of the most common sites for the generation of biologically active isothiazolones.[45−47] A widely used method to produce *N*-substituted isothiazolones is through chlorine-induced oxidative cyclization of 3,3′-dithiodipropioamides (Scheme 1), a method documented by Szamborski and co-workers in 1971.[47] Utilizing this methodology, we sought to produce nitroxide-functionalized isothiazolones.

Scheme 1. Synthesis of *N*-Substituted Isothiazolones from 3,3′-Dithiodipropionic Acid 3[a]



[a]*Reagents and conditions:* (a) (i) $SOCl_2$, pyridine, anhydrous toluene, 85 °C, and O/N, (ii) 4-amino-TEMPO **4**, $^{i}Pr_2NEt$, anhydrous dichloromethane (DCM), R.T., and 2 h. (b) SO_2Cl_2, anhydrous toluene, 0 °C, 20 min, then R.T., and 36 h. Yields: **6** (1% isolated), **7** (1% isolated), and **8**−**11** (could not be isolated in appreciable amounts, detected only by mass spectrometry).

Here, 3,3′-dithiodipropionic acid **3** was converted to the corresponding acid chloride *in situ* and then added to 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO **4**) to generate 3,3′-dithiodipropioamide **5** (Scheme 1). Subsequent cyclization of **5** with sulfuryl chloride gave the desired isothiazolone-TEMPO products **6** and **7** but only in trace amounts (isolated yields of 1% for each compound) (Scheme 1). Further analysis of the crude reaction mixture by mass spectrometry identified the presence of additional oxidized and reduced derivatives **8**−**11** of hybrids **6** and **7** (Scheme 1).

To probe the low yield of desired isothiazolone hybrids **6** and **7** from the oxidative cyclization of 3,3′-dithiodipropioamide **5**, we investigated the stability of the nitroxide moiety in the presence of sulfuryl chloride. Treatment of a DCM solution of 2,2,6,6-tetramethylpiperidin-1-yl-oxyl (TEMPO)

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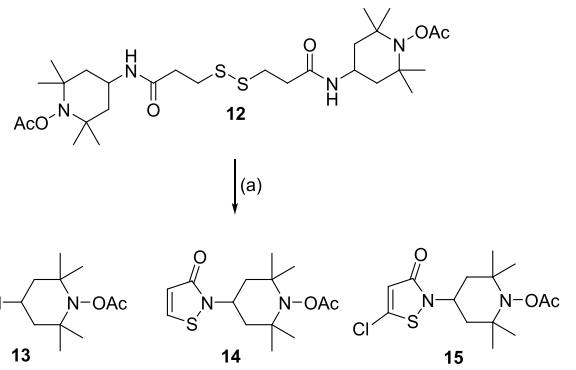
with sulfuryl chloride followed by the addition of 2-propanol gave the corresponding hydroxylamine (R_2N-OH) and acetone (both species were detected by ^1H NMR spectroscopy and mass spectrometry; electron paramagnetic resonance (EPR) spectroscopy showed a loss of the typical hyperfine triplet signal for the nitroxide radical). This observation revealed that, in the presence of sulfuryl chloride, the nitroxide is easily oxidized to its corresponding oxoammonium ion which can act as a potent oxidizing agent.

To circumvent the undesired reactivity of the nitroxide with sulfuryl chloride, the nitroxide moiety of 3,3′-dithiodipropioamide **5** was protected as an acetate following standard literature procedures.[31] Subsequent treatment of 3,3′-dithiodipropioamide **12** with sulfuryl chloride (Table 1, entry 2) gave

Table 1. Optimization of the Sulfuryl Chloride-Mediated Cyclization of 3,3′-Dithiodipropioamide 12 in Toluene to Form N-Substituted Isothiazolones[a]



entry	SO_2Cl_2 (equiv)	SO_2Cl_2 addition time (min)	reaction time (h)	isolated yields (%) 13	14	15
1	2	120	36	10	20	0
2	3	20	36	20	20	31
3	3	360	36	39	14	14
4	5	20	36	23	13	55

[a]Reagents and conditions: (a) SO_2Cl_2 and anhydrous toluene, see below for reaction conditions and corresponding yields of **13**–**15**.

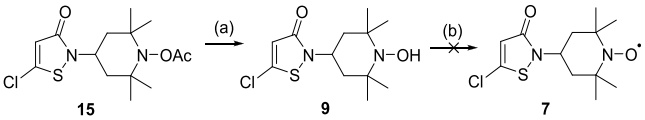
the acetyl-protected hybrids **13**, **14**, and **15** in modest isolated yields (20–31%). As sulfuryl chloride-mediated cyclization of 3,3′-dithiodipropioamide **12** generated products **13**–**15** in different proportions, we investigated a range of reaction conditions to optimize the formation of the desired products **14** and **15**.

The rate of addition and the equivalents of sulfuryl chloride used were found to significantly alter the final product ratios (Table 1). As such, if isothiazolone **14** was desired, a 2 h addition rate of sulfuryl chloride (2 equiv) should be adopted (Table 1, entry 1), while faster addition rates with higher equivalents of sulfuryl chloride will favor the 5-chloro isothiazolone **15** (Table 1, entry 4). Interestingly, the amount of isothiazolidinone **13** remained relatively constant (~20%) regardless of the equivalents of sulfuryl chloride used. However, extending the addition rate of sulfuryl chloride to 6 h resulted in a product ratio shift to favor the formation of isothiazolidinone **13** (39%, Table 1, entry 3). The literature suggests that a likely mechanistic pathway for *N*-substituted isothiazol-3(2*H*)-one formation via the chlorination-cyclization of 3,3′-dithiodipropioamides involves chlorination of an isothiazolidinone such as **13** followed by dehydrochlorination.[47] Thus we were intrigued to determine if isothiazolidinone **13** was in fact an intermediate in the reaction pathway to isothiazolones **14** and **15** or simply a dead-end side product.

In our hands, the treatment of isothiazolidinone **13** with sulfuryl chloride did not convert **13** into either of the desired isothiazolone products **14** and **15**. Likewise, treatment of isothiazolone **14** or **15** with sulfuryl chloride did not yield the 5-chloro isothiazolone **15** or the related 4,5-dichloro isothiazolone. These findings suggest that isothiazolidinone **13** is in fact a dead-end side product and imply that the 5-chloro substituent must be introduced prior to the cyclization step to form the isothiazolidinone core (a finding also reached by Lewis for the formation of the analogous 5-chloro isothiazolones).[47]

With the protected isothiazolones **14** and **15** now in hand, we attempted to remove the acetyl protecting groups via base-mediated hydrolysis, utilizing previously documented conditions.[31] However, treatment of isothiazolone **14** or **15** with NaOH did not cleave the acetyl protecting groups (as assessed by thin-layer chromatography (TLC) and mass spectrometry). As an alternative, acid-mediated hydrolysis of isothiazolone **15** gave the corresponding hydroxylamine derivative **9** (Scheme 2), which was surprisingly stable and did not convert to the

Scheme 2. Acid-Mediated Ester Hydrolysis of Compound 15[a]



[a]*Reagents and conditions*: (a) 2 M HCl, MeOH, 45 °C, and overnight; (b) air, PbO_2, *m*CPBA, and Na_2WO_4/H_2O_2.

desired nitroxide **7** when bubbled with air for several days. The observed stability of the hydroxylamine presumably arises from the electron-withdrawing nature of the isothiazolone ring which renders the radical more reduction-prone.[48] Attempts to expose **9** to stronger oxidizing conditions such as treatment with *m*CPBA, PbO_2, or Na_2WO_4/H_2O_2 favored oxidation of the sulfur atom in the isothiazolone ring rather than formation of nitroxide **7**. Consequently, we sought an alternative method for generating our desired isothiazolone–nitroxide hybrids.

We investigated the use of the acid-mediated ring closing reaction of *N*-substituted (*Z*)-3-(benzylsulfinyl) propenamides[45] to form the desired isothiazolone hybrid **6** (alternative synthetic procedures to the 5-chloro isothiazolones are limited). In this approach, (*Z*)-3-(benzylsulfanyl)-propenoic acid **16** was activated by conversion to the corresponding phosphinic ester in situ and allowed to react directly with amine-functionalized nitroxides (4-amino-TEMPO **4** or 4-amino-1,1,3,3,-tetramethylisoindolin-1-oxyl (4-amino-TMIO, **17**) (Scheme 3). Amides **18** and **19** were isolated in high yields of 90 and 85% respectively. Oxidation of amide **18** with *m*CPBA gave the corresponding sulfoxide **20** in high yield (92%); however, the final cyclization of **20** in the presence of trifluoroacetic anhydride (TFAA) was unsuccessful. The nitroxide moiety of **20** may have reacted directly with the cyclization reagent, TFAA, as **20** was no longer present following TLC analysis, and product **6** could not be detected by mass spectrometry. Furthermore, attempted cyclization of **20** with alternative reagents (such as thionyl chloride[49] and

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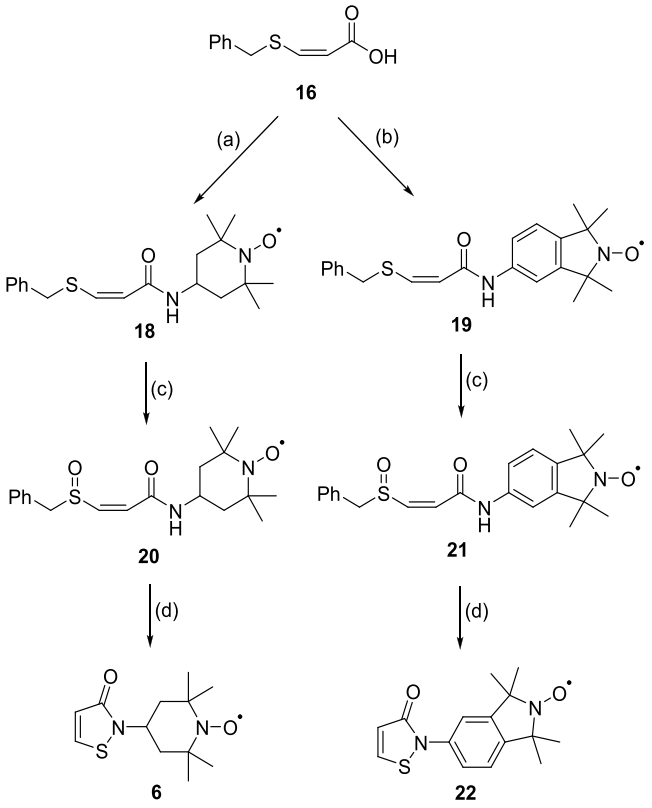
Scheme 3. Alternative Synthetic Pathway to Isothiazolone−Nitroxide Hybrids 6 and 22[a]



[a]*Reagents and conditions:* (a) (i) Ph_2POCl, ethylmorpholine, DCM, -10 °C, and 1 h; (ii) 4-amino-TEMPO **4**, DCM, R.T., and O/N; (b) (i) Ph_2POCl, ethylmorpholine, DCM, -10 °C, and 1 h; (ii) 4-amino-1,1,3,3-tetramethylisoindolin-1-oxyl **17**, DCM, R.T., and O/N, (c) *m*CPBA, DCM, −10 °C, and 10 min.; (d) AcCl, lutidine, DCM, R.T., O/N. Yields: **18** (90%), **19** (85%), **20** (92%), **21** (70%), **6** (70%), and **22** (60%).

trifluoromethanesulfonic anhydride[50]) failed to give the desired product **6**.

The cyclization mechanism of (*Z*)-3-(benzylsulfinyl) propenamides, detailed by Hedger and colleagues,[45] indicates that the role of the cyclization reagent is to convert the oxygen atom of the sulfoxide into an ester leaving group. Thus, we envisioned that the addition of acetyl chloride to the reaction could potentially generate the required ester leaving group without reacting with the nitroxide moiety. Pleasingly, treatment of sulfoxide **20** with acetyl chloride generated the desired isothiazolone−nitroxide hybrid **6** in good yield (70%). Using the same methodology, amide **19** was converted smoothly to the corresponding sulfoxide **21** in good yield (70%) and then cyclized with acetyl chloride to form the isoindoline nitroxide-based hybrid **22** in moderate yield (60%).

Biological Evaluation. With our isothiazolone−nitroxide hybrids **6** and **22** in hand, we sought to investigate their antibacterial activity. As our isothiazolone−nitroxide hybrids were designed as antibiotics to target *S. aureus* cells, we initially screened hybrids **6** and **22** against methicillin-susceptible *S. aureus* (MSSA). Hybrids **6** and **22** both exhibited moderate activity against MSSA (minimum inhibitory concentration (MIC) = 140 and 35 μM, respectively) (Table 2). The TEMPO-based hybrid **6** was at least 2-fold more active against MSSA than the comparable compound, MIT **1** (MIC = 280 μM), while the TMIO-based hybrid **22** was at least 8-fold more active than MIT **1** (Table 2). As a control and to confirm the essentiality of the free radical nitroxide, we also examined hybrid **14** (acetyl-protected derivative of hybrid **6**) for activity against MSSA. Unlike hybrid **6**, conjugate **14** was completely devoid of activity (MIC >1000 μM), confirming that the free radical nitroxide is required for antimicrobial activity. To further explore the antistaphylococcal activity of hybrid **22**, we determined its MIC against MRSA and VRSA. Hybrid **22** retained moderate activity against MRSA with an MIC similar to that observed for MSSA (MIC = 35 μM) (Table 2), indicating that it is impervious to existing resistance mechanisms present in this WHO high-priority pathogen under planktonic test conditions. Intriguingly, hybrid **22** showed markedly improved activity against VRSA (MIC = 8.75 μM) and was at least 32-fold more active than MIT **1** against this pathogen (Table 2). Hybrids **6** and **22** were further screened for antimicrobial activity against other clinically important pathogens, including *E. coli*, *P. aeruginosa*, and *E. faecalis* (Table 2); however, antibacterial activity was only evident against *E. faecalis* (MIC = 310 and 140 μM, respectively). Interestingly, it appears that the addition of the bulky nitroxide to the isothiazolone core may have significantly impacted the ability of the resulting hybrids **6** and **22** to enter Gram-negative cells. This finding supports our original hypothesis that adding a nitroxide to the core structure of an isothiazolone would prevent diffusion into some cells while facilitating entry into others.

Table 2. MIC and Minimum Biofilm Eradication Concentration (MBEC) Values for MIT 1 and Isothiazolone Hybrids 6 and 22

	MIC (μM)			MBEC (μM)	
strain	**6**	**22**	**1**	**22**	**1**
MSSA	140	35	280	35	280
MRSA		35	280	280	>280
VRSA		8.75	280	70	>280
P. aeruginosa	2500	>2500	174		
E. coli	2500	>2500	174		
E. faecalis	310	140	87		

While hybrids **6** and **22** exhibited moderate-to-potent antibacterial activity against planktonic *S. aureus* cells, human infections involving this pathogen often involve recalcitrant *S. aureus* communities known as biofilms.[51] Biofilms are notoriously difficult to treat and are considered one of the leading factors contributing to the failure of antibiotic treatment and persistence of chronic infections.[52] Hence, we considered whether the increased antistaphylococcal activity of hybrid **22** would also extend to eradicating established biofilms. Hybrid **22** was subsequently assessed for biofilm eradication of *S. aureus* biofilms established using the MBEC device (formerly the Calgary Biofilm Device). Treatment with hybrid **22** at a concentration of only 35 μM resulted in complete eradication (99.9%) of MSSA biofilms (MBEC = 35 μM), while VRSA biofilms could also be completely eradicated with 70 μM hybrid **22** (Table 2). While antibiofilm activity was not observed against MRSA, our results importantly indicate that the potency of hybrid **22** is retained against both planktonic and biofilm *S. aureus*, a finding which is extremely rare for most of the currently prescribed antibiotics.[53] Furthermore, the potency of hybrid **22** is comparable to that of other promising biofilm eradication agents currently being

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investigated for the treatment of *S. aureus* biofilms, such as halogenated phenazines (MBEC, < 10 μM)[54] and quaternary ammonium compounds (MBEC, 25 μM).[21]

With the antibacterial activity of hybrid **22** confirmed, we next sought to explore its cytotoxicity for human cells. We assessed hybrid **22** for toxicity against human epithelial T24 cells, using a lactate dehydrogenase (LDH) assay. No cytotoxicity was observed following 24 h of cell exposure to hybrid **22** at concentrations of up to 560 μM (IC_{50} > 560 μM). Importantly, when the comparable compound MIT **1** was examined under the same conditions, significant cytotoxicity was evident at concentrations as low as 35 μM. This finding is in close agreement with a recent study that investigated the toxicity of MIT **1** and several other isothiazolone derivatives against human epithelial-like HepG2 cells.[55] Xu and colleagues produced dose−response curves for several isothiazolones, including MIT **1**, and reported the IC_{50} of MIT **1** as 73 μM, while other derivatives ranged from 32 to 58 μM.[55] Taken together, these results strongly suggest that the addition of a nitroxide to the isothiazolone core can significantly reduce its human cytotoxicity.

CONCLUSIONS

Isothiazolone-based nitroxides were studied for their potential as antistaphylococcal agents for the treatment of human infections. To prepare the desired isothiazolone-TEMPO hybrids **6** and **7**, chlorine-induced oxidative cyclization of 3,3′-dithiodipropioamides was first investigated. The reaction of 3,3′-dithiodipropioamide **12** with sulfuryl chloride gave the acetyl-protected hybrids **14** and **15** in yields of 20 and 31%, respectively. Base-mediated hydrolysis was unable to remove the acetyl protecting groups of **14** or **15**. Acid-mediated hydrolysis of **15** gave the corresponding hydroxylamine derivative **9**, but it could not be converted to the desired nitroxide **7**. As an alternative synthetic strategy, the acid-mediated ring closing reaction of *N*-substituted (*Z*)-3-(benzylsulfinyl) propenamide was examined. Treatment of the phosphinic ester of (*Z*)-3-(benzylsulfanyl)-propenoic acid **16** with 4-amino-TEMPO **4** or 4-amino-TMIO **17** gave the desired amides **18** and **19** in high yields of 90 and 85%, respectively. Oxidation of **18** and **19** with *m*CPBA gave the corresponding sulfoxides **20** and **21** in high yield (92 and 70% respectively), which could be cyclized with acetyl chloride to form the desired isothiazolone−nitroxide hybrids **6** and **22** in good yield (70 and 60% respectively). Evaluation of the antibacterial activity of hybrids **6** and **22** revealed that hybrid **22** displayed better activity (MIC = 35 μM) than the parent isothiazolone MIT **1** (MIC = 280 μM) against planktonic MSSA. Improvement in the activity of hybrid **22** over MIT **1** was also observed against the drug-resistant strain (VRSA) (MICs = 8.75 and 280 μM, respectively). The activity of hybrid **22** over MIT **1** was also retained against *S. aureus* biofilms, but its potency was strain-specific. Hybrids **6** and **22** were also screened against other bacterial species (*E. coli*, *P. aeruginosa*, and *E. faecalis*) with antibacterial activity only evident for *E. faecalis*. No toxicity was observed for **22** up to concentrations of 560 μM against human epithelial T24 cells, while MIT **1** displayed significant cytotoxicity at concentrations as low as 35 μM. These results suggest that the addition of a nitroxide to the isothiazolone core improves its antibacterial and antibiofilm potency while reducing human cell toxicity.

EXPERIMENTAL SECTION

Materials. Synthetic reactions of an air-sensitive nature were carried out under an atmosphere of ultrahigh-purity argon. Anhydrous DCM was obtained from the solvent purification system, Pure Solv Micro, by Innovative Technologies. All other reagents were purchased from commercial suppliers and used without further purification. 3,3′-Dithiodipropionic acid **3**, methylisothiazolone (MIT) **1**, and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO) **4** were purchased from Sigma-Aldrich. (*Z*)-3-(Benzylsulfanyl)-propenoic acid **16**[45] and 4-amino-1,1,3,3,-tetramethylisoindolin-1-oxyl **17**[56] were prepared using documented procedures.

Methods and Instrumentation. All ^{1}H NMR and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively, on a Bruker Avance 600 instrument. Spectra were obtained in the following solvents: $CDCl_3$ (reference peaks: ^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.2 ppm) and $CDOD_3$ (reference peaks: ^{1}H NMR: 3.31 ppm; ^{13}C NMR: 49.0 ppm). All NMR experiments were performed at room temperature. Chemical shift values (δ) are reported in parts per million (ppm) for all ^{1}H NMR and ^{13}C NMR spectral assignments. ^{1}H NMR spectroscopy multiplicities are reported as: s = singlet, br. s = broad singlet, d = doublet, dd = doublet of doublets, and m = multiplet. Coupling constants are reported in Hz. All spectra are presented using MestReNova 11.0. Spectra reported for nitroxides typically contain broadened signals (or signals broadened to the baseline) because of their paramagnetic nature. The purity of all final compounds was determined to be 95% or higher using a Dionex Ultimate 3000 RSLC coupled to a Thermo Fischer Scientific Orbitrap Elite mass spectrometer, equipped with an Agilent prep C18 scalar column (10 μm, 4.6 × 150 mm, eluting 10−80% MeCN (0.1% formic acid) with a coeluent of water (0.1% formic acid) over 20 min). EPR spectra were obtained with the aid of a miniscope MS 400 Magnettech EPR spectrometer. Column chromatography was performed using a LC60A 40−63 Micron DAVISIL silica gel. TLC was performed on Merck Silica Gel 60 F_{254} plates. TLC plates were visualized under a UV lamp (254 nm) and/or by development with a phosphomolybdic acid stain.

Bacterial Strains, Culture Conditions, and Human Cell Culture. *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, methicillin-sensitive *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 33591, and vancomycin-resistant *Staphylococcus aureus* HIP11714 were grown routinely in Lysogeny broth (LB) medium with shaking (200 rpm) at 37 °C. MIC assays were conducted in Mueller Hinton (MH) medium (OXOID, Thermo Fisher). Biofilms were grown in LB medium, and biofilm challenges (antimicrobial susceptibility testing) were performed in MH medium. Human bladder epithelial cell line T24 (ATCC HTB-4) was cultured in McCoy's 5A modified medium (Life Technologies, Gibco, Australia) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Gibco, Australia) at 37 °C in a humidified atmosphere of 5% CO_2 until 90% confluency was reached.

Synthesis of 3,3′-Dithiobis(*N*-(2,2,6,6-tetramethylpiperidin-4-yloxyl)propanamide) 5. 3,3′-Dithiodipropionic acid **3** (100 mg, 0.476 mmol, 1 equiv), $SOCl_2$, (0.138 mL, 1.904 mmol, 4 equiv), and pyridine (0.1 mL, 1.24 mmol, 2.6 equiv) were added to anhydrous toluene (5 mL) under an atmosphere of argon and heated at reflux for 2 h. Excess $SOCl_2$

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was removed in vacuo to give a light-yellow oil. This crude product was dissolved in anhydrous DCM (5 mL) under an atmosphere of argon. To this stirring mixture, 4-amino-TEMPO **4** (180 mg, 1.05 mmol, 2.2 equiv) and *i*-Pr_2NEt (0.33 mL, 1.9 mmol, 4 equiv) were added, and the solution was allowed to stir for 2 h at room temperature followed by 1 h at 30 °C. The final reaction mixture was diluted with H_2O and adjusted to pH 12 with 2 M NaOH. The organic phase was separated, and the aqueous phase was re-extracted with DCM (3 × 20 mL). The combined extracts were dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo to afford the crude product. Purification was achieved via column chromatography (SiO_2, 95% chloroform, and 5% methanol). Data for **5**: Light orange solid (165 mg, 0.319 mmol, 67%). M.p. >200 °C (dec.). ^{1}H NMR (600 MHz, $CDCl_3$) (*note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum) δ = 5.56 (br s, 2H, 2 × C(*O*)N**H**), 4.34 (br s, 2H, 2 × C(*O*)NHC**H**), 3.06 (br s, 4H, 2 × SC**H**$_2$CH$_2$), 2.63 (br s, 4H, 2 × SCH$_2$C**H**$_2$), 1.1−1.8 (m, 32H, 8 × C**H**$_3$, 4 × C**H**$_2$). HRMS (ESI): *m*/*z* calcd. for $C_{24}H_{45}N_4O_4S_2$ $[M + H]^+$ 517.2877 found 517.2867. LC−MS: R_t = 7.58 mins, area 99%. EPR: *g* = 2.0058, a_N = 1.6061 mT.

Synthesis of 3,3′-Dithiobis(*N*-(4-acetoxy-2,2,6,6-tetramethylpiperidine)propanamide) 12. Compound **5** (110 mg, 0.214 mmol, 1 equiv) was added to a suspension of Pd/C (50 mg, 0.0054 mmol, 2.5 mol %) in anhydrous tetrahydrofuran (25 mL) under an atmosphere of H_2 (using a balloon) and stirred for 30 min. The solution was cooled to 0 °C, and Et_3N (0.15 mL, 1.08 mmol, 5 equiv) and acetyl chloride (0.08 mL, 1.08 mmol, 5 equiv) were added. The resulting solution was stirred for 30 min at 0 °C followed by 1 h at room temperature. The reaction mixture was then filtered through celite, and the solvent was removed in vacuo to afford the crude product. Purification was achieved via column chromatography (SiO_2, 95% chloroform, and 5% methanol). Data for **12**: white solid (112 mg, 0.186 mmol, 87%). M.p. >200 °C (dec.). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 5.87 (d, *J* = 7.9 Hz, 1H, NH), 4.24 (dd, *J* = 8.1, 4.1 Hz, 1H, NHC**H**), 2.97 (t, *J* = 6.9 Hz, 2H, SC**H**2), 2.56 (t, *J* = 6.9 Hz, 2H, SCH$_2$C**H**$_2$), 2.10 (s, 3H, C(*O*)C**H**$_3$), 1.88 (dd, *J* = 12.7, 3.6 Hz, 2H, NHCHC**H**$_2$), 1.61 (dd, *J* = 12.4, 3.6 Hz, 2H, NHCHC**H**$_2$), 1.26 (s, 6H, CC**H**$_3$), and 1.08 (s, 6H, CC**H**$_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 170.6, 170.4, 60.4, 45.0, 41.2, 35.9, 34.4, 32.1, 21.3, 19.4. HRMS (ESI): *m*/*z* calcd. for $C_{28}H_{51}N_4O_6S_2$ $[M + H]^+$ 603.3250 found 603.3225. LC−MS: R_t = 12.42 mins, area 100%.

General Procedure for the Synthesis of *N*-Substituted Isothiazolones 6−11 and 13−15 from 3,3′-Dithiodipropioamide 5 or 12. The specific 3,3′-dithiodipropioamide (1 equiv) was suspended in anhydrous toluene (10 mL) and cooled to 0 °C. SO_2Cl_2 (2−5 equiv, see Table 1) was dissolved in anhydrous toluene (1 mL) and then added dropwise over 20−360 min (see Table 1) at 0 °C followed by stirring at room temperature for 36 h. The final reaction mixture was filtered, and the filter paper was washed with DCM (3 × 10 mL). The solvents were removed in vacuo to yield the crude product mixture. Purification was achieved via column chromatography (SiO_2, 95% chloroform, and 5% methanol).

*Compounds **6−11**.* Compounds **6−11** were prepared using the general procedure above with compound **5** (50 mg, 0.097 mmol, 1 equiv) and SO_2Cl_2 (39.3 mg, 0.291 mmol, 3 equiv).

*2-(2,2,6,6-Tetramethylpiperidin-4-yloxyl)isothiazol-3(2H)-one **6**.* Light orange solid (<1 mg). ^{1}H NMR (600 MHz, $CDCl_3$) (*note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum) δ = 8.14 (s, 1H, C(*O*)CH=C**H**S), 6.62 (s, 1H, C(*O*)C**H**=CHS). LTQ-MS: *m*/*z* calcd. for $C_{12}H_{20}N_2O_2S$ $[M + H]^+$ 256.12 found 256.17. EPR: *g* = 1.334, a_N = 1.593 mT.

*5-Chloro-2-(2,2,6,6-tetramethylpiperidin-4-yloxyl)-isothiazol-3(2H)-one **7**.* Light orange solid (<1 mg). ^{1}H NMR (600 MHz, $CDCl_3$) (*note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum) δ = 6.64 (s, 1H, C(*O*)CH=C**H**S). LTQ-MS: *m*/*z* calcd. for $C_{12}H_{19}ClN_2O_2S$ $[M + H]^+$ 290.10 found 290.17. EPR: *g* = 2.0058, a_N = 1.5721 mT.

*2-(2,2,6,6-Tetramethylpiperidin-1-ol)isothiazol-3(2H)-one **8**.* LTQ-MS: *m*/*z* calcd. for $C_{14}H_{21}N_2O_2S$ $[M + H]^+$ 257.13 found 257.17.

*5-Chloro-2-(2,2,6,6-tetramethylpiperidin-1-ol)isothiazol-3(2H)-one **9**.* LTQ-MS: *m*/*z* calcd for $C_{12}H_{20}ClN_2O_2S$ $[M + H]^+$ 291.10 found 291.17.

*5-Chloro-2-(2,2,6,6-tetramethylpiperidin-1-ol)isothiazol-3(2H)-one 1-oxide **10**.* LTQ-MS: *m*/*z* calcd for $C_{12}H_{20}ClN_2O_3S$ $[M + H]^+$ 307.10 found 307.17.

*2-(2,2,6,6-Tetramethylpiperidin-1-ol)isothiazol-3(2H)-one 1-oxide **11**.* LTQ-MS: *m*/*z* calcd. for $C_{12}H_{21}N_2O_3S$ $[M + H]^+$ 273.13 found 273.17.

*Compounds **13−15**.* Compounds **13−15** were prepared using the general procedure above with compound **12** (50 mg, 0.083 mmol, 1 equiv) and SO_2Cl_2 (33.6 mg, 0.247 mmol, 3 equiv).

*2-(4-Acetoxy-2,2,6,6-tetramethylpiperidine)isothiazolidin-3-one **13**.* White solid (17.4 mg, 0.058 mmol, 34.9%). M.p. >200 °C (dec.). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 4.52 (m, 1H, C(*O*)NC**H**), 3.29 (ddd, *J* = 17.2, 10.8, 7.9 Hz, 1H, C(*O*)C**H**$_2$), 3.09 (ddd, *J* = 13.1, 10.7, 8.4 Hz, 1H, C(*O*)C**H**$_2$), 2.99 (ddd, *J* = 13.1, 7.9, 1.0 Hz, 1H, C(*O*)CH$_2$C**H**$_2$), 2.80 (ddd, *J* = 17.2, 8.4, 1.1 Hz, 1H, C(*O*)CH$_2$C**H**$_2$), 2.15−1.74 (m, 4H, 2 × NCHC**H**$_2$), 2.10 (s, 3H, NOC(*O*)C**H**$_3$), 1.25 and 1.11 (s, 12H, NCHCH$_2$C**H**$_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 175.7, 60.8, 60.6, 47.3, 46.3, 44.4, 43.9, 32.0, 27.6, 21.1, 20.9, 19.2. LRMS (ESI): *m*/*z* calcd. for $C_{14}H_{24}N_2O_3S$ + H^+ $[M + H]^+$ 301.2; found 301.4.

*2-(4-Acetoxy-2,2,6,6-tetramethylpiperidine)isothiazol-3-2(H)-one **14**.* Light yellow solid (6.2 mg, 0.021 mmol, 14%). M.p. >200 °C (dec.). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 8.05 (d, *J* = 6.2, 1H, C(*O*)CH=C**H**), 6.25 (d, *J* = 6.2 Hz, 1H, C(*O*)C**H**=CH), 4.90 (m, 1H, NC**H**), 2.12 (s, 3H, NOC(*O*)C**H**$_3$), 1.98 and 1.94 (m, 4H, 2 × NCHC**H**$_2$), 1.32 and 1.13 (s, 12H, NCHCH$_2$C**H**$_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 168.9, 139.1, 115.1, 60.7, 45.6, 44.7, 32.0, 21.3, 19.2. HRMS (ESI): *m*/*z* calcd. for $C_{14}H_{22}N_2O_3S$ + H^+ $[M + H]^+$ 299.1424; found 299.1418. LC−MS: R_t = 8.64 mins, area 100%.

*5-Chloro-2-(4-acetoxy-2,2,6,6-tetramethylpiperidine)-isothiazol-3-2(H)-one **15**.* (6.4 mg, 0.020 mmol, 14%). M.p. >200 °C (dec.). ^{1}H NMR (600 MHz, $CDCl_3$) δ = .6.24 (s, 1H, C(*O*)C**H**=CCl), 4.89 (m, 1H, NC**H**), 2.11 (s, 3H, NOC(*O*)C**H**$_3$), 1.93 and 1.69 (m, 4H, 2 × NCHC**H**$_2$), 1.29 and 1.11 (s, 12H, 2 × NCHCH$_2$C**H**$_3$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 167.0, 146.5, 115.2, 66.8, 60.7, 45.9, 44.5, 37.5, 32.0, 21.3, 21.2, 21.0, 19.3. HRMS (ESI): *m*/*z* calcd. for

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$C_{14}H_{21}ClN_2O_3S$ + H^+ $[M + H]^+$ 333.1034; found 333.1030. LC−MS: R_t = 11.52 mins, area 100%.

Synthesis of 5-Chloro-2-(2,2,6,6-tetramethylpiperidin-1-ol)isothiazol-3(2*H*)-one 9 from 5-Chloro-2-(4-acetoxy-2,2,6,6-tetramethylpiperidine)isothiazol-3-2(*H*)-one 15. Hydrochloric acid (2 M aqueous, 5 equiv) was added to a solution of compound **15** (20 mg, 0.06 mmol, 1 equiv) in methanol (5 mL), and the resulting solution was stirred overnight at 45 °C. The reaction mixture was cooled to room temperature and diluted with deionized water before being quenched with saturated sodium hydrogen carbonate (5 mL) and extracted with chloroform (3 × 20 mL). The combined organic extracts were dried over anhydrous sodium sulfate, and the solvent was removed in vacuo to afford crude product **9** (5 mg). ^{1}H NMR (600 MHz, D_2O) (**note crude reaction mixture*) δ = 6.19 (br. s, 1H, C(*O*)C**H**=CCl), 4.26 (m, 1H, NC**H**), 2.14 and 1.76 (m, 4H, 2 × NCHC**H**$_2$), 1.45 and 1.39 (s, 12H, 2 × NCHCH$_2$C**H**$_3$). LTQ-MS: *m/z* calcd. for $C_{12}H_{20}ClN_2O_2S$ $[M + H]^+$ 291.10 found 291.16.

General Procedure for the Synthesis of (*Z*)-3-(Benzylthio)-*N*-(2,2,6,6-tetramethylpiperidin-4-yloxyl)-acrylamide 18 and (*Z*)-3-(Benzylthio)-*N*-(1,1,3,3-tetramethylisoindolin-2-yloxyl)acrylamide 19. Diphenylphosphinic chloride (1.2 equiv) was added to a solution of (*Z*)-3-(benzylsulfanyl)-propenoic acid **16** (1 equiv) and *N*-ethylmorpholine (2.2 equiv) in anhydrous DCM under an atmosphere of argon at −10 °C (acetone and ice bath), and the mixture was stirred for 30 min. The specific amine (1.2 equiv) was dissolved in anhydrous DCM (0.5 mL) and added dropwise to the stirring solution at −10 °C followed by stirring at room temperature overnight. The reaction solvent was removed in vacuo, and the remaining residue was taken up in diethyl ether (50 mL) before being washed with 2 M hydrochloric acid (3 × 20 mL), then 2 M sodium hydroxide (3 × 20 mL), followed by saturated sodium hydrogen carbonate (1 × 20 mL), and finally brine (1 × 20 mL). The remaining organic solution was dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo to afford the crude product. Purification was achieved via column chromatography (SiO_2, 99% chloroform, and 1% methanol).

(*Z*)-3-(*Benzylthio*)-*N*-(*2,2,6,6-tetramethylpiperidin-4-yloxyl*) *acrylamide* ***18***

It was prepared using the above general procedure with compound **16** (190 mg, 0.975 mmol, 1 equiv), 4-amino-TEMPO **4** (200 mg, 1.17 mmol, 1.2 equiv), diphenylphosphinic chloride (223 μL, 1.17 mmol, 1.2 equiv), ethylmorpholine (272 μL, 2.145 mmol, 2.2 equiv), and anhydrous DCM (10 mL). Data for compound **18**: Orange solid (305 mg, 0.88 mmol, 90%). M.p. >200 °C (dec.). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum*) δ = 7.39−6.85 (m, 5H, Ar-**H**), 5.57 (br s, 1H, SC**H**=CH), 4.72 (br s, 1H, SCH=C**H**), 3.85 (s, 2H, SC**H**$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 164.5, 144.0, 136.1, 127.7, 127.4, 126.1, 38.5. HRMS (ESI): *m/z* calcd. for $C_{19}H_{28}N_2O_2S$ $[M + H]^+$ 348.1866 found 348.1868. LC−MS: R_t = 12.69 mins, area 100%. EPR: g = 2.0058, a_N = 1.5933 mT.

(*Z*)-3-(*Benzylthio*)-*N*-(1,1,3,3-tetramethylisoindolin-2-yloxyl)*acrylamide* ***19***

It was prepared using the above general procedure with compound **16** (190 mg, 0.975 mmol, 1 equiv), 4-amino-TMIO **17** (240 mg, 1.17 mmol, 1.2 equiv), diphenylphosphinic chloride (223 μL, 1.17 mmol, 1.2 equiv), ethylmorpholine (272 μL, 2.145 mmol, 2.2 equiv), and anhydrous DCM (10 mL). Data for compound **19**: yellow solid (316 mg, 0.83 mmol, 85%). M.p. >200 °C (dec.). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum*) δ = 7.41−7.30 (br m, 5H, Ar-**H**), 7.04 (br s, 1H, SC**H**=CH), 5.92 (br s, 1H, SCH=C**H**), 4.00 (s, 2H, Ar-C**H**$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 137.0, 128.8, 128.6, 128.3, 127.3, 39.6. HRMS (ESI): *m/z* calcd for $C_{22}H_{26}N_2O_2S$ $[M + H]^+$ 382.1710 found 382.1698. LC−MS: R_t = 14.56 mins, area 96.66%. EPR: g = 2.0056, a_N = 1.4881 mT.

General Procedure for the Synthesis of (*Z*)-3-(Benzylsulfide)-*N*-2,2,6,6-tetramethylpiperidin-4-yloxyl)acrylamide 20 and (*Z*)-3-(Benzylsulfide)-*N*-(1,1,3,3-tetramethylisoindolin-2-yloxyl)acrylamide 21. *m*CPBA (1 equiv) was dissolved in DCM (1 mL) and added dropwise to a mixture containing the specific sulfide (1 equiv) dissolved in DCM (10 mL) at −10 °C (acetone/ice bath). The resulting mixture was allowed to stir for 10 min at −10 °C before being quenched with aqueous sodium hydrogen sulfite (10 mL). The organic phase was separated and washed with saturated sodium hydrogen carbonate (3 × 10 mL) and then brine (1 × 10 mL). The organic phase was dried over anhydrous sodium sulfate, and the solvent was removed in vacuo to afford the crude final product, which was used directly in the subsequent reaction.

(*Z*)-3-(*Benzylsulfide*)-*N*-*2,2,6,6-tetramethylpiperidin-4-yloxyl*) *acrylamide* ***20***

It was prepared using the above general procedure with *m*CPBA (55 mg, 0.24 mmol, 1 equiv) and compound **18** (83 mg, 0.24 mmol, 1 equiv). Data for compound **20** (**note crude product*): Orange solid (80 mg).^{1}H NMR (600 MHz, $CDCl_3$) (**note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum*) δ = 7.83 (s, 1H, C(*O*)N**H**CH), 7.42 (m, 5H, Ar-**H**), 6.50 (br s, 1H, SC**H**=CH), 5.70 (br s, 1H, SCH=C**H**), 4.36 and 4.24 (br s, 2H, S(*O*)C**H**$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 153.1, 132.1, 130.6, 130.2, 128.9, 128.8, 128.3, 128.0, 59.5.

(*Z*)-3-(*Benzylsulfide*)-*N*-(*1,1,3,3-tetramethylisoindolin-2-yloxyl*)*acrylamide* ***21***

It was prepared using the above general procedure with *m*CPBA (55 mg, 0.24 mmol, 1 equiv) and compound **19** (92 mg, 0.24 mmol, 1 equiv). Data for compound **21** (**note crude product*): orange solid (68 mg). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum*) δ = 8.84 (s, 1H, C(*O*)N**H**CH), 7.42 (m, 5H, Ar-**H**), 6.51 (br s, 1H, SC**H**=CH), 5.37 (br s, 1H, SCH=C**H**), 4.47 and 4.33 (s, 2H, S(*O*)C**H**$_2$).^{13}C NMR (150 MHz, $CDCl_3$) δ = 160.6, 153.0, 130.1, 129.6, 128.2, 128.1, 59.5.

General Procedure for the Synthesis of *N*-Substituted Isothiazolones, 2-(2,2,6,6-Tetramethylpiperidin-4-yloxyl)isothiazol-3(2*H*)-one 6, and 2-(1,1,3,3-Tetramethylisoindolin-2-yloxyl)isothiazol-3(2*H*)-one 22 from (*Z*)-3-(Benzylsulfinyl)-*N*-ethylpropenamines. Acetyl chloride (5 equiv) was diluted in anhydrous DCM (1 mL) and added dropwise to a stirring mixture of the specific (*Z*)-3-(benzylsulfinyl)-*N*-ethylpropenamines (1 equiv) and lutidine (5 equiv) in anhydrous DCM (5 mL) under an atmosphere of argon at 0 °C. The final reaction mixture was stirred at 0 °C for

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5 min and then at room temperature overnight. The solvent was removed *in vacuo*, and purification was performed via column chromatography (SiO_2, 95% chloroform and 5% methanol).

2-(2,2,6,6-Tetramethylpiperidin-4-yloxyl)isothiazol-3(2H)-one **6**

It was prepared according to the procedure described above, using acetyl chloride (50 μL, 0.688 mmol, 5 equiv), compound **20** (crude) (50 mg, 0.138 mmol, 1 equiv), and lutidine (80 μL, 0.688 mmol, 5 equiv). The obtained data matched those reported earlier for compound **6** (25.5 mg, 0.1 mmol, 70%).[13] ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum*) δ = 8.15 (s, 1H, C(*O*)CH=C**H**S), 6.63 (s, 1H, C(*O*)C**H**=CHS). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 168.4, 137.7, 114.3, 68.5, 43.4, 41.3, 27.8, 20.5. HRMS (ESI): *m/z* calcd. for $C_{12}H_{26}N_2O_2S$ $[M + H]^+$ 256.1240 found 256.1233. LC−MS: R_t = 7.25 mins, area 96.63%.

2-(1,1,3,3-Tetramethylisoindolin-2-yloxyl)isothiazol-3(2H)-one **22**

It was prepared according to the procedure described above, using acetyl chloride (98.5 μL, 1.38 mmol, 5 equiv), compound **21** (crude) (110 mg, 0.28 mmol, 1 equiv), and lutidine (160 μL, 1.38 mmol, 5 equiv). Data for compound **22**: yellow solid (49 mg, 0.17 mmol, 60%). M.p. >200 °C (dec.) ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound contains a free radical (i.e., is paramagnetic), which can cause some signals to appear broadened/absent in the NMR spectrum*) δ = 8.24 (s, 1H, C(*O*)CH=C**H**S), 6.43 (s, 1H, C(*O*)C**H**=CHS). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 167.1, 139.5, 123.5, 114.2. HRMS (ESI): *m/z* calcd. for $C_{15}H_{18}N_2O_2S$ $[M + H]^+$ 290.1083 found 290.1079. LC−MS: R_t = 9.27 mins, area 100%. EPR: g = 2.0058, a_N = 1.4813 mT.

MIC Susceptibility Assays for Compounds 1, 6, and 22. The MICs for compounds **1**, **6**, and **22** were determined by the broth microdilution method, in accordance with the 2015 (M07-A10) Clinical and Laboratory Standards Institute (CLSI). In a 96-well plate, 11 twofold serial dilutions of each compound were prepared to a final volume of 100 μL in MH medium. At the time of inoculation, 5×10^5 bacterial colony forming units (CFUs), prepared from fresh overnight MH cultures were added to each well. The MIC for a compound was defined as the lowest concentration that prevented visible bacterial growth after 18 h of static incubation at 37 °C. MIC values were also confirmed by spectrophotometric analysis at OD_{600nm} in a BMG Spectrostar plate reader. Compounds **1**, **6**, and **22**, were tested in the concentration range of 1120−1.09 μM. Working solutions of compounds **1**, **6**, and **22** were prepared in MH medium that had been inoculated with bacteria at 5×10^6 CFU mL^{-1}. Negative controls containing DMSO at the highest concentration required to produce a 1120 μM final concentration for compounds **1**, **6**, and **22** were also prepared and serially diluted (11 dilutions total) in the same method as the antimicrobial agents. The MIC values for compounds **1**, **6**, and **22** were obtained from two independent experiments, each consisting of at least three biological replicates. MIC values were determined as the lowest concentration that resulted in no visible growth after 24 h.

MBEC Susceptibility Assay for Compounds 1 and 22. Biofilms were grown using an MBEC device purchased from Innovotech Inc. (Canada) and used unmodified. The device consists of a two-part reaction vessel. The top component contains 96 identical pegs protruding down from the lid, which fits into a standard flat bottom 96-well plate (bottom component). Biofilm cultivation was achieved following a previously documented methodology.[13,57] Overnight cultures of each bacterial species prepared in LB were diluted to ~10^6 CFU mL^{-1} via spectrophotometry (OD_{600}), in LB medium. The enclosed flat bottom 96-well plate was inoculated with ~10^5 CFU (150 μL) of each bacterial strain per well. The peg lid was returned to the inoculated microtiter plate, and the complete MBEC device was incubated at 150 rpm, 37 °C, and 95% relative humidity for 24 h. Establishment of mature biofilms at this stage of the assay was determined by removing at least three individual pegs from the device, placing them in fresh LB media and sonicating for 30 min at <20 °C, which sufficiently disrupts biofilms and dislodges cells into the recovery media (LB). Recovered cells were enumerated by serial dilution and plating on LB agar. For treatment of established biofilms, the peg lid containing 24 h biofilms was rinsed for 10 s in PBS (96-well plate, 200 μL in each well) to remove loosely adherent planktonic cells before being transferred to a new flat bottom 96-well plate (challenge plate), which contained 2-fold serial dilutions of compounds **1** and **22** (concentration range between 1120 and 1.09 μM) in MH medium (total volume 200 μL per well). The complete CBD was then incubated at 120 rpm, 37 °C, and 95% relative humidity for 24 h. The lid was removed from the challenge plate and rinsed twice for 10 s in PBS (96-well plate, 200 μL in each well). The rinsed lid with attached pegs containing the treated biofilms was transferred to a new 96-well plate containing fresh LB recovery media (for viable CFU enumeration by plating). To assist the transfer of any remaining viable cells into the recovery media, the device was sonicated for 30 min (<20 °C). The peg lid was then discarded, and 50 μL from each well was serially diluted and spotted on LB agar plates for CFU enumeration. The remainder recovery plate was then covered and incubated at 37 °C and 95% relative humidity for 24 h. MBEC values were determined as the lowest concentration that resulted in no CFU after 24 h of growth.

LDH Release Assay for Cytotoxicity Assessment of Compounds 1 and 22. The cytotoxicity of compounds **1** and **22** against human T24 urinary bladder epithelial cells was examined utilizing the standard Pierce LDH cytotoxicity assay kit (Life Technologies, Australia), and assays were performed according to the manufacturer's instructions. Briefly, T24 cells were seeded at a density of 7500 cells/100 μL in a 96-well tissue culture plate and after 24 h incubation at 37 °C in a humidified atmosphere of 5% CO_2. The stock solutions of compounds **1** and **22** (in DMSO) were then diluted in PBS and added to the T24 cells to give a final concentration of 560 μM. The treated samples were incubated for 24 h under the same conditions. Lysis buffer 10× was used for maximum LDH release (positive control), and cells treated with DMSO/PBS (4.5% DMSO final concentration) or sterile water served as negative controls. After 24 h, 50 μL of the supernatant was transferred into a new 96-well plate, mixed with 50 μL of the reaction mixture (LDH assay kit), and incubated at room temperature (protected from light) for 30 min before the stop solution (50 μL) was added. The plate was then centrifuged (1000 × g) for 5 min to remove air bubbles, and the absorbance at 490 and 680 nm was measured using a Spectrostar plate reader (BMG).

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■ ASSOCIATED CONTENT

Supporting Information

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Synthesis, characterization and purity determinations of synthesized compounds, NMR spectra, and HPLC-MS data for compounds **5**, **6**, **12**, **14**, **15**, **18**, **19**, and **20**−**22** (PDF)

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Author Contributions

A.D.V. performed all the synthetic chemistry and most of the microbiology experiments and drafted the original manuscript. S.H. performed some of the microbiology experiments and reviewed and edited the manuscript. J.H. assisted with mass spectrometry evaluation of several compounds and reviewed and edited the manuscript. M.T. and K.E.F.-S. conceptualized the work, acquired funding, supervised the project, and reviewed and edited the manuscript.

Notes

The authors declare no competing financial interest.

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