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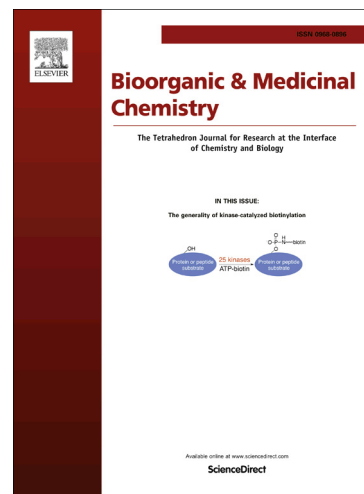Huy X. Ngo, Sanjib K. Shrestha, Keith D. Green, Sylvie Garneau-Tsodikova

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Development of ebsulfur analogues as potent antibacterials against methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Antibiotic resistance is a worldwide problem that needs to be addressed. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the dangerous "ESKAPE" pathogens that rapidly evolve and evade many current FDA-approved antibiotics. Thus, there is an urgent need for new anti-MRSA compounds. Ebselen (also known as 2-phenyl-1,2-benzisoselenazol-3(2H)-one) has shown promising activity in clinical trials for cerebral ischemia, bipolar disorder, and noise-induced hearing loss. Recently, there has been a renewed interest in exploring the antibacterial properties of ebselen. In this study, we synthesized an ebselen-inspired library of 33 compounds where the selenium atom has been replaced by sulfur (ebsulfur derivatives) and evaluated them against a panel of drug sensitive and drug resistant *S. aureus* and non-*S.aureus* strains. Within our library, we identified three outstanding analogues with potent activity against all *S. aureus* strains tested (MIC values mostly $\leq$2 μg/mL), and numerous additional ones with overall very good to good antibacterial activity (1-7.8 μg/mL). We also characterized the time-kill analysis, anti-biofilm ability, hemolytic activity, mammalian cytotoxicity, membrane-disruption ability, and reactive oxygen species (ROS) production of some of these analogues.

1. Introduction

Only two years after its introduction on the market in 1959, methicillin had experienced resistance by *Staphylococcus aureus*.[1] Ever since, methicillin-resistant *S. aureus* (MRSA) strains have spread worldwide and have become resistant to many additional FDA approved antibiotics causing great harm to infected patients. In 2011, the Centers for Disease Control (CDC) estimated that the national incidence of invasive MRSA infections was 80,461 cases including 650 cases of death, which was the highest mortality rate among bacterial infections.[2] *S. aureus* is a Gram-positive bacterium residing mostly on the skin and nasal lining of up to one-third of healthy individuals and can be transferred from one host to another by skin contact. Typically, the bacterium causes no symptoms. However, once the skin layer is broken due to scratches or cuts, *S. aureus* may lead to many problems varying from mild acne to life-threatening conditions such as bacteremia, pneumonia, endocarditis, and osteomyelitis.[3] Furthermore, MRSA is one of the "ESKAPE" pathogens, which also include *Enterococcus faecium, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter* species. The "ESKAPE" pathogens are termed that way because of their ability to escape the therapeutic effects of many known antibiotics and are responsible for the majority of hospital infections. In fact, the Infectious Diseases Society of America (IDSA) has expressed concerns about the empty pipeline for novel antibacterials that can target these pathogens.[4]

Thus, there is a need for new drug candidates to combat MRSA and the rest of the "ESKAPE" pathogens.

In recent years, a number of novel scaffolds with promising activities and mechanism of actions against MRSA have been described. Among them, the compound 5-nitro-2-phenyl-(1H)-indole was discovered as a NorA, efflux pump, inhibitor at IC_{50} values lower than 5.0 μM.[5] We also reported 6"-thioether tobramycin and kanamycin B analogues with long linear alkyl chains disrupting bacterial cell membranes and displaying good activity against *S. aureus* strains.[6-8] Another compound, AFN-125, was found to selectively inhibit *S. aureus* enoyl-ACP reductase, and has even been tested in clinical trials.[9] These recent advancements have certainly contributed towards our efforts of eradicating MRSA. However, the concerns about MRSA and "ESKAPE" pathogens have not been completely alleviated and novel scaffolds with potent antibacterial activities are still urgently needed.

Ebselen (also known as 2-phenyl-1,2-benzisoselenazol-3(2H)-one) was developed by Daiichi Pharmaceuticals in 1997 for cerebral ischemia in Japan, but failed during phase 3 clinical trial due to insufficient efficacy.[10,11] Since then, there has been a renewed interest in this compound; in fact, ebselen is currently being evaluated in clinical trials for treatments of bipolar disorder[12] and noise-induced hearing loss.[13] Clinical applications of ebselen are hypothesized to be related to its ability to

covalently bind to cysteine residues on targeted proteins or its antioxidant activity *via* mimicking glutathione peroxidase.[14,15] In addition, ebselen was found to inhibit the growth of various Gram-positive and Gram-negative bacterial strains.[16] In 2014, the crystal structure of antigen 85C, a putative drug target in *Mycobacterium tuberculosis* (*Mtb*), was solved and revealed that ebselen covalently inhibit the antigen 85 complex and hence, explained its activity against *Mtb*.[17] From a high-throughput screen, ebselen was discovered to target the cysteine protease domain within the major virulence factors A and B of *Clostridium difficile*.[18] Futhermore, ebselen and ebsulfur (**2a**) were found to inhibit bacterial thioredoxin reductase, suggesting that they may be useful agents against bacteria lacking the glutathione redox system.[19] Finally, ebselen especially caught our attention because it demonstrated potent bactericidal activity against many clinical isolates of drug-resistant *S. aureus* and was effective in a murine model of MRSA skin infection.[20] These findings prompted us to investigate the antibacterial activity of our own library of ebselen-inspired compounds.

Although no clear evidence has yet been established during clinical studies, there were initial concerns about selenium toxicity of ebselen as some systemic accumulation was observed.[11] In lieu of this potential adverse effect, we decided to study ebsulfur, in which the selenium of ebselen is replaced by a sulfur atom. Additionally, in terms of synthesis, the ebsulfur scaffold or 2-phenyl-1,2-benzisothiazol-3(2H)-one is readily accessible *via* a convenient 2-step synthesis, which allows for simple scale-up and derivatization. Herein, we synthesized and evaluated ebsulfur (**2a**) and 32 of its analogues (**2b-4n**) against a panel of methicillin-sensitive *S. aureus* (MSSA), MRSA, and other bacterial strains. We also performed the time-kill analysis, established the anti-biofilm ability, hemolytic activity, membrane-disruption ability, and ROS production of some of these analogues.

2. Results and discussion

2.1. Chemistry

To synthesize the desired ebsulfur (**2a**) and its derivatives **2b-3o**, we first prepared the common intermediate compound **1** in 80% yield by refluxing 2,2'-dithio-dibenzoic acid with thionyl chloride (Scheme 1). Compound **1** was then treated with a variety of aniline analogues and triethylamine in dichloromethane to afford compounds **2a-o** in 9-51% yield, as shown in procedure **A**. We initially tried to use procedure **A** for the preparation of ebsulfur analogues with aliphatic amines, but were unsuccessful as the undesired 2,2'-dithio-dibenzamide products were typically the major products observed. Thus, the undesired 2,2'-dithio-dibenzamide products were converted to the desired products **3a-o** in 14% to quantitative yield by subsequent addition of *N*-chlorosuccinimide to the reaction mixture, as shown in procedure **B**. During our synthesis, we also noticed that extra-long reaction time or poor-quality anhydrous dichloromethane led to the formation of the oxidized sulfoxide byproducts. On silica gel, the oxidized byproducts actually displayed retention times that were similar to the desired product and could be mistakenly isolated. Thus, we carefully monitored our reactions by TLC and verified their masses either by LRMS or HRMS. To test the effect of oxidation of the sulfur on the biological activity of our ebsulfur (**2a**) derivatives, we randomly selected three compounds (**3e**, **f**, and **n**) for which we let the reaction go longer to isolate the corresponding oxidized derivatives **4e**, **f**, and **n**. All new molecules were characterized by ^{1}H and ^{13}C NMR as well as by mass spectrometry and were confirmed to be ≥95% purity.
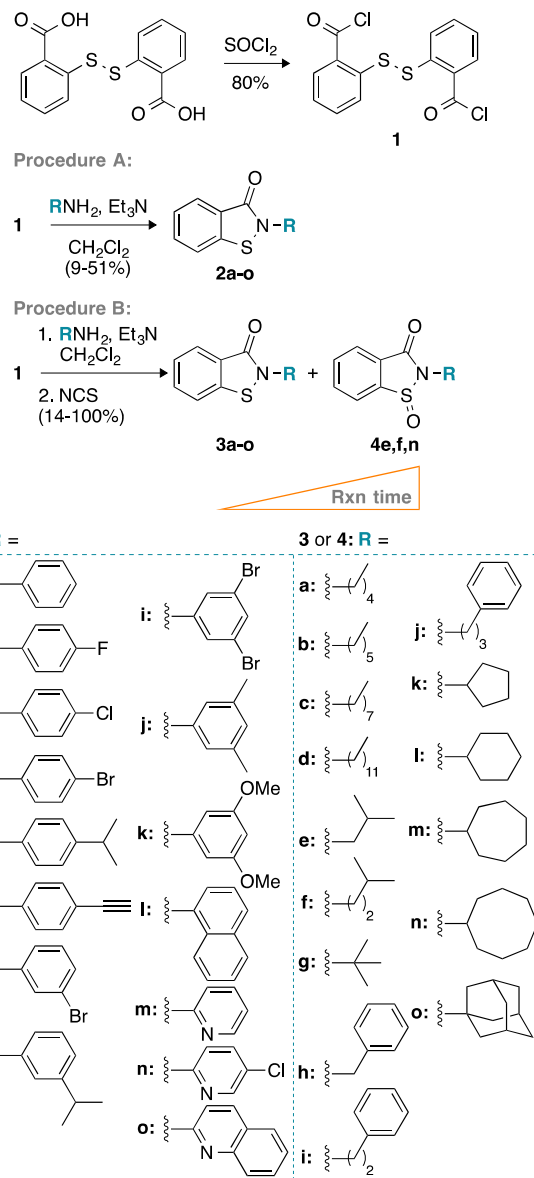
Scheme 1. Synthetic scheme for the preparation of compounds **2a-o**, **3a-o**, and **4e**, **4f**, and **4n** following two different experimental procedures (**A** and **B**).

2.2. Evaluation of compounds 2a-4n as antibacterial agents

The antibacterial activity of **2a-4n** was evaluated against a panel of *S. aureus* strains comprised of three MSSA strains (**A-C**) and 25 MRSA strains (**D-AB**) (Table 1).[21] We also tested the activity of all these analogues against non-*S. aureus* strains such as *S. epidermidis*, *E. faecalis*, *E. faecium*, VRE, *L. monocytogenes*, and *M. smegmatis* (**AC-AI**) (Table 2). Furthermore, the controls amikacin (AMK), ebselen, and ebsulfur (**2a**), along with our best compound, **2h**, were tested against a panel of additional bacterial strains, which included *A. baumannii*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. enterica*, and *S. epidermidis* (Table 3). The strains tested ranged from completely resistant to the control AMK (≥125 μg/mL, **AD**, **AE**, and **AF**) to very susceptible to AMK (≤0.50 μg/mL, **B**, **T**, and **AI**). Minimum inhibitory concentration (MIC) values were determined using the broth double dilution method.

2.2.1. Evaluation of compounds 2a-4n against various S. aureus strains

We commenced our study by evaluating ebselen against a panel of 28 *S. aureus* strains (Table 1). Ebselen displayed

excellent (≤0.50 µg/mL), very good (1-2 µg/mL), and good (3.9-7.8 µg/mL) activity against 5 (**D**, **E**, **J**, **U**, and **X**), 16 (**A**, **C**, **F**, **H**, **I**, **K**, **M-Q**, **S**, **V**, **W**, **Y**, and **Z**), and 7 (**B**, **G**, **L**, **R**, **T**, **AA**, and **AB**) of these *S. aureus* strains, respectively. We then investigated the replacement of the selenium atom by sulfur by synthesizing and testing ebsulfur (**2a**). Compound **2a** was found to have similar MIC values to those of ebselen against all *S. aureus* strains tested. As replacement of the selenium atom by sulfur could result in decreased toxicity, we decided to use compound **2a** as our model compound for further derivatization and evaluation.

Previous literature on the antibacterial properties of ebselen suggested that the 1,2-benzisothiazol-3(2H)-one core could be required for antibacterial activity.[16,19] We also thought of possibly removing the annulated benzene ring of the core to generate 1,2-isothiazolin-3-one analogues. However, we shied away from these analogues once we realized that there were reports suggesting that these compounds could be allergenic and neurotoxic against humans.[22,23] Thus, we decided to keep the 1,2-benzisothiazol-3(2H)-one core intact and hypothesized that the phenyl group adjacent to the core scaffold would be a good site for our investigation. In search of chemical modifications to increase the biological activity of **2a**, we first replaced the phenyl ring by the following moieties: substituted phenyl and other aromatic rings (**2b-o**), alkyl chains (**3a-g**), alkyl chains with a terminal phenyl group (**3h-j**), and aliphatic rings (**3k-o**). In general, all of our analogues displayed moderate to excellent activity against *S. aureus* (15.6 to ≤0.25 µg/mL), except for compounds **4e**, **4f**, and **4n**, which were found to be completely inactive (>125 µg/mL) against all *S. aureus* strains tested. In addition, compounds **2i**, **2n**, **2o**, and **3o** were not evaluated due to solubility issues in liquid Mueller-Hinton medium.

Most of the compounds with mono- (**2b**, **2c**, **2d**, **2f**, and **2g**) and disubstitutions (**2i** and **2j**) at the 3- and 4-positions of the phenyl ring were found to display good to excellent activity (3.9 to ≤0.25 µg/mL), but the activity was very similar to that of **2a**. The three overall best compounds of this series of analogues (**2e**, **2h**, and **2k**) were found to have very good to excellent activity (≤2.0 µg/mL) across all *S. aureus* strains tested (Table 1). Among these three, the relatively bulkier 4-isopropylphenyl (**2e**) and 3-isopropylphenyl (**2h**) analogues were our two best compounds and displayed up to 16-fold improvement in MIC values when compared to the parent compound **2a**. We also noticed that the substitution pattern (*p- vs m*-isopropylphenyl (**2e** *vs* **2h**) or *p- vs m*-bromophenyl (**2d** *vs* **2g**)) did not have a substantial effect on the MIC values of these compounds (mostly within 2 fold dilutions). Intrigued by this result, we synthesized and tested more analogues with bulky substituents on the phenyl ring. Surprisingly, the 2,3-dimethoxyphenyl analogue (**2k**) also yielded very good to excellent MIC values (2 to ≤0.25 µg/mL). We decided to add even more bulkiness to the scaffold by synthesizing the naphthyl analogue (**2l**), which displayed very good to excellent MIC values (2 to ≤0.25 µg/mL). This result further suggested that adding different bulky groups to the phenyl ring is a favorable strategy to increase activity.

To further understand the structure-activity-relationship (SAR) of our ebsulfur scaffold, we decided to introduce a heteroatom into the benzene ring. Previously, the 3-chloropyridyl replacement of the phenyl ring in the ebselen scaffold was reported to greatly reduce toxicity in mammalian HEK293T cell line (IC$_{50}$ >160 µM).[24] We applied this knowledge to our ebsulfur scaffold and synthesized the pyridyl (**2m**), 3-chloropyridyl (**2n**), as well as the bulky quinolinyl (**2o**) analogues. However, only **2m** was soluble enough for biological testing, but yielded inferior

MIC values (15.6 to 2 µg/mL) relative to the parent compound **2a**, suggesting that introduction of a nitrogen atom into the phenyl ring may not be the way to pursue.

We next investigated the effect of replacing the phenyl ring by linear alkyl chains, which were previously shown to improve the antibacterial activity of another class of antibiotics, the aminoglycosides.[6-8,25,26] Compounds **3a-d** were synthesized to contain *n*-pentyl, *n*-hexyl, *n*-octyl, and *n*-dodecyl side chains instead of the typical phenyl ring found in **2a**. Among these compounds, the *n*-octyl analogue (**3c**) displayed the best MIC values (3.9 to ≤0.25 µg/mL). Compounds **3a**, **3b**, and **3d** displayed MIC values (62.5 to 0.25 µg/mL) that were similar to that of the parent compound **2a**. Inspired by our best compounds in series **2** containing an isopropyl moiety, compounds **2e** and **2h**, we decided to explore the effect of branched alkyl chains by synthesizing and testing compounds **3e-g**. These compounds displayed mostly good to moderate activity (3.9 to 15.6 µg/mL). However, they were definitely inferior when comparing to compounds **2e** and **2h**. Additionally, we were also able to isolate the sulfoxide analogues of these compounds (**4e** and **4f**) and evaluated them. We were surprised to find that these oxidized compounds completely lost their antibacterial activity against *S. aureus*. This finding was consistent with previous reports that the S-N bond is essential for biological activity by covalently binding to cysteine residues of targeted enzymes.[17,19]

In an attempt to further understand the SAR of the phenyl ring, we explored whether having this ring directly attached to (compound **2a**) or at a distance from the 1,2-benzisothiazol-3(2H)-one core made any difference. We synthesized compounds **3h-j** with 1-3 carbon linkers separating the phenyl ring and the core. These compounds displayed good to excellent activity (3.9 to ≤0.25 µg/mL). Lastly, to confirm that the aromaticity of the substituent is not required for antibacterial activity, we synthesized a series of compounds containing different-sized aliphatic rings (**3k-o**). We found that compound **3l** with a cyclohexyl ring displayed very similar MIC values (7.8 to 0.5 µg/mL) to its aromatic counterpart **2a**. We noted that all of these compounds retained good to excellent antibacterial activity against *S. aureus*, with the exception of the adamantyl derivative **3o**, which could not be tested due to solubility issues.

2.2.2. Evaluation of compounds 2a-4n against various non-S. aureus strains

To further examine the antibacterial spectrum of compounds **2a-4n**, we also tested them against a panel of non-*S. aureus* strains. Overall, we found that many of our ebsulfur (**2a**) analogues were more specific towards *S. aureus* strains. We did observe that a few analogues actually displayed moderate to excellent activity (15.6 to ≤0.25 µg/mL) against certain non-*S. aureus* strains (Table 2). Interestingly, we found that ebselen still displayed good to excellent activity against many non-*S. aureus* strains (7.8 to ≤0.25 µg/mL), in contrary to what was previously observed when ebselen was tested against a different panel of bacterial strains.[16] We were especially enlightened to find that our best compounds, **2e** and **2h**, still retained their excellent activity (1 to ≤0.25 µg/mL) against selected strains such as *S. epidermidis* (**AC**), VRE (**AG**), *L. monocytogenes* (**AH**), and *M. smegmatis* (**AI**).

To gain a better understanding of the antibacterial spectrum of our ebsulfur (**2a**) analogues, we decided to test them against a biofilm-forming *S. epidermidis* (**AC**), another Gram-positive bacterium of the genus *Staphylococcus*. As expected, other than the previously inactive oxidized compounds **4e**, **4f**, and **4n**, we found that our entire library of analogues still retained moderate

to excellent activity that was similarly observed during our evaluation of these compounds against the panel of *S. aureus* strains presented in Table 1.

Next, we explored the activity of our analogues against examples of the genus *Enterococcus*. Against the *E. faecalis* strains (**AD** and **AE**) our ebsulfur analogues mostly showed moderate to poor activity (≥15.6 μg/mL), except in the case of compound **2k**, which still displayed good to very good activity (7.8 to 2 μg/mL) (Table 2). When comparing the activity of our compounds against *E. faecalis* (strains **AD** and **AE**) and *E. faecium* (strain **AF**), we observed a slightly improved activity (lower MIC values) against *E. faecium*, with compounds **2a**, **2d**, **2f**, **2h**, **2k**, **3b-f**, **3g**, **3h**, and **3m-n** displaying good to very good activity (7.8 to 2 μg/mL). Surprisingly, against the vancomycin-resistant *Enterococcus* (VRE) strain (**AG**), all of our analogues (except for **4e**, **4f**, and **4n**) displayed good to excellent activity (7.8 to ≤0.25 μg/mL).

To continue our study, we explored the activity of our library against *L. monocytogenes* (**AH**) and *M. smegmatis* (**AI**). We found that most of our analogues (**2a-h**, **2k-m**, **3a-f** (except **3e,f** against strain **AH**), and **3h-m**) displayed good to excellent activity (7.8 to 0.5 μg/mL) against these strains.

Intrigued by our results against the non-*S. aureus* panel, we further investigated the activity of our best compound, **2h**, along with ebselen and ebsulfur (**2a**) against some additional non-*S. aureus* strains (*A. baumannii*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. enterica*, and an additional non-biofilm-forming *S. epidermidis*). However, we found that these compounds were mostly inactive against these strains (Table 3).

2.3. Evaluation of time-kill curve of compounds 2a, 3b, and 3c

To gain more insights into the antibacterial activity kinetics of our ebsulfur analogues, we performed time-kill assays[27] of compounds **3b** and **3c** on MRSA S22 (strain **W**) over a period of 24 h and compared that to ebselen, ebsulfur (**2a**), and AMK (Fig. 1). At sub-MIC concentration (0.5× MIC), as expected, we observed that strain **W** grew well in the presence of all these compounds. Unlike the control (untreated bacteria), we found that all of the compounds were bacteriostatic up until 3 to 6 h. At MIC concentration, we observed similar bacteriostatic profiles as described previously in our experiment at sub-MIC. We decided to conduct another study at 4× MIC, where we were surprised to find that ebselen was bactericidal and led to complete cell death at 24 h. Compounds **2a**, **3b**, and **3c** reduced bacterial load by 1.5 orders of magnitude, but they still remained bacteriostatic. These results are interesting and may suggest that the selenium atom has a role in promoting the bactericidal effect. However, it should be noted that the time-kill curve assays were conducted under different conditions than those used in the MIC broth double-dilution assays (inoculum size, air flow, and volumes of medium). These differences could lead to a change in the effective or therapeutic doses and thus, potentially explain why the 4× MIC dosages were required for strong efficacy. It is well established that increasing the inoculum size will require a different MIC to achieve therapeutic efficacy. To investigate whether the inoculum size affected our MIC values, we re-performed the MIC broth double-dilution assay on eight compounds (AMK, ebselen, **2a**, **2g**, **2h**, and **3a-c**) with the inoculum size used in time-kill assays (1×10^{6} CFU/mL) and found that the MIC values remained identical. Therefore, the selenium atom might indeed play a role in the bactericidal effect.
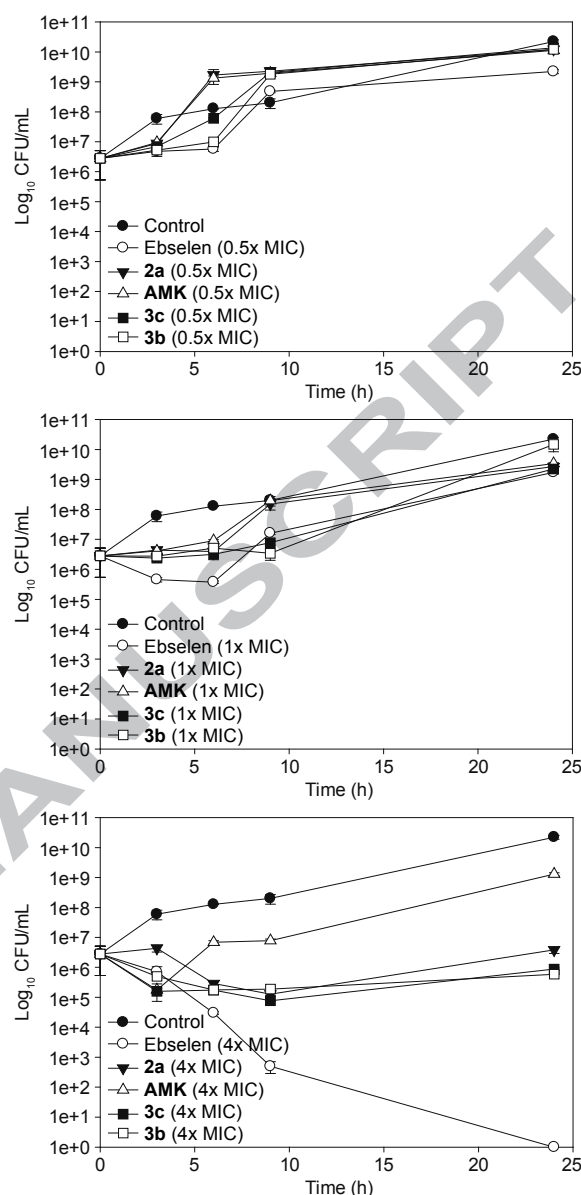
Fig. 1. Representative time-kill studies of ebselen, AMK, **2a**, **3c**, and **3b** against MRSA S22 (strain **W**). Bacterial cells were either treated with ebselen (white circle), AMK (white triangle), **2a** (black inverted triangle), **3c** (black square), and **3b** (white square) at 0.5× MIC (top panel), 1× MIC (middle panel) and 4× MIC (bottom panel), respectively or no drug (black circle).

2.4. Activity against biofilms of S. aureus and S. epidermidis

Having established that our compounds displayed activity against biofilm-forming *S. aureus* (strain **A**) and *S. epidermidis* (strain **AC**), we wanted to explore whether these compounds could also reduce the biofilm mass of these strains. Bacterial biofilm is a matrix of bacterial cells that are more tolerant to most antibacterial compounds and are highly associated with chronic persistent infections.[28] Recently, ebselen was reported to display potent biofilm reduction properties against established biofilms of *S. aureus* and *S. epidermidis*. When compared to the biofilm reduction activity of conventional antibiotics (linezolid, mupirocin, vancomycin, and rifampicin), the activity of ebselen was found to be significantly superior.[20] We were interested in finding out whether our ebsulfur analogues also possess the same anti-biofilm property, which would make them highly desirable in combating pathogenic bacteria associated with chronic infectious diseases. To evaluate the biofilm reduction activity,[20] ebselen, ebsulfur (**2a**), as well as some of our best compounds (**2e**, **2h**, **2k**, and **3c**) were tested against the established biofilm of

two biofilm-forming strains of *S. aureus* ATCC 6538 (strain **A**) and *S. epidermidis* ATCC 35984 (strain **AC**) at 125x, 62.5x, 31.3x, 15.6x, 7.8x, 3.9x, and 1x MIC (Fig. 2). Due to the observed ceiling effect (where the biofilm reduction plateaus), for each compound, we are reporting the lowest MIC folds at which the plateau begins.

Against the established biofilm of strain **A**, we found that ebselen (at 15.6 μg/mL or 7.8x MIC) reduced approximately 20% of the biofilm when comparing to the biofilm of the control (untreated biofilm) (Fig. 2A). This is different from previous finding about the biofilm reduction of this MRSA strain by ebselen, which was reported to be approximately 60% at 2 μg/mL.[20] Ebsulfur (**2a**) (at 31.3 μg/mL or 3.9x MIC) displayed superior biofilm reduction, approximately 40%. Compound **2e** also resulted in ~50% biofilm reduction at a much higher concentration of 125 μg/mL or 62.5x MIC. Meanwhile, compound **2h** (at 62.5 μg/mL or 15.6x MIC) only reduced the biofilm by ~10%. Compound **3c** was also found to result in low to no biofilm reduction. However, we found that compound **2k** displayed 40% biofilm reduction at 3.9 μg/mL or 3.9x MIC, which is the lowest concentration tested in our assay.
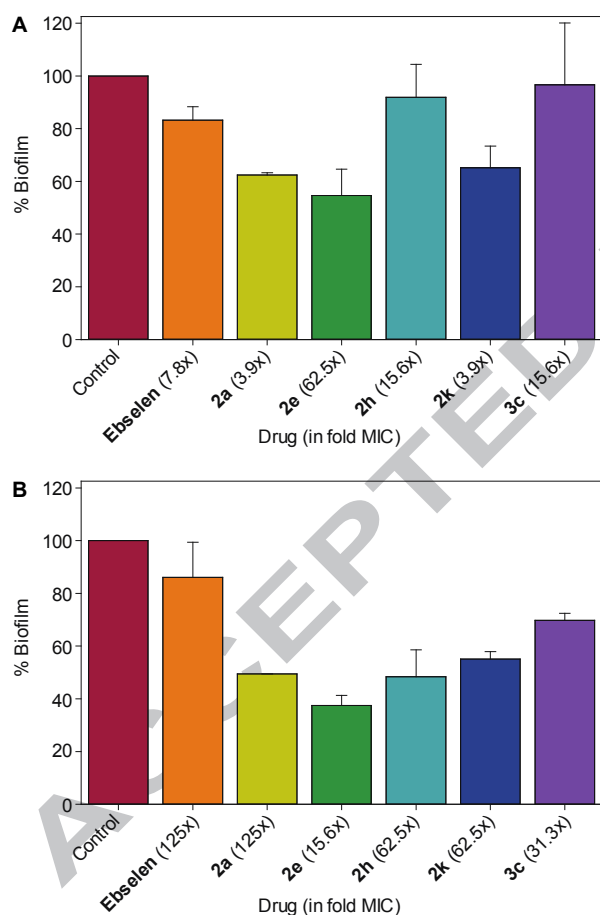

Fig. 2. Bar graphs showing the ability of selected compounds to reduce the amount of biofilm observed for **A.** *S. aureus* ATCC 6538 (strain **A**) and **B.** *S. epidermidis* ATCC 35984 (strain **AC**). The fold MIC for the optimal reduction for each compound is shown into parenthesis beside the compound name/number.

Against the biofilm of strain **AC**, ebselen (at 31.3 μg/mL or 125x MIC) was found to result in ~20% reduction of the biofilm (Fig. 2B). Ebsulfur (**2a**) was found to be slightly more effective, reducing the biofilm by ~50%, but at a much higher concentration (125 μg/mL or 125x MIC). Compounds **2e** and **2h** were found to have similar biofilm reduction activity (~60-50%) at 3.9 and 15.6 μg/mL (15.6x and 62.5x MIC), respectively.

Compound **2k** displayed about 50% biofilm reduction at 15.6 μg/mL or 62.5x MIC. Finally, we found that compound **3c** displayed about 40% biofilm reduction at 31.3 μg/mL or 31.3x MIC.

Overall, when compared to ebselen, we found that ebsulfur (**2a**) and compound **2e** displayed more potent biofilm reduction, but at higher concentrations. Compounds **2h** and **3c** were found to be active against the biofilm of strain **AC**, but not against the biofilm of strain **A**. Finally, compound **2k** displayed good biofilm disruption (40-50%) against both biofilm-forming strains at relatively low concentrations (3.9 and 15.6 μg/mL).

2.5. Evaluation of the hemolytic potential of compounds **2a**, **2h**, and **3c**

As red blood cells are some of the more fragile mammalian cells, we were interested in assessing our analogues for their general mammalian cell cytotoxicity and establishing whether these compounds would be suitable for any applications in humans. Hemolytic assays[29,30] were performed by testing ebselen, compounds **2a**, **2h**, and **3c**, as well as AMK (as a negative control) at various concentrations against murine red blood cells (mRBCs) (Fig. 3). We observed that our compounds tested did not show any significant hemolytic activity (≤20% hemolysis) up to 7.8 μg/mL. This result is relatively encouraging knowing that against *S. aureus* strains, compound **2h** was found mostly to display MIC values ranging from ≤0.25 to 1 μg/mL, while compound **3c** mostly displayed MIC values ranging from ≤0.25 to 2 μg/mL. Another interesting observation was that **2a** and its analogues **2h** and **3c** were less hemolytic when compared to ebselen (100% hemolytic at 15.6 μg/mL). Amongst the ebsulfur analogues tested, compound **2h** was found to be the least hemolytic.
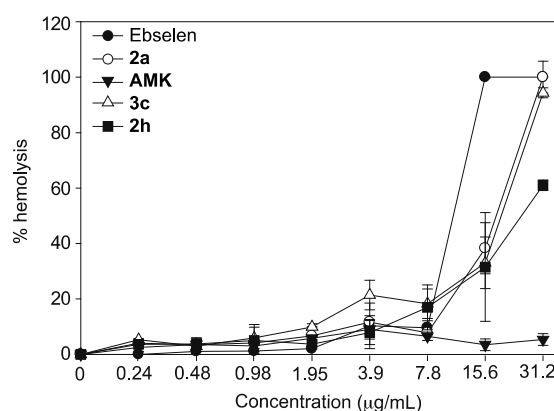
Fig. 3. Hemolytic activity of ebselen (black circle), **2a** (white circle), AMK (black inverted triangle), **3c** (white triangle), and **2h** (black square) on mouse red blood cells. The positive control (untreated) was found to display no hemolysis while the negative control (1% Triton X) was found to display 100% hemolysis (not shown on the graph).

2.6 Evaluation of mammalian toxicity potential of ebselen and compounds **2e**, **2h**, **2k**, **2l**, **3c**, **3i**, and **3k**

Although the hemolysis data was encouraging, we realized that a more extensive *in vitro* cytotoxicity study would still be needed to further understand the mammalian toxicity potential of our compounds.[30] Given that many current antibiotics are nephrotoxic, we selected the human embryonic kidney cell line HEK-293 as the model mammalian cell line for our cytotoxicity evaluation (Fig. 4). Ebselen was used as the control because it was shown to be safe for human use.[13] Compounds **2e**, **2h**, and **2l** all did not display any significant cytotoxicity (>20% cell death) up until 10 μg/mL, which was very similar to what was found with ebselen. On the other hand, compounds **2k**, **3c**, **3i**, and **3k**

were slightly more toxic against HEK-293 with >20% cell death observed at 1.25 μg/mL. Collectively, it was intriguing and encouraging that our best analogues **2e** and **2h** displayed stronger selectivity for bacterial cells. Depending on the MRSA strains, there could be up to 40-fold difference when compared to their MIC values.
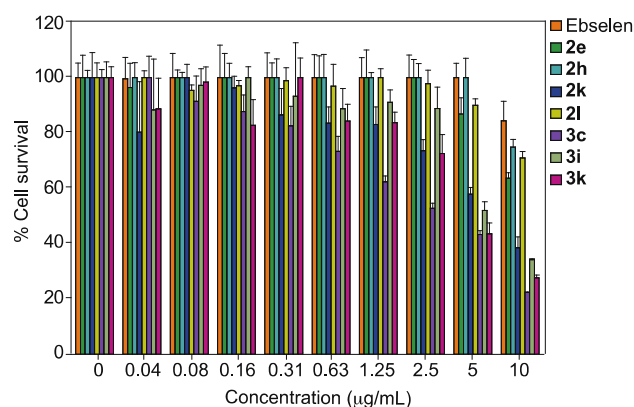
Fig. 4. Mammalian cytotoxicity of ebselen (orange bars), **2e** (green bars), **2h** (teal bars), **2k** (blue bars), **2l** (yellow bars), **3c** (purple bars), **3i** (light green bars), and **3k** (pink bars). Untreated cells were used as positive control and cells treated with 1% Triton X were used as negative control (not shown on graph).

2.7. Evaluation of ebselen and compounds 2a and 3b,c as bacterial membrane disruptors

Based on previous literature on membrane-disrupting cationic amphiphilic aminoglycosides,[6-8] we suspected that our linear alkyl ebsulfur analogues (**3b,c**) could potentially exert their antibacterial activity by their ability to disrupt bacterial cell membranes. Thus, we tested compounds **3b,c** along with **2a** and ebselen against *S. epidermidis* ATCC 35984 (strain **AC**) at their 1x and 4x MIC values and evaluated them for membrane disruption. We also used a tobramycin derivative with a linear alkyl chain of 14 carbons in length attached in a thioether linkage to the 6"-position (C$_{14}$-TOB) and AMK as positive and negative controls, respectively. We used propidium iodide (PI) staining to visualize any damage to the bacterial cell membrane. If the bacterial cell membrane was compromised, the PI would penetrate and stain the cells red. Based on our results, we saw no sign of membrane disruption from any of our compounds (Fig. 5). Thus, membrane disruption is likely not a viable antibacterial mechanism of action for these analogues. We then decided to switch our attention to study another possible mechanism of action for these compounds, the production of ROS.

2.8. Detection of reactive oxygen species (ROS) production

In bacteria lacking glutathione and glutaredoxin such as *S. aureus* and others, the thioredoxin system is crucial for ROS regulation and thus, bacterial survival and proliferation.[31-33] Ebselen was identified previously to inhibit bacterial thioredoxin reductase.[19] To test the hypothesis that our analogues also inhibit thioredoxin reductase, we treated *S. epidermidis* ATCC 35984 (strain **AC**) with ebselen, compounds **2a** and **3b,c** at various concentrations (1x and 4x their respective MIC values). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)[34] was then used to detect and visualize ROS production (Fig. 6). We found that ebselen and all of our analogues led to production of ROS and that inhibition of thioredoxin reductase is possibly the target responsible for antibacterial activity, which will be the subject of future studies.

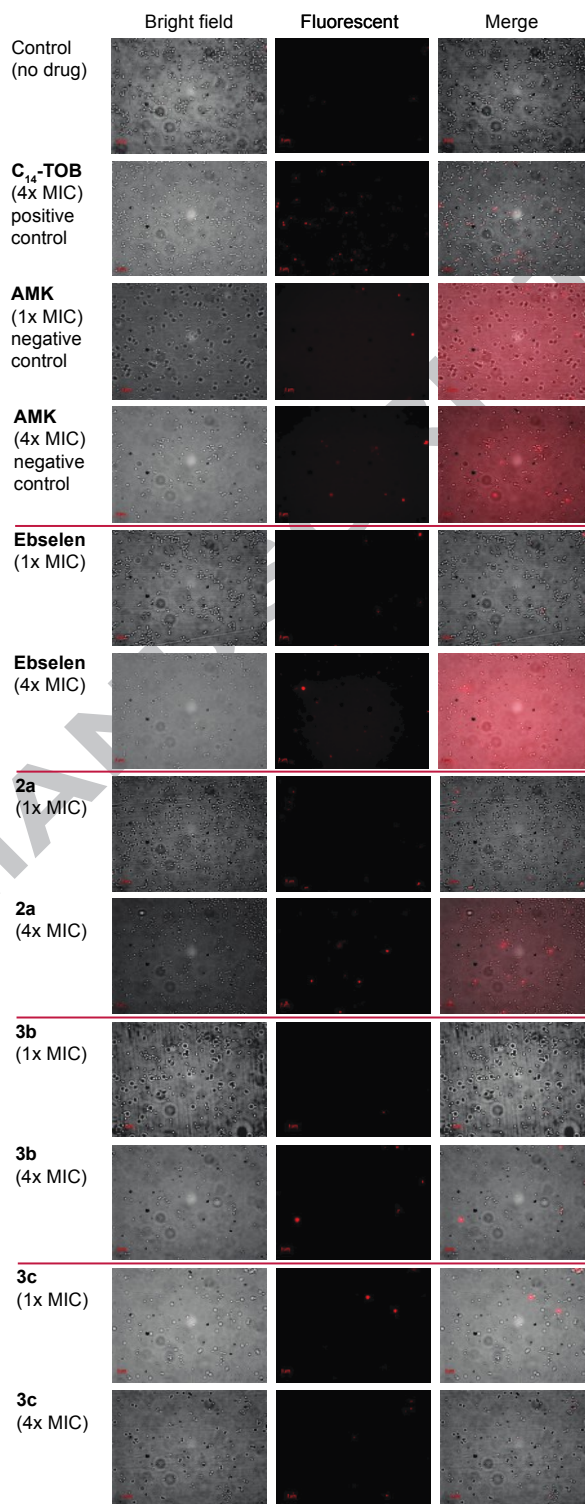**Fig. 5.** Effect of ebsulfur (**2a**) and its analogues **3b** and **3c** on cell membrane integrity of *S. epidermidis* ATCC 35984 (strain **AC**). Bacterial cells were treated with no drug or AMK (negative controls), C$_{14}$-TOB (positive control), or ebselen, **2a**, **3b**, and **3c**, at their 1× and 4× respective MIC values. Propidium iodine (PI) dye was used to monitor the uptake by bacterial cells.
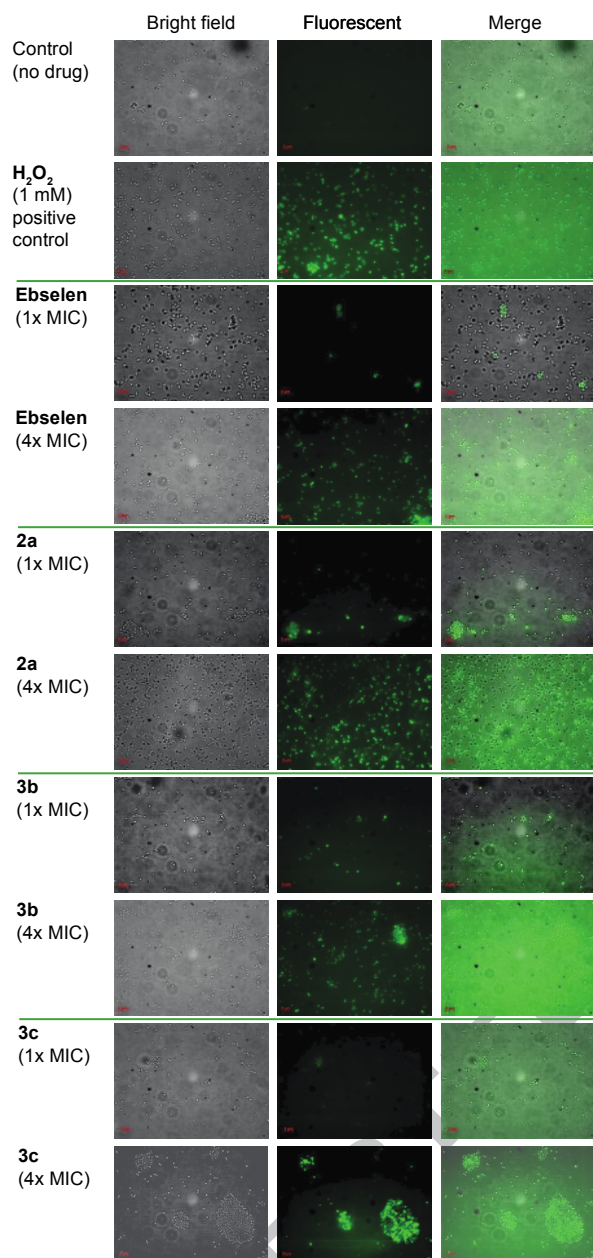
	Bright field	Fluorescent	Merge
Control (no drug)			
H$_2$O$_2$ (1 mM) positive control			
Ebselen (1x MIC)			
Ebselen (4x MIC)			
2a (1x MIC)			
2a (4x MIC)			
3b (1x MIC)			
3b (4x MIC)			
3c (1x MIC)			
3c (4x MIC)			

Fig. 6. Effect of ebsulfur (**2a**) and its analogues **3b** and **3c** on intracellular ROS production by *S. epidermidis* ATCC 35984 (strain **AC**). Bacterial cells were treated with no drug (negative control), 1 mM of H$_2$O$_2$ (positive control), or ebselen, **2a**, **3b**, and **3c**, at their 1× and 4× respective MIC values for 1 h at 37 °C. After staining with DCFH-DA (40 μg/mL), the samples were analyzed using a Zeiss Axovert 200M fluorescence microscope.

3. Conclusions

We synthesized an ebselen-inspired (ebsulfur) library comprised of 33 molecules based on previous reports of the antibacterial activity of ebselen against drug-resistant *S. aureus* clinical isolates. Our SAR analysis suggested that replacing the selenium atom with the sulfur atom in the 1,2-benzisoselenazol-3(2H)-one core did not significantly alter antibacterial activity. However, oxidizing the sulfur atom of the 1,2-benzisothiazol-3(2H)-one core, as in compounds **4e**, **4f**, and **4n**, completely obliterated antibacterial activity. This finding demonstrated that the stereoelectronic nature of the S-N bond is indeed important for biological activity, which is consistent with previous reports.[17,19] During our search for analogues with improved antibacterial activity, we identified three compounds (**2e**, **2h**, and

2k) with remarkably potent activity (MIC values mostly ≤2 μg/mL) against *S. aureus* clinical isolates. We then evaluated our compounds for their antibacterial spectrum by testing them against non-*S. aureus* strains. Our analogues were generally more selective towards *Staphylococcus* strains, but some of them also displayed good activities against VRE, *L. monocytogenes*, and *M. smegmatis*. Compounds with biofilm reduction are currently in high demand. Our evaluation showed that albeit at high concentrations, our analogues were able to reduce *Staphylococcal* established biofilms. We showed that the antibacterial activity of our compounds was highly correlated with ROS production. Lastly, we assessed our compounds for general mammalian toxicity by testing them against murine RBCs and HEK293. The mammalian toxicities of our most potent compounds were found to be acceptable. With further optimizations, these ebsulfur analogues could potentially have clinical utilities in our fight against bacterial resistance.

4. Experimental section / Supplementary material

Details of all experimental procedures for (i) synthesis of and characterization of compounds **1-4n**, (ii) determination of MIC values, as well as (iii) time-kill curves, (iv) biofilm disruption, (v) hemolysis assays, (vi) mammalian cytotoxicity, (vii) cell membrane permeabilization, and (viii) detection of ROS production are included in the Supplemental Information. The Supplemental Information also includes all ^{1}H and ^{13}C NMR spectra (Figs. S1-S57) for the molecules generated.

Acknowledgments

References

(1) Barber, M. *J. Clin. Pathol.* **1961**, *14*, 385.
(2) Dantes, R.; Mu, Y.; Belflower, R.; Aragon, D.; Dumyati, G.; Harrison, L. H.; Lessa, F. C.; Lynfield, R.; Nadle, J.; Petit, S.; Ray, S. M.; Schaffner, W.; Townes, J.; Fridkin, S.; Emerging Infections Program-Active Bacterial Core Surveillance, M. S. I. *JAMA Int. Med.* **2013**, *173*, 1970.
(3) Harris, A. **2016**, Methicillin-resistant *Staphylococcus aureus* (MRSA) (Beyond the Basics). In UpToDate post TW (ed), *UpToDate*. Waltham, MA.
(4) Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J. *Clin. Infect. Dis.* **2009**, *48*, 1.
(5) Lepri, S.; Buonerba, F.; Goracci, L.; Velilla, I.; Ruzziconi, R.; Schindler, B. D.; Seo, S. M.; Kaatz, G. W.; Cruciani, G. *J. Med. Chem.* **2016**.
(6) Herzog, I. M.; Green, K. D.; Berkov-Zrihen, Y.; Feldman, M.; Vidavski, R. R.; Eldar-Boock, A.; Satchi-Fainaro, R.; Eldar, A.; Garneau-Tsodikova, S.; Fridman, M. *Angew. Chem.* **2012**, *51*, 5652.
(7) Fosso, M. Y.; Shrestha, S. K.; Green, K. D.; Garneau-Tsodikova, S. *J. Med. Chem.* **2015**, *58*, 9124.
(8) Shrestha, S. K.; Fosso, M. Y.; Green, K. D.; Garneau-Tsodikova, S. *Antimicrob. Agents Chemother.* **2015**, *59*, 4861.
(9) Hafkin, B.; Kaplan, N.; Murphy, B. *Antimicrob. Agents Chemother.* **2015**. DOI: 10.1128/AAC.01741/15.
(10) Yamaguchi, T.; Sano, K.; Takakura, K.; Saito, I.; Shinohara, Y.; Asano, T.; Yasuhara, H. *Stroke* **1998**, *29*, 12.
(11) Parnham, M. J.; Sies, H. *Biochem. Pharmacol.* **2013**, *86*, 1248.
(12) Singh, N.; Halliday, A. C.; Thomas, J. M.; Kuznetsova, O. V.; Baldwin, R.; Woon, E. C.; Aley, P. K.; Antoniadou, I.; Sharp, T.; Vasudevan, S. R.; Churchill, G. C. *Nat. Commun.* **2013**, *4*, 1332.
(13) Lynch, E.; Kil, J. *Semin. Hear* **2009**, *30*, 047.
(14) Abdul-Hay, S. O.; Bannister, T. D.; Wang, H.; Cameron, M. D.; Caulfield, T. R.; Masson, A.; Bertrand, J.; Howard, E. A.; McGuire, M. P.; Crisafulli, U.; Rosenberry, T. R.; Topper, C. L.; Thompson, C. R.;

Schurer, S. C.; Madoux, F.; Hodder, P.; Leissring, M. A. *ACS Chem. Biol.* **2015**, *10*, 2716.

(15) Bhowmick, D.; Srivastava, S.; D'Silva, P.; Mugesh, G. *Angew. Chem.* **2015**, *54*, 8449.

(16) Nozawa, R.; Yokota, T.; Fujimoto, T. *Antimicrob. Agents Chemother.* **1989**, *33*, 1388.

(17) Favrot, L.; Lajiness, D. H.; Ronning, D. R. *J. Biol. Chem.* **2014**, *289*, 25031.

(18) Bender, K. O.; Garland, M.; Ferreyra, J. A.; Hryckowian, A. J.; Child, M. A.; Puri, A. W.; Solow-Cordero, D. E.; Higginbottom, S. K.; Segal, E.; Banaei, N.; Shen, A.; Sonnenburg, J. L.; Bogyo, M. *Sci. Transl. Med.* **2015**, *7*, 306ra148.

(19) Lu, J.; Vlamis-Gardikas, A.; Kandasamy, K.; Zhao, R.; Gustafsson, T. N.; Engstrand, L.; Hoffner, S.; Engman, L.; Holmgren, A. *FASEB J.* **2013**, *27*, 1394.

(20) Thangamani, S.; Younis, W.; Seleem, M. N. *Sci. Rep.* **2015**, *5*, 11596.

(21) Gajadeera, C.; Willby, M. J.; Green, K. D.; Shaul, P.; Fridman, M.; Garneau-Tsodikova, S.; Posey, J. E.; Tsodikov, O. V. *J. Antibiotics* **2015**, *68*, 153.

(22) Schnuch, A.; Geier, J.; Uter, W.; Frosch, P. J. *Brit. J. Dermatol.* **1998**, *138*, 467.

(23) Du, S.; McLaughlin, B.; Pal, S.; Aizenman, E. *J. Neurosci.* **2002**, *22*, 7408.

(24) Lu, J.; Vodnala, S. K.; Gustavsson, A. L.; Gustafsson, T. N.; Sjoberg, B.; Johansson, H. A.; Kumar, S.; Tjernberg, A.; Engman, L.; Rottenberg, M. E.; Holmgren, A. *J. Biol. Chem.* **2013**, *288*, 27456.

(25) Benhamou, R. I.; Shaul, P.; Herzog, I. M.; Fridman, M. *Angew. Chem.* **2015**, *54*, 13617.

(26) Bera, S.; Zhanel, G. G.; Schweizer, F. *J. Med. Chem.* **2010**, *53*, 3626.

(27) Motyl, M.; Dorso, K.; Barrett, J.; Giacobbe, R. *Curr. Protoc Pharmacol.* **2006**, *Chapter 13*, Unit13A 3.

(28) Bjarnsholt, T. *Acta Pathol. Microbiol. Immunol. Scand. A.* **2013**, *121*, 1.

(29) Dartois, V.; Sanchez-Quesada, J.; Cabezas, E.; Chi, E.; Dubbelde, C.; Dunn, C.; Granja, J.; Gritzen, C.; Weinberger, D.; Ghadiri, M. R.; Parr, T. R., Jr. *Antimicrob. Agents Chemother.* **2005**, *49*, 3302.

(30) Shrestha, S. K.; Fosso, M. Y.; Garneau-Tsodikova, S. *Sci. Rep.* **2015**, *5*, 17070.

(31) Uziel, O.; Borovok, I.; Schreiber, R.; Cohen, G.; Aharonowitz, Y. *J. Bacteriol.* **2004**, *186*, 326.

(32) Gustafsson, T. N.; Sahlin, M.; Lu, J.; Sjoberg, B. M.; Holmgren, A. *The J. Biol. Chem.* **2012**, *287*, 39686.

(33) Scharf, C.; Riethdorf, S.; Ernst, H.; Engelmann, S.; Volker, U.; Hecker, M. *J. Bacteriol.* **1998**, *180*, 1869.

(34) Li, Y.; Chang, W.; Zhang, M.; Li, X.; Jiao, Y.; Lou, H. *PloS one* **2015**, *10*, e0128693.

Table 1: MIC values[a] (in µg/mL) determined for all compounds and for the control antibacterial agent (AMK) against various *S. aureus* strains.

Cpd #	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Ebselen	2	3.9	2	0.5	≤0.25	2	7.8	2	1	0.5	2	3.9	1	2
2a	7.8	3.9	3.9	0.5	2	7.8	7.8	3.9	2	2	7.8	3.9	2	3.9
2b	3.9	2	3.9	1	3.9	2	3.9	2	3.9	1	3.9	3.9	2	2
2c	3.9	2	2	1	1	1	3.9	2	1	1	3.9	3.9	1	2
2d	2	2	2	0.5	3.9	2	3.9	2	2	1	3.9	3.9	2	2
2e	2	2	≤0.25	≤0.25	≤0.25	1	2	≤0.25	0.5	0.5	1	1	0.5	≤0.25
2f	7.8	3.9	1	1	3.9	2	7.8	2	2	2	7.8	7.8	2	2
2g	7.8	7.8	3.9	2	7.8	3.9	7.8	3.9	3.9	3.9	7.8	3.9	3.9	3.9
2h	3.9	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25
2j	7.8	7.8	3.9	1	7.8	7.8	7.8	3.9	3.9	3.9	15.6	7.8	7.8	7.8
2k	1	0.5	≤0.25	≤0.25	≤0.25	1	2	0.5	≤0.25	0.5	2	2	≤0.25	1
2l	>125	2	0.5	0.5	1	0.5	2	2	0.5	1	2	15.6	0.5	2
2m	7.8	7.8	7.8	3.9	15.6	7.8	15.6	3.9	7.8	7.8	7.8	7.8	7.8	7.8
3a	7.8	3.9	3.9	1	0.5	2	3.9	2	2	2	3.9	7.8	1	0.5
3b	7.8	3.9	3.9	2	2	3.9	3.9	3.9	3.9	3.9	3.9	7.8	2	0.5
3c	1	1	1	0.5	0.5	1	1	1	0.5	1	1	2	0.5	≤0.25
3d	62.5	3.9	3.9	2	0.25	1	15.6	3.9	1	2	15.6	3.9	1	0.5
3e	7.8	125	15.6	>125	7.8	15.6	7.8	7.8	7.8	7.8	7.8	7.8	7.8	3.9
3f	3.9	>125	3.9	>125	3.9	7.8	3.9	3.9	3.9	3.9	3.9	7.8	3.9	2
3g	15.6	15.6	15.6	3.9	7.8	15.6	15.6	7.8	7.8	15.6	15.6	15.6	7.8	7.8
3h	2	3.9	1	1	0.5	2	2	3.9	1	1	2	3.9	1	1
3i	3.9	1	2	1	1	1	2	2	2	2	2	3.9	1	0.5
3j	3.9	2	2	1	0.5	2	3.9	2	2	2	3.9	3.9	2	0.5
3k	7.8	3.9	2	3.9	2	3.9	3.9	3.9	3.9	3.9	3,9	3,9	3.9	2
3l	7.8	3.9	3.8	2	2	3.9	7.8	2	3.9	3.9	2	3.9	3.9	2
3m	7.8	3.9	3.9	1	2	3.9	2	2	2	3.9	2	3.9	1	0.5
3n	2	>125	2	>125	3.9	3.9	3.9	3.9	3.9	3.9	2	3.9	2	1
4e	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
4f	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
4n	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
AMK	2	≤0.25	62.5	31.3	31.3	3.9	62.5	2	2	7.8	31.3	31.3	3.9	31.3

Cpd #	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB
Ebselen	1	1	1	7.8	1	3.9	≤0.25	1	1	0.5	2	2	3.9	3.9
2a	3.9	3.9	2	7.8	3.9	3.9	≤0.25	3.9	2	7.8	3.9	3.9	3.9	3.9
2b	3.9	2	2	3.9	3.9	3.9	≤0.25	3.9	2	2	2	3.9	2	2
2c	2	2	1	3.9	3.9	3.9	≤0.25	1	2	1	2	2	3.9	2
2d	3.9	2	2	3.9	2	2	≤0.25	3.9	2	2	7.8	3.9	3.9	3.9
2e	0.5	1	1	2	0.5	1	≤0.25	≤0.25	0.5	≤0.25	0.5	1	2	1
2f	7.8	3.9	3.9	7.8	3.9	3.9	0.5	3.9	2	2	2	3.9	3.9	7.8
2g	3.9	7.8	7.8	7.8	3.9	3.9	0.5	3.9	>125	3.9	3.9	7.8	3.9	7.8
2h	0.5	0.5	0.5	1	≤0.25	0.5	≤0.25	≤0.25	1	≤0.25	0.5	0.5	≤0.25	≤0.25
2j	7.8	7.8	3.9	7.8	7.8	7.8	≤0.25	3.9	3.9	7.8	3.9	7.8	7.8	7.8
2k	1	0.5	≤0.25	2	1	2	≤0.25	1	0.5	≤0.25	0.5	0.5	≤0.25	2
2l	0.5	0.5	2	2	1	2	≤0.25	1	1	0.5	0.5	2	2	2
2m	7.8	7.8	7.8	15.6	7.8	15.6	2	7.8	7.8	7.8	7.8	7.8	7.8	15.6
3a	2	2	2	2	3.9	2	≤0.25	2	3.9	3.9	3.9	3.9	3.9	3.9
3b	3.9	2	2	3.9	3.9	3.9	≤0.25	3.9	3.9	3.9	7.8	7.8	3.9	7.8
3c	1	1	1	1	1	1	≤0.25	1	1	1	2	3.9	2	3.9
3d	1	1	2	3.9	3.9	2	1	1	3.9	1	3.9	7.8	7.8	7.8
3e	7.8	7.8	7.8	7.8	7.8	7.8	2	7.8	7.8	7.8	7.8	7.8	7.8	7.8
3f	3.9	3.9	7.8	3.9	7.8	3.9	1	3.9	7.8	3.9	7.8	7.8	7.8	7.8
3g	15.6	15.6	15.6	15.6	7.8	15.6	2	15.6	15.6	15.6	15.6	15.6	15.6	15.6
3h	2	1	1	1	2	1	≤0.25	1	2	1	2	2	2	2
3i	1	1	1	2	2	2	0.5	1	3.9	2	3.9	2	2	3.9
3j	2	2	2	2	2	2	≤0.25	2	3.9	2	2	3.9	2	3.9
3k	3.9	3.9	3.9	3.9	3.9	3.9	0.5	3.9	7.8	3.9	3.9	3.9	3.9	7.8
3l	3.9	3.9	3.9	3.9	3.9	3.9	0.5	7.8	7.8	3.9	7.8	7.8	7.8	7.8
3m	2	2	3.9	2	3.9	2	0.5	1	3.9	3.9	3.9	7.8	15.6	7.8
3n	2	2	3.9	0.5	2	2	1	2	7.8	3.9	7.8	7.8	7.8	7.8
4e	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
4f	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
4n	>125	>125	>125	>125	>125	>125	>125	>125	>125	125	>125	>125	>125	>125
AMK	15.6	15.6	31.3	31.3	15.6	0.5	7.8	31.3	7.8	31.3	62.5	62.5	62.5	62.5

Bacterial strains: **A** = *S. aureus* ATCC 6538, **B** = *S. aureus* ATCC 25923, **C** = *S. aureus* ATCC 29213, **D** = *S. aureus* ATCC 33591, **E** = MRSA1, **F** = MRSA2, **G** = MRSA BRS3, **H** = MRSA C1, **I** = MRSA C2, **J** = MRSA C7, **K** = MRSA C14, **L** = MRSA C16, **M** = MRSA C19, **N** = MRSA G1, **O** = MRSA G6, **P** = MRSA G12, **Q** = MRSA G14, **R** = MRSA MRSA NRS4, **S** = MRSA NRS51, **T** = MRSA NRS77, **U** = MRSA S14, **V** = MRSA S17, **W** = MRSA S22, **X** = MRSA S24, **Y** = MRSA USA100, **Z** = MRSA USA200, **AA** = MRSA USA300, **AB** = MRSA USA600.

Table 2: MIC values[a] (in µg/mL) determined for all compounds and for the control antibacterial agent (AMK) against various non-*S. aureus* bacterial strains.

Cpd #	AC	AD	AE	AF	AG	AH	AI
Ebselen	0.25	0.5	≤0.25	0.5	1	1	7.8
2a	1	31.3	15.6	7.8	1	2	3.9
2b	2	62.5	>125	31.3	2	2	1
2c	1	62.5	>125	31.3	0.5	1	3.9
2d	2	7.8	15.6	7.8	2	2	0.5
2e	0.25	15.6	62.5	15.6	≤0.25	0.5	0.5
2f	2	7.8	<125	7.8	2	3.9	3.9
2g	3.9	15.6	>125	15.6	7.8	3.9	3.9
2h	0.25	>125	>125	3.9	≤0.25	1	1
2j	3.9	15.6	15.6	15.6	3.9	7.8	3.9
2k	0.25	2	7.8	3.9	0.5	2	2
2l	0.25	>125	>125	>125	0.5	2	1
2m	3.9	>125	>125	>125	7.8	7.8	7.8
3a	3.9	7.8	>125	15.6	1	3.9	2
3b	2	>125	>125	7.8	2	3.9	2
3c	1	>125	>125	2	0.5	1	2
3d	0.25	>125	>125	3.9	1	15.6	7.8
3e	7.8	7.8	15.6	7.8	3.9	>125	3.9
3f	3.9	7.8	15.6	7.8	2	>125	3.9
3g	15.6	15.6	125	7.8	3.9	3.9	3.9
3h	1	31.3	>125	2	1	1	2
3i	2	>125	>125	>125	1	3.9	2
3j	2	15.6	>125	15.6	0.5	2	3.9
3k	2	>125	>125	>125	2	3.9	2
3l	2	>125	>125	>125	2	3.9	2
3m	2	>125	>125	7.8	2	3.9	7.8
3n	3.9	15.6	15.6	15.6	1	>125	3.9
4e	>125	>125	>125	>125	>125	>125	>125
4f	>125	>125	>125	>125	>125	>125	>125
4n	>125	>125	>125	>125	>125	>125	>125
AMK	15.6	125	>125	125	62.5	3.9	≤0.25

Bacterial strains: AC = *S. epidermidis* ATCC 35984, **AD** = *E. faecalis* ATCC 29212, **AE** = *E. faecalis* ATCC 49533, **AF** = *E. faecium* BM4105-RF, **AG** = VRE, **AH** = *L. monocytogenes* ATCC 19115, **AI** = *M. smegmatis* MC2-155.

Table 3: MIC values[a] (in µg/mL) determined for all compounds **2a**, **2h**, and for the control antibacterial agent (AMK) against additional non-*S. aureus* bacterial strains.

Strain	Ebselen	2a	2h	AMK
A. baumannii ATCC 19606	15.6	15.6	31.3	7.8
E. cloacae ATCC 13047	>125	125	>125	1
E. coli MC1061	7.8	31.3	15.6	2
K. pneumoniae ATCC 27736	>125	62.5	>125	1
P. aeruginosa ATCC 27853	>125	62.5	>125	31.3
S. enterica ATCC 14028	>125	125	>125	7.8
S. epidermidis ATCC 12228	>125	125	>125	31.3

Graphical Abstract

Development of ebsulfur analogues as potent antibacterials against methicillin-resistant *Staphylococcus aureus*

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