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Sulfenate Esters of Simple Phenols Exhibit Enhanced Activity against Biofilms

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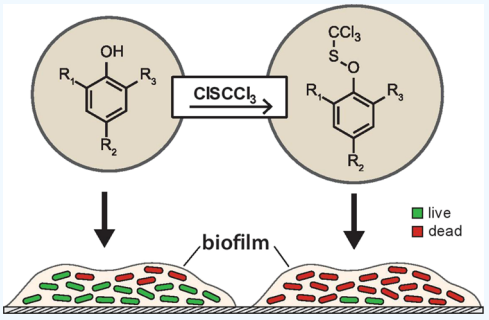
ABSTRACT: The recalcitrance exhibited by microbial biofilms to conventional disinfectants has motivated the development of new chemical strategies to control and eradicate biofilms. The activities of several small phenolic compounds and their trichloromethylsulfenyl ester derivatives were evaluated against planktonic cells and mature biofilms of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Some of the phenolic parent compounds are well-studied constituents of plant essential oils, for example, eugenol, menthol, carvacrol, and thymol. The potency of sulfenate ester derivatives was markedly and consistently increased toward both planktonic cells and biofilms. The mean fold difference between the parent and derivative minimum inhibitory concentration against planktonic cells was 44 for *S. epidermidis* and 16 for *P. aeruginosa*. The mean fold difference between the parent and derivative biofilm eradication concentration for 22 tested compounds against both *S. epidermidis* and *P. aeruginosa* was 3. This work demonstrates the possibilities of a new class of biofilm-targeting disinfectants deploying a sulfenate ester functional group to increase the antimicrobial potency toward microorganisms in biofilms.



1. INTRODUCTION

Biofilms are multicellular communities that form when planktonic cells adhere to a surface via cell adhesion structures such as pili or flagella.[1,2] The attached cells begin to secrete extracellular DNA, proteins, and polysaccharides to form an extracellular polymeric substance (EPS), which traps nutrients while providing protection from antimicrobials, disinfectants, and host immune defences.[3−7] In the biofilm interior, cells experience slow growth rates or become dormant and are able to persist when other cells in the biofilm are killed. These persistent cells are able to regenerate the biofilm, resulting in chronic infection, and contribute greatly to the refractory characteristics of biofilms.[8−12] Reactive antimicrobial agents may be retarded in their penetration if they are neutralized as they diffuse into the biofilm.[13−17] These factors all contribute to increased tolerance toward antibacterial agents and disinfectants.[11,13,18−22] It is traits such as these and biofilms' prominence in hospitals that lead to elevated efforts to control biofilms with small molecules.[23]

Over the last 2 decades, the number of hospital-acquired infections has increased by 36% in the US, further stressing the need for novel disinfectants.[24] Routine disinfectants that are currently used in hospitals include hydrogen peroxide, sodium hypochlorite, chlorine, and quaternary ammonium salts, although many of these have serious shortcomings when treating biofilms. For example, several studies have shown that *Pseudomonas aeruginosa* and *Escherichia coli* biofilms exhibit resistance toward hydrogen peroxide.[25−27] Bacterial strains prevalent in hospitals such as *Staphylococcus aureus* and *P. aeruginosa* have also been shown to exhibit tolerance toward many quaternary ammonium salts such as benzalkonium chloride, benzyldimethyltetradecylammonium chloride, and didecyldimethylammonium bromide.[28−30] Chlorine and chlorine dioxide have been shown to have limited potency toward biofilms because of their inability to fully penetrate through the robust EPS, thus being unable to reach the inner layers of the biofilm.[31,32] Essential oils such as thymol and eugenol are used as environmentally friendly disinfectants to control *S. aureus* biofilms, although they are used at high concentrations in order to be effective.[33]

Phenols are a well-studied class of organic compounds which have been shown to demonstrate varying degrees of antimicrobial activity[34−36] and were chosen here because of a wide variety of structurally diverse phenols being previously evaluated for biological activity.[37−45] Among these activities, phenols have been shown to disrupt the cell membrane causing cell lysis, resulting in cell death.[40,44,46,47] Phenols have also been shown to attack cytoplasmic targets by denaturing proteins and deactivating enzymes, thereby binding to them to

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form inoperative complexes.[42,45] The majority of phenols selected for this study were done so for their previously known antimicrobial activity toward planktonic cells. The essential oils thymol, menthol, carvacrol, and eugenol were chosen for their inhibitory and antimicrobial properties against a wide range of taxonomically diverse bacteria.[48−51] These essential oils were also chosen because of being found in several edible herbs.[52,53] Halogenated phenols were chosen because of their extensive evaluation and high activity.[39,54] Select alkylphenols were chosen for their antimicrobial and antifouling activity.[55,56] Several alkoxyphenols were selected as a variety of alkoxy phenols studied for antimicrobial activity.[57,58] Two non-phenolic compounds were also chosen for this study, menthol for its structural similarity to thymol and 5-fluoro-2-((trichloromethyl)thio)isoindoline-1,3-dione (**21b**) for its similarity to the fungicide folpet.

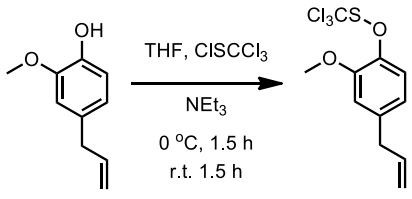
The trichloromethylanesulfenimide group that has been employed here on select phenols is the active antimicrobial pharmacophore in the broad-spectrum commercial fungicides captan and folpet. captan and folpet are phthalimide derivatives and are commonly used for protection of fruit and vegetable crops. Their activity is attributed to their reactivity with thiols and they are active against a wide range of fungal diseases.[59,60] Significantly, these compounds have been shown not to possess carcinogenic, mutagenic, or teratogenic threats to humans.[61]

In this study, the trichloromethylsulfenate esters of a variety of phenolic compounds were synthesized and evaluated against both planktonic cells and biofilms. The bacteria chosen for evaluation were *P. aeruginosa*, a Gram-negative bacterium, and *Staphylococcus epidermidis*, a Gram-positive bacterium. These bacteria were also selected for their prevalence in hospitals[62−64] as well as their propensity to form biofilms.[65−67] Although the concept of biofilms was presented as early as the 1960s, the study of behavioral variations in biofilms such as nutrient uptake, gene expression, and increased tolerance did not arise until more recently.[68−71] Biofilm research is an emerging field which has been rapidly gaining interest in light of new technologies in 3D modeling, imaging, antibiofilm strategies, and analytical tools[72−74] as well as recent research emphasizing clinical relevance.[69,75] These also shed light on the need for novel strategies for the treatment and eradication of biofilms including antibacterial small natural molecules, peptides, and lipids.[76−81]

2. RESULTS AND DISCUSSION

In this study, 25 sulfenate esters of small phenols were synthesized. This synthesis was accomplished by treating each phenol with trichloromethyl hypochlorothioite in either tetrahydrofuran or diethyl ether with triethylamine at 0 °C for 1.5 h and then allowing the reaction mixture to stir for 1.5 h at room temperature (Scheme 1).

Scheme 1. Representative Synthesis, Using Eugenol (8a)



Sulfenate esters were more potent than their parent compounds 92 % of the time. For example, on average, trichloromethylsulfenate esters were nine times more potent than parent compounds against *S. epidermidis* and 17 times more potent toward *P. aeruginosa* in planktonic assays (Figure 1). Against biofilms, sulfenate esters were on average four times more potent toward *S. epidermidis* and 3.8 times more potent toward *P. aeruginosa*. It was also observed that toward biofilms, phenols and sulfenates were less potent compared to planktonic cells, a phenomenon that has widely been observed in previous studies.[3,67,70,82−84] The relative potencies of the precursor phenols and the corresponding sulfenate esters against both planktonic cells and biofilms will be discussed in turn.

2.1. Disinfectant Activities in the Planktonic State.

2.1.1. Parent Phenols. The most potent parent phenols against planktonic cells were 4-heptyloxyphenol (**7a**), 4-chloro-2-methylphenol (**16a**), 3,4-dichlorophenol (**14a**), 2,4-dimethylphenol (**3a**), 6-(1-methylethyl)-3-methylphenol (thymol) (**1a**), and 3-(1-methylethyl)-6-methylphenol (carvacrol) (**4a**) against both *S. epidermidis* and *P. aeruginosa* (Figure 1). Compounds **16a** and **14a** both possess at least one chlorine group on the aromatic ring, and although *p*-fluorophenol (**15a**) also possesses a halogen on the aromatic ring, it was significantly less potent against both *S. epidermidis* and *P. aeruginosa* (Figure 1). This is congruous with previous studies demonstrating that chlorine, which is more electron-withdrawing than fluorine, increases the potency of the parent phenols to a greater extent.[39] Compounds **1a**, **3a**, and **4a** all have either isopropyl or methyl groups in both the ortho and para positions, whereas **7a** has a para heptyloxy group (Figure 1). In contrast to **1a**, **3a**, and **4a**, compound **5a** (2,6-diisopropylphenol) has two isopropyl groups in the ortho positions and possesses a significantly lower potency. This is likely due to the higher degree of steric hindrance around the phenolic hydroxyl.

Compound **7a** had the lowest minimum inhibitory concentration (MIC) toward both bacteria, making it of particular interest as it is considerably more active than the corresponding 4-methoxy and 2,4-dimethoxy derivatives, **9a** and **10a** (Table 1), which are less lipophilic. In light of this observation, two additional compounds in this series were synthesized and examined to evaluate the influence of lipophilicity on activity, these being 4-(benzyloxy)phenol (**11a**) and 4-(2-(2-methoxyethoxy)ethoxy)phenol (**12a**). Compound **12a** was chosen for its near identical side chain length, when compared to **7a**, and the increased hydrophilicity imparted by the oxygens within the side chain. Significantly, both of these structural alterations led to a marked decrease in activity compared to **7a** (Table 1). In addition, **24a**, which differs from **7a** solely by possessing a sulfur in place of oxygen, was evaluated and was found to be less potent than **7a** against both bacteria.

Based on these results, a second SAR study was conducted with compounds **17a**, **19a**, and **20a**, which all possess an amide chain in the para position (Table 2). Compounds **17a** and **19a** were chosen to compare the amide chain length, as **17a** has a 4-butanamide group whereas **19a** has a 4-heptanamide group, which is predicted to increase lipophilicity. Compound **20a** was chosen for the comparison of nitrogen placement in the amide side chain vis a vis "amide inversion". Accordingly, compound **19a** (*N*-(4-hydroxyphenyl) heptanamide) has the amide nitrogen on the aromatic ring, whereas **20a** (*N*-hexyl-4-

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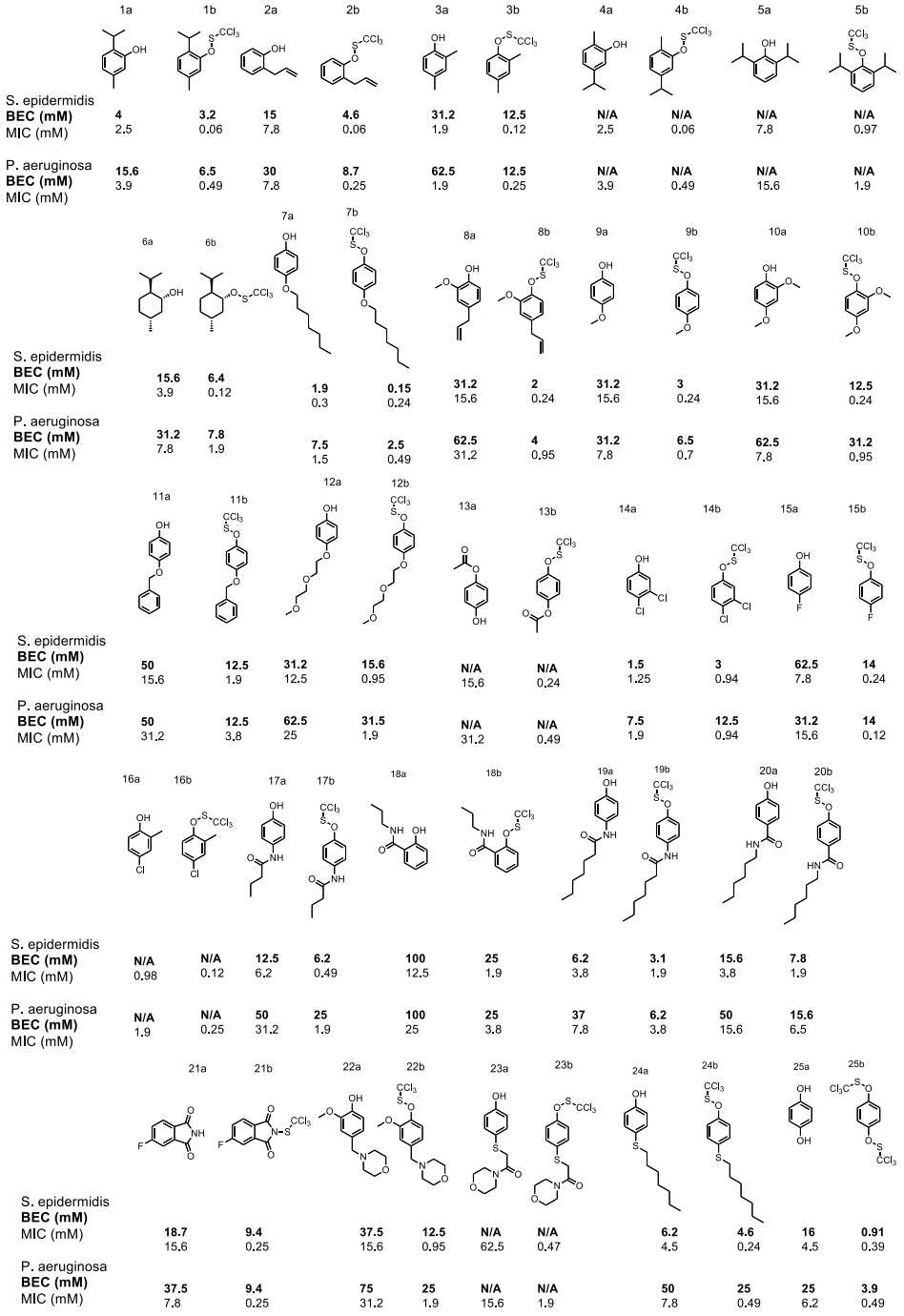

Figure 1. Parent phenols and corresponding sulfenate esters and their MICs and BECs.

Table 1. MICs of Phenols 7a, 9a, 10a, 11a, 12a, and 24a

	MICs (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
7a	0.3	1.5
9a	15.6	7.8
10a	15.6	7.8
11a	15.6	31.2
12a	12.5	25
24a	4.5	7.8

Table 2. MICs of Phenols 17a, 18a, 19a, 20a

	MICs (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
17a	6.2	31.2
18a	12.5	25
19a	3.8	7.8
20a	3.8	15.6

hydroxybenzamide) has the carbonyl of the amide group attached to the aromatic ring. This structural alteration was performed to assess the effects of electron-donating and electron-withdrawing amide groups of the same length. An additional compound, **18a**, was also evaluated for similar reasons to compare to **17a**, although here the butanamide group was placed in the 2-position.

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Phenol **19a** was the most potent compound in this series overall, although **20a** shared the same potency against *S. epidermidis* (Table 2). It was observed that **19a** was more potent than **17a** toward both bacteria, continuing the trend seen earlier that a longer alkyl chain length does increase the potency toward planktonic cells. Between **17a** and **18a**, **17a** was the more potent isomer against *S. epidermidis* but not against *P. aeruginosa*. This evaluation also suggests that the length of the alkyl chain has a greater affect than nitrogen placement with respect to the aromatic ring.

(−)-Menthol (**6a**) was selected because of its structural similarities to thymol (**1a**), and as it is nonphenolic. Menthol, like thymol, has been studied for its antimicrobial activity.[85,86] It should be noted that **6a** was far less active than **1a** toward both bacterial strains used in this study.

2.1.2. Trichloromethylsulfenate Esters. It was observed that, in general, more potent parent phenols produced more potent sulfenate esters, although this trend could not reliably be used to predict activity in all cases. The most potent sulfenates against *S. epidermidis* were, in descending order, **1b**, **2b**, **4b**, **3b**, and **16b**. For *P. aeruginosa*, the most potent sulfenates were **15b**, **2b**, **3b** followed by **16b**. As seen with the parent phenols, the sulfenate esters **3b** and **16b** are among the most potent disinfecting agents toward planktonic cells of both bacteria (Figure 1). Likewise, **1a**, **3a**, and **4a** were also among the most potent toward planktonic *S. epidermidis*. In contrast, sulfenates **2b** and **15b** showed a significant potency when their parent phenols did not. Conversely, phenols **7a** and **14a** showed exceptional potency toward both bacteria, whereas the corresponding sulfenates **7b** and **14b** did not share this characteristic (Figure 1).

In consonance with the prior SAR study, the series of sulfenate esters **7b**, **9b**, **10b**, **11b**, **12b**, and **24b** showed a similar trend to the corresponding phenols toward planktonic cells. Derivatives **7b**, **9b**, **10b**, and **24b** shared the highest potency toward *S. epidermidis*, whereas **7b** and **24b** were the most potent toward *P. aeruginosa* in this series (Table 3).

Table 3. MICs of Sulfenates 7b, 9b, 10b, 11b, 12b, and 24b

	MICs (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
7b	0.24	0.49
9b	0.24	0.7
10b	0.24	0.95
11b	1.9	3.8
12b	0.95	1.9
24b	0.24	0.49

Sulfenate **11b** was least potent toward both bacteria, as was seen with phenols. It was interesting to note that, in contrast to sulfenates, the 4-alkoxyphenol **7a** was significantly more potent than its 4-(thioalkyl) counterpart, **24a** (Table 1).

In the SAR study involving amides, a trend consistent with parent compounds was not observed. Compound **17b** was the most potent sulfenate toward both bacteria, whereas the most potent parents were **19a** and **20a** against *S. epidermidis* and **19a** toward *P. aeruginosa* (Table 4). Similarly, **20b** was the least potent sulfenate in the amide series, whereas the least potent parent was **18a** toward *S. epidermidis* and **17a** toward *P. aeruginosa*. This further demonstrates that trends in parent compounds cannot necessarily be used to predict trends in their corresponding sulfenate ester derivatives.

Table 4. MICs of Sulfenates 17b, 18b, 19b, and 20b

	MICs (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
17b	0.49	1.9
18b	1.9	3.8
19b	1.9	3.8
20b	1.9	6.5

Compounds **6b** and **21b** were evaluated separately against planktonic cells. Compound **6b** was among the six most potent sulfenates against *S. epidermidis*, sharing an MIC with **3b** and **16b** (Figure 1). Compound **21b** was among the five most potent compounds toward *P. aeruginosa*, sharing an MIC with **2b**, **3b**, and **16b**.

MICs of sulfenate esters were, in general, statistically significantly lower than the MIC of the parent phenols. A two-tailed *t*-test was performed on four select phenol/sulfenate ester pairs; **3a**/**b**, **10a**/**b**, **13a**/**b**, and **16a**/**b**. Compounds **10a**/**b** and **13a**/**b** were chosen because of the large discrepancy in potency observed between the parent phenol and the sulfenate. Compounds **3a**/**b** and **16a**/**b** were chosen because the potency between parent phenols and sulfenate esters was the least dramatic of the 25 compound pairs evaluated. The *p*-value of **2a**/**b** against *S. epidermidis* was calculated to be 0.043 and against *P. aeruginosa*, 0.0028. The *p*-value of **10a**/**b** against *S. epidermidis* is 0.039 and against *P. aeruginosa* is 0.031. The *p*-value of **13a**/**b** against *S. epidermidis* is 0.0039 and against *P. aeruginosa* was calculated to be 2.8×10^{-5}. The *p*-values for **16a**/**b** were 0.00051 and 0.0028 against *S. epidermidis* and *P. aeruginosa*, respectively.

2.2. Disinfectant Activity against Biofilms. For comparison, the antiparasitic drug nitazoxanide and the antibiotics metronidazole and tobramycin were evaluated for activity toward *S. epidermidis* and *P. aeruginosa* biofilms. Nitazoxanide is an antidiarrheal commonly used to treat strains of *Cryptosporidium*, *Blastocystis*, and *Giardia* and is believed to interfere with pyruvate:ferredoxin oxidoreductase enzyme-dependent electron transfer reaction.[87−89] Nitazoxanide has also been shown to inhibit biofilm formation in *S. epidermidis*[90] and enteroaggregative *E. coli*[91] as well as decrease the viability of *Clostridioides difficile* biofilms.[92] However, the efficacy of the drug to eradicate biofilms has yet to be evaluated, as it is here. The biofilm eradication concentration (BEC) of nitazoxanide was found to be 50 mM toward *S. epidermidis* and 3.12 mM toward *P. aeruginosa*.

Metronidazole is a nitroimidazole used to treat a variety of bacterial and parasitic infections and is most commonly used to treat infections related to inflammatory disorders of the gastrointestinal tract. Metronidazole is often used to treat Gram-negative, Gram-positive, and Gram-variable anaerobic bacteria, as well as protozoans such as Giardia lamblia.[93] It has been shown to exhibit lower activity toward biofilms such as that of *Helicobacter pylori*[94] and *Clostridium difficile*[95,96] although it has also been evaluated in tandem with several other antibiotics, resulting in improved activity against *Enterococcus faecalis* and *Candida albicans* biofilms.[97] Against *S. epidermidis*, the BEC of metronidazole was 6.25 and against *P. aeruginosa* it was 50 mM.

Tobramycin is an antibiotic that inhibits protein synthesis, used to treat Gram-negative bacteria. Tobramycin is commonly used to treat bacterial pneumonia and bacterial eye infections, and has been extensively studied against

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biofilms.[98,99] A study by Høiby et al. showed that the inhibitory properties toward *P. aeruginosa* biofilms were lower than that toward planktonic cells, concluding that biofilms are tolerant to the clinically recommended dose of the antibiotic.[100] Sans-Serramitjana et al. evaluated the antimicrobial activity of nanoencapsulated tobramycin, finding that nanoencapsulation did improve the ability of tobramycin to eradicate *P. aeruginosa* biofilms and thus suggesting the strategy of lipid carriers to deliver the drug, overcoming drug resistance to tobramycin.[101] Tobramycin was in part chosen because of the known antimicrobial resistance of *P. aeruginosa*, making it a valuable comparison to this series of novel antimicrobials.[102−104] Tobramycin was found to have a BEC of 18 mM toward *S. epidermidis* and 0.6 mM toward *P. aeruginosa*.

2.2.1. Parent Phenols. Based on the observations from planktonic cell assays, 18 parent phenols and the corresponding sulfenate esters were chosen for evaluation against biofilms. Compounds **1a**, **3a**, **7a**, and **14a** were chosen for their high potency toward planktonic cells (Figure 1). Compounds **2a**, **8a**, **15a**, **10a**, **17a**, and **22a** were selected because of the large increase in potency between the moderately active parent phenols and the corresponding sulfenate esters (Figure 1). Compounds **7a**, **9a**, **10a**, **11a**, **12a**, **24a**, **17a**, **18a**, **19a**, and **20a** were selected for the purpose of continuing the two SAR studies conducted with planktonic cells. Compound **25a** was chosen as the corresponding sulfenate ester possesses two trichloromethylsulfenate ester groups. Additionally, the non-phenolic compounds **6a** and **21a** were chosen, **6a** as an aliphatic alcohol bearing a structural similarity to **1a** and **21a** for its similarity to the imide corresponding to the commercial fungicide folpet. Neither compound showed a significant activity toward either bacterium.

As a continuation of the previous SAR study involving phenols **7a**, **9a**, **10a**, **11a**, **12a**, and **24a** against planktonic cells, this series was subsequently evaluated against biofilms (Table 5). In accordance with planktonic trends, **7a** was the most potent phenol in this series toward both bacteria.

Table 5. BECs of Phenols 7a, 9a, 10a, 11a, 12a, and 24a

	BEC (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
7a	1.9	7.5
9a	31.2	31.2
10a	31.2	62.5
11a	50	50
12a	31.2	62.5
24a	6.2	50

In the SAR study involving amides, **19a** was the most potent phenol toward both bacteria. In contrast, **18a** was the least potent compound toward both bacteria, whereas in planktonic assays **17a** was least potent toward *P. aeruginosa*. In both SAR studies, the more potent phenols against planktonic cells did in general have lower BECs as well, though the trend in potency was not always predictable for all compounds (Table 6).

Among the additional compounds selected for biofilm evaluations, the majority were alkyl phenols, along with two halophenols and hydroquinone (Table 7). The most potent phenols toward *S. epidermidis* biofilms were, in descending order, **14a**, **7a**, **1a**, **19a**, and **24a**. Against *P. aeruginosa*, the most potent phenols were **14a**, **7a**, **1a**, **25a**, and **2a**, whereas here, **14a** and **7a** shared the same BEC. Out of these seven

Table 6. BECs of Phenols 17a, 18a, 19a, and 20a

	BEC (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
17a	12.5	50
18a	100	100
19a	6.2	37
20a	15.6	50

Table 7. BECs for Allyl- and Halo-Phenols and Hydroquinone

	BEC (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
1a	4	15.6
2a	15	30
3a	31.2	62.5
8a	31.2	62.5
14a	1.5	7.5
15a	62.5	31.2
22a	37.5	75
25a	16	25

compounds, only three (**1a**, **7a**, and **14a**) were among the most active against planktonic cells. These results further reveal that the activity toward planktonic cells cannot be reliably used to predict the potency toward biofilms.

2.2.2. Sulfenate Esters. The most potent sulfenate esters toward *S. epidermidis* were **7b**, **25b**, **8b**, **9b**, and **14b**. For *P. aeruginosa*, the most active sulfenates were **25b**, **8b**, **19b**, **9b**, and **1b**. Interestingly, out of these seven compounds, none were among the most potent toward planktonic cells, which was unexpected as it differs from the trend observed with parent phenols. However, there were similarities between most potent phenols and sulfenates toward biofilms. For example, the phenols corresponding to sulfenates **1b**, **7b**, **14b**, **19b**, and **25b** were among the most potent parents.

In consonance with the previous SAR study involving 4-alkoxyphenols, sulfonates **7b**, **9b**, **10b**, **11b**, **12b**, and the 4-(heptylthio)phenyl sulfenate **24b** were evaluated toward biofilms (Table 8). Sulfenate **7b** was the most potent

Table 8. BECs for Sulfenates 7b, 9b, 10b, 11b, 12b, and 24b

	BEC (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
7b	0.15	2.5
9b	3	6.5
10b	12.5	31.2
11b	12.5	12.5
12b	15.8	31.2
24b	4.6	25

compound against biofilms in this series. Against planktonic cells however, **7b** had the same MIC as **9b**, **10b**, and **24b** against *S. epidermidis* and **24b** against *P. aeruginosa* (Table 3). This observation supports the finding that long, saturated alkoxy chains generally increase the potency more so than a diethylene glycol-derived chain or a benzyl group. It is also noteworthy that the replacement of the oxygen by sulfur (e.g. **7b** → **24b**) results in a substantial decrease in activity.

The SAR study involving amides showed that **19b** was the most potent derivative toward both bacteria, which is not

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congruent with what was observed with planktonic cells, where **17b** was the most active (Table 9). Compound **18b** was the least potent sulfenate in this series against biofilms, whereas **20b** was the least potent toward planktonic cells.

Table 9. BEC for Sulfenates 17b, 18b, 19b, and 20b

	BEC (mM)	
compounds	*S. epidermidis*	*P. aeruginosa*
17b	6.2	25
18b	25	25
19b	3.1	6.2
20b	7.8	15.6

Sulfenates selected for biofilm evaluations, which were not part of the two preceding SAR studies, are assembled in Table 10. Among these, monosulfenates **8b** and **1b** were the most

Table 10. BECs for Allyl- and Halo-Sulfenates as Well as the Bis(sulfenate) 25b

	BEC (Mm)	
compounds	*S. epidermidis*	*P. aeruginosa*
1b	3.2	6.5
2b	4.6	8.7
3b	12.5	12.5
8b	2	4
14b	3	12.5
15b	14	14
22b	12.5	25
25b	0.91	3.9

active toward both strains of bacteria. It is not surprising that bis(sulfenate) **25b** showed excellent activity toward biofilms as well. Sulfenate **22b**, which contains a basic morpholine group, exhibited a low potency against both bacterial strains. In this case, it had been hoped that the presence of a basic amine might increase the permeability by way of protonation, resulting in enhanced solubility. Nonphenolic sulfenates **6b** and **21b** were also evaluated toward biofilms (Figure 1). Neither compound showed a significant activity, with **6b** showing only half the potency of **1b** toward *S. epidermidis*.

2.3. Comparison of Phenols and Sulfenates. Among parents and sulfenates chosen for the alkoxy and alkylthio side chains' SAR study, (**7a/b**, **9a/b**, **10a/b** **11a/b**, **12a/b**, and **24b**), it was shown that the more potent phenols did typically produce more potent sulfenate esters when evaluated against biofilms. The exception to this was **12a**, which has a lower BEC than **11a**, whereas **11b** has a lower BEC than **12b** against *S. epidermidis* biofilms. It is therefore evident that **7a/b** were the most potent compounds in this series overall. In the amide SAR study, between **17a/b** and **19a/b**, it was observed that **19a/b** was typically more potent than **17a/b** with the exception of **19b** being less potent toward planktonic *P. aeruginosa*. This relationship demonstrates that an increasing alkyl chain length, as with **7a/b**, will in general increase the potency of phenols and sulfenates. Between the isomers **19a/b** and **20a/b**, **19a/b** were generally the more potent isomers in both planktonic and biofilm assays, although possessing the same MICs toward *S. epidermidis*.

Overall, a correlation between an increased potency toward planktonic cells leading to an increased potency in biofilms was observed through evaluation of phenols and corresponding sulfenate esters, with the exception of **14a/b** and **7a/b** against *P. aeruginosa* biofilms (Figure 1). This type of relationship has been previously described by others as well,[105] although it has also been observed that activity toward planktonic cells cannot reliably be used to predict the same compound potency against biofilms. This has been demonstrated most recently by Walsh et al. (2019)[106] and is further supported here by the foregoing results.

The BECs of sulfenate esters when compared to their corresponding parent phenols were generally statistically significantly lower. A two-tailed *t*-test was performed on four select phenol/sulfenate ester pairs; **8a/b**, **9a/b**, **12a/b**, and **17a/b**. Compounds **8a/b** and **9a/b** were chosen because of the large discrepancy in potency observed between the parent phenol and the sulfenate. Compounds **12a/b** and **17a/b** were chosen because the potency between parent phenols and sulfenate esters was the least dramatic of the 25 compound pairs evaluated. The *p*-value of **8a/b** against *S. epidermidis* was calculated to be 0.00012 and against *P. aeruginosa*, 0.044. The *p*-value of **9a/b** against *S. epidermidis* is 0.044 and against *P. aeruginosa* is 0.0091. The *p*-value of **12a/b** against *S. epidermidis* is 0.0038 and against *P. aeruginosa* was calculated to be 0.019. The *p*-value for **17a/b** was 0.019 against both *S. epidermidis* and *P. aeruginosa*.

2.4. Comparison of Sulfenates and Known Antibacterial Compounds. The sulfenate esters which showed the highest potency toward biofilms were **7b** toward *S. epidermidis*, with a BEC of 0.15 mM (Table 8), and **25b** toward *P. aeruginosa* with, a BEC of 3.9 mM (Table 10). Among the commercially available antimicrobials evaluated here, metronidazole exhibited the highest potency toward *S. epidermidis* biofilms with a BEC of 6.25 mM and tobramycin toward *P. aeruginosa* with a BEC of 0.6 mM. Against *S. epidermidis*, nine of 19 sulfenate esters had a lower BEC than metronidazole, 18 out of 19 had a lower BEC than tobramycin, and all 19 had a lower BEC than nitazoxanide. Against *P. aeruginosa*, tobramycin and nitazoxanide had a lower BEC than all sulfenate esters, although all 19 sulfenate esters had a lower BEC than metronidazole.

As *Staphylococci* and *Pseudomonas* are both facultative anaerobes and metronidazole is most affective toward anaerobic bacteria, it was predicted that the majority of sulfenate esters would be more potent toward both bacteria. Tobramycin is typically used to treat Gram-negative infections, and, as shown here, was significantly more potent toward *P. aeruginosa* than sulfenate esters. Nitazoxanide is used to treat both Gram-positive and -negative bacteria, although is more often used to treat anaerobes. Sulfenate esters were statistically more potent toward *S. epidermidis* than *P. aeruginosa*; so it is no surprise that the majority of sulfenates showed greater potency toward *S. epidermidis* but not toward *P. aeruginosa* when compared to known antimicrobials.

2.5. Analysis of Sulfenate Degradation. Sulfenate esters are expected to hydrolyze to the parent phenols in aqueous solutions via cleavage of the S−O bond. In a study to determine the hydrolytic stability of sulfenates, the decomposition of (4-fluorophenoxy)trichloromethylsulfane (**15b**) in water was monitored via ^{19}F NMR (Figure 2). In this study, the gradual appearance of 4-fluorophenol (**15a**) (^{19}F NMR δ: −125.1 ± 0.1) was clearly revealed.

After 12 h (C), there were no signs of decomposition of the sulfenate (**15b**). However, after 24 h (D), the sulfenate ester (**15b**) had begun to hydrolyze to the parent phenol. A

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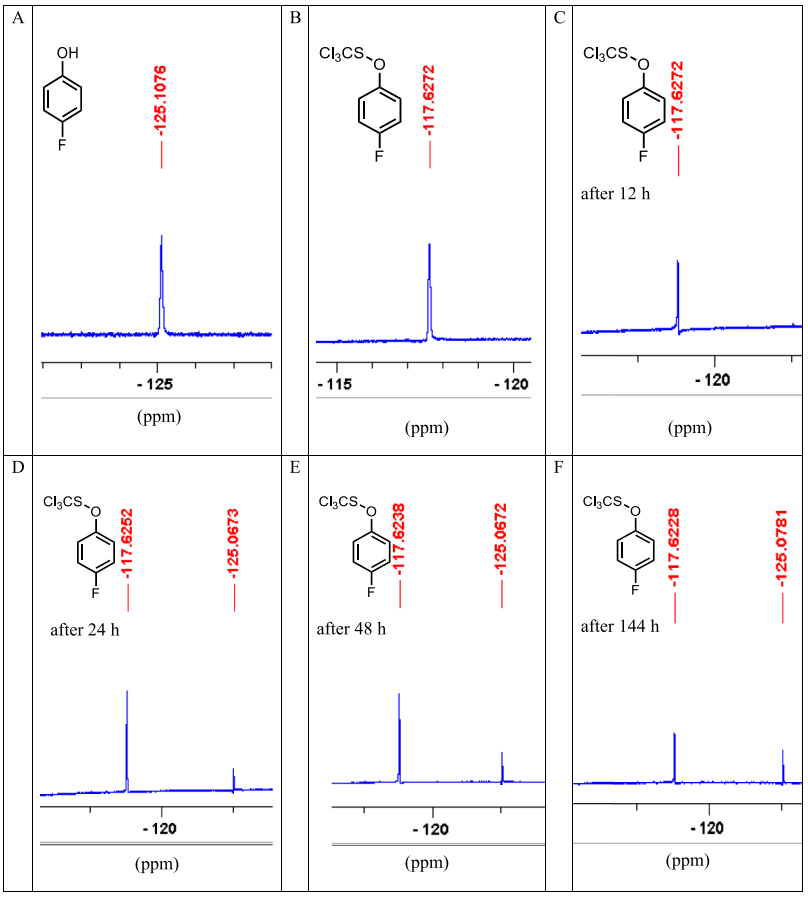

Figure 2. (A) ^{19}F NMR of *p*-fluorophenol (**15a**) in D_2O; (B) ^{19}F NMR of (4-fluorophenoxy) (trichloromethyl)sulfane (**15b**) in D_2O at 0 h; (C) ^{19}F NMR of (4-fluorophenoxy)(trichloromethyl)sulfane in D_2O after 12 h; (D) ^{19}F NMR of (4-fluorophenoxy) (trichloromethyl)sulfane in D_2O after 24 h; (E) ^{19}F NMR of (4-fluorophenoxy) (trichloromethyl)sulfane in D_2O after 48 h; (F) ^{19}F NMR of (4-fluorophenoxy) (trichloromethyl)sulfane in D_2O after 144 h.

continuation of this decomposition was observed (E and F), and after 144 h, the sulfenate ester approached a 1:1 ratio with the corresponding phenol (F). This shows that while sulfenate esters do hydrolyze in the presence of water, they are stable for up to 12 h. This is crucial as the biological assays employed here require a 12 h exposure time for each phenol and sulfenate ester derivative in planktonic and biofilm assays. Accordingly, sulfenates should be robust for the entirety of the exposure time.

3. CONCLUSIONS

This study has shown that sulfenate esters generally exhibit a significant increase in potency toward planktonic cells and biofilms of *S. epidermidis* and *P. aeruginosa* when compared to their phenolic counterparts. For example, it was found that on average sulfenates were nine times more potent than the parent phenols against *S. epidermidis* and 17 times more potent toward *P. aeruginosa* in planktonic assays. Against biofilms, sulfenates were four times more potent toward both *S. epidermidis* and *P. aeruginosa*. The findings presented here also reveal that the most potent compounds toward planktonic cells are not always the most potent toward biofilms. Likewise, the most potent parent phenols do not consistently produce the most potent sulfenate esters. SAR studies have shown that placement, configuration, and alkyl chain length of functional groups do affect the potency of the parent phenols as well as the derivatized sulfenates. An additional study, the monitored hydrolysis of **15b** by ^{19}F NMR, has shown that the stability of a representative sulfenate ester in aqueous solution is approximately 24 h. Further experimentation to determine clinical significance could be conducted with biofilms' eradications measurements being determined with biofilms grown on different surfaces, such as metal and plastic, to mimic those found in clinical settings.

4. MATERIALS AND METHODS

4.1. Synthetic Reagents and Bacteria. All organic reagents for chemical synthesis were purchased from commercial sources and used as received without further purification. *P. aeruginosa* (PA01) and *S. epidermidis* (35984) were obtained from American Type Culture Collection (ATCC). All bacteria were subcultured onto tryptic soy agar (TSA) plates and incubated at 37 °C for 24 h. Single colonies were transferred from the plates and inoculated into 25 mL tryptic soy broth (TSB) in Erlenmeyer flasks. Cultures were incubated 37 °C for 24 h and 10 μL of culture was transferred into 25 mL of TSB. The absorbance was read at 600 nm (OD_{600}) using a spectrophotometer, adjusted to an OD of 0.05 and standardized to 10^6 to 10^7 CFU/mL.

4.2. Synthesis. *4.2.1. Preparation of (2,4-Dimethylphenoxy)trichloromethyl Sulfane (**3b**).* A 25 mL round-bottomed flask equipped with a magnetic stirring bar was charged with 2,4-dimethylphenol (610 mg, 5 mmol, 1 equiv) and anhydrous diethyl ether (10 mL). The mixture was

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cooled to 0 °C and anhydrous triethylamine (0.77 mL, 5.5 mmol, 1.1 equiv) was added. To the stirred mixture was added trichloromethyl hypochlorothioite (0.57 mL, 5.25 mmol, 1.05 equiv) dropwise. The reactant mixture was stirred at 0 °C for 1.5 h and allowed to warm to room temperature and stirred for an additional 12 h. To the resulting mixture was added pentane (5 mL), which was then filtered through Celite and washed with *t*-butyl methyl ether (3 × 5 mL). The solvents were evaporated in vacuo to provide the title compound 1.03 g (76%) as a yellow oil. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.23 (dd, J = 843, 2.62 Hz, 1H), 7.00 (dd, J = 2.62, 0.45 Hz, 1H), 6.98 (dd, J = 8.43, 0.45 Hz, 1H), 2.39 (s, 3H), 2.31 (s, 3H). **C^{13} NMR** (500 MHz, $CDCl_3$): δ 16.16 (CH_3), 20.55 (CH_3), 116.19 (C), 121.49 (CH), 130 (C), 132.02 (C), 133.81 (CH), 136.73 (CH), 156.05 (C). ^{1}H and ^{13}C NMR was used to confirm the purity of all sulfenate esters. Details can be found in the Supporting Information.

4.3. Efficacy of Phenols and Derivatives on Inhibiting Planktonic Cells. MICs of all compounds evaluated here were determined using a 96-well plate assay previously described by Xie.[35] Ninety-six-well plates were inoculated with bacterial culture, prepared as stated in Section 2.1, followed by exposure to the phenol or the sulfenate. The plates were incubated at 37 °C for 12 h. A plate reader was used to analyze bacterial inhibition. Samples were diluted in dimethyl sulfoxide (DMSO) and DMSO controls were conducted as the negative control. Experiments were done in biological triplicate with technical duplicates. Tests for statistical significance were calculated with a two-tailed *t*-test assuming unequal variances. All compounds were readily soluble in DMSO and no solvent carriers were used in this procedure.

4.4. Efficacy of Phenols and Derivatives on Biofilms. In methods similar to those published by Walsh. et al.,[106] both strains were cultured as described above and biofilms were grown in Costar polystyrene 96-well plates at 37 °C. After 24 h of incubation, the planktonic-phase cells were gently removed, and the wells were washed three times with phosphate-buffered saline (PBS). Wells were filled with 150 μL dilutions of the compound being evaluated. The 96-well plates were incubated for an additional 12 h at 37 °C. The media was gently removed and each well filled with 150 μL of PBS and the biofilm broken up through stirring with sterile, wooden rods. Three tenfold dilutions of each sample were drop-plated on TSA plates and incubated for 24 h. The BEC was determined to be the lowest concentration at which no bacterial growth occurred. This procedure was modeled on previously reported procedures according to Pitts.[107] Two negative controls were conducted with 150 μL of PBS and with 150 μL of DMSO in the absence of disinfecting agents. Positive disinfectant controls were conducted using nitazoxanide, metronidazole, and tobramycin. Experiments were done in biological triplicate with technical duplicates. All compounds were readily soluble, and no solvent carriers were used in this procedure.

4.5. Measuring Rate of Hydrolysis of Sulfenate Derivatives. (4-Fluorophenoxy)trichloromethylsulfane (**15b**) (13 mg, 0.05 mmol) was dissolved in water (1 mL). An aliquot was taken every 12 h and dissolved in D_2O in an NMR tube. ^{19}F NMR was performed to measure the appearance of the parent compound, *p*-fluorophenol (**15a**), in the sample. A 0 h ^{19}F NMR of the sulfane derivative (**15b**) was taken, as was that of the pure parent compound (**15a**) for reference (Figure 2). Technical triplicates were done.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04392.

General procedure for the characterization of all the compounds (PDF)

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Notes

The authors declare no competing financial interest.

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